Development of *in-vitro* and *in-vivo* bioassays to elucidate the mechanism of action of a synthetic novel compound N-phenethyl-1-phenyl-pentan-3-amine (FK70)

Kayatri Govindaraju

Abstract

Hypertension, while being the most common condition of the circulatory system, it has been the greatest attributor to the burden of cardiovascular diseases. Despite the availability of a broad-spectrum pharmacological and non-pharmacological means to control blood pressure, the average blood pressure values over the past decades have remained unchanged. Moreover, in some countries, the trend has been increasing. Such occurrence precipitates a real demand for the discovery and development of newer drugs to treat this condition. In an earlier project, Schwarzinicine A (Sch A), a phenylethylamine alkaloid, was isolated from the leaves of *Ficus Schwarzii*. Sch A was shown to have similar vasorelaxation effect as dobutamine and isoprenaline (β-adrenoceptor agonists), and verapamil (a calcium channel blocker) in the rat aorta. Sch A, as compared with these molecules, was more effective in its ability to relax the blood vessel. N-phenethyl-1-phenyl-pentan-3-amine, (FK70) is a novel compound synthesized based on the main pharmacophore of Sch A as an alternative compound to study the mechanism of action of the novel compound. The primary goal of this study was to elucidate the mechanism of action of FK70 as a vasorelaxant. Initially, the primary pharmacological effects of FK70 were examined on different biological systems using rat isolated smooth muscle tissues (aorta, trachea, and bladder), porcine coronary artery (PCA), and guinea pig ileum. FK70 exhibited relaxation effect in all the tissue types tested, with the higher relaxation effect in the vascular smooth muscle tissues. Subsequently, an evaluation of the pharmacological actions of FK70 in rat aorta were performed. The effects of FK70 were tested in the presence of endothelium and nitric oxide (NO), alpha- and beta-adrenoceptors, potassium channels, and calcium channels. The findings showed that the aortic relaxation by FK70 was not affected by all modulators as mentioned earlier, except it inhibited extracellular calcium entry as suggested from the reduced contraction responses of potassium chloride (KCl) and G-protein coupled receptors (GPCR) linked contractile agents as well as the contractions by calcium chloride in calcium-free Krebs solution. Next, the role of extracellular and stored calcium (Ca^{2+}) in contractile agents mediated responses were examined in rat aorta. The findings showed that (1) the role of stored and extracellular Ca^{2+} in G_q-protein activation (PE and 5-HT) are not the same and (2) the contractile agents (PE, 5-HT, and KCl) depends mostly on extracellular Ca^{2+} to cause a contraction in the rat aorta. To demonstrate the role of Ca^{2+} on FK70 effect, FK70 was tested with known calcium channel blockers, nifedipine (an L-type Ca^{2+} channel blocker) and SKF 96365 (a non-selective TRPC blocker) in normotensive rats (SD) and spontaneously hypertensive rats (SHR). The study exhibited that the relaxation effect of FK70 is comparable to that of known calcium channel blocker, SKF 96365 and the most important mechanism that seemed to be involved in FK70-induced relaxation is inhibition of extracellular Ca^{2+} influx in both SD and SHR. Finally, the effects of

FK70 was examined *in-vivo* using *Caenorhabditis elegans* (*C. elegans*). The study allowed the assessment of both calcium inhibiting property and toxicity effect of FK70. The evaluation of pharyngeal pumping further confirmed the calcium blocking property of FK70 and prolonged exposure to FK70 did not induce a toxic effect on the lifespan of *C. elegans.* Taken together, FK70 exhibited an inhibitory role on calcium -dependent contraction in all the animal models tested. Hence the potential of developing FK70 as an alternative treatment for resistance hypertensive is promising.

To my beloved father, the reason for who I am today and to whom I dedicate this thesis.

To my sister Blomy, who always encourages me to go on every adventure, especially this one!

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Publications

1) **Govindaraju K**, Lee MK, Ting KN, Mbaki Y (2018). Intracellular stored calcium plays a minor role in G_q-coupled receptor-mediated contraction in rat airway smooth muscle. *Life Sciences, medicine and Biomedicine* **2**: 13.

2) Chan ZY, **Govindaraju K**, Krishnan P, Low YY, Chong KW, Yong KT, Ting KN, Lee KH (2019). Diphenethylpiperidine alkaloids with tracheal smooth muscle relaxation activity from *Hippobroma longiflora* (L.) G. Don. *Phytochemistry Letters* **30**: 93-98.

3) **Govindaraju K**, Lee MK, Ting K, Tan C, Ng S, Loh H, Then S and Mohankumar SK (2019). Lignosus rhinocerotis water-soluble sclerotial extract relaxes rat isolated aortae.. *Front. Pharmacol. Conference Abstract: International Conference on Drug Discovery and Translational Medicine 2018 (ICDDTM '18) "Seizing Opportunities and Addressing Challenges of Precision Medicine".* doi: 10.3389/conf.fphar.2018.63.00083

Abbreviations

($[Ca^{2+}]_I$	Intracellular calcium
IL-6	Interleukin 6
MLC	Myosin light chain
O^{2-}	Superoxide anion
5-HT	5-hydroxytrptamine
ABPM	Ambulatory blood pressure measurement
ACE	Angiotension-converting enzyme
ACEI	Angiotension-converting enzyme inhibitor
ACME	Absorption, distribution, metabolisation, and excretion
ADP	Adenosine diphosphate
Akt	Protein kinase B
ANG II	Angiotensin II
ANP	Atrial natriuretic peptide
ARB	Angiotensin II receptor blockers
AT 1	Angiotensin 1 type 1
AT_1R	Angiotensin 1 receptors
ATP	Adenosine triphosphate
BK_{Ca}	Ca^{2+}-activated K^+ channel
BP	Blood pressure
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CCB	Calcium channel blocker
cGMP	Cyclic guanosine monophosphate
CNP	C-natriuretic peptide
CNS	Central nervous system
CO	Carbon monoxide
COPD	Chronic obstructive pulmonary disease
COX	Cycloxygenase
CVD	Cardiovascular disease
DAG	Diacylglycerol
ECL	Extracellular loop
EDHF	Endothelium-derived hyperpolarising factor
EDRF	Endothelium-derived relaxing factor
EET	Epoxyeicosatrienoic acids
ER	Endoplasmic reticulum

ET-1	Endothelin 1
ET_A / ET_B	Endothelin A/B
FK70	N-phenethyl-1-phenyl-pentan-3-amine
GPCR	G-protein coupled receptor
GTP	Guanine tri-phosphatases
H^+	Proton
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen sulfide
ICL	Intracellular loop
IGF-1	Ins-like growth factor
IL1	Inner labial
Ins	Insulin
IP_3	Inositol triphosphate
IP_3R	Inositol triphosphate receptor
K^+	Potassium ion
K_{ATP}	ATP-sensitive K^+ channel
K_{ir}	Inward rectifier K^+ channel
K_v	Voltage-dependent K^+ channel
KH_2PO_4	Potassium dihydrogen phosphate
K_2HPO_4	Dipotassium phosphate
miRNA	microRNA
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
MYPT-1	Myosin phosphatase targeting subunit 1
Na^+	Sodium ion
NADPH	Nicotinamide adenine dinucleotide phosphate
NCX	Na^+/Ca^{2+} exchanger
NF-κB	Nuclear Factor kappa B
NICE	National Institute for Health and Care excellence
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NOS	Nitric oxide synthase
$ONOO^-$	Peroxynitrite
ORCa	Store-independent outwardly rectifying Ca^{2+} channel
PDE	Phosphodiesterases
PE	Phenylephrine
PGX	Prostacyclin

PI3K	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol 4,5 biphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PKG	cGMP-dependent protein kinases
PLC	Phospholipase C
PVR	Pulmonary vascular resistance
RAAS	Renin angiotensin-aldosterone system
RH	Resistant hypertension
ROCC	receptor operated calcium channel
ROCK	Rho associated protein kinase
ROS	Reactive oxygen species
Sch A	Schwarzinicine A
SMOC	Second messenger operated calcium channel
SNP	Sodium nitroprusside
SOCC	Store operated calcium channel
SR	Sarcoplasmic reticulum
STIM 1	Stromal interaction molecule
STOC	Spontaneous transient outward currents
SVR	Systemic vascular resistance
TM	Transmembrane
TNF-α	Tumor necrosis factor-alpha
TRP	Transient receptor potential
TRPC	Transient receptor potential-canonical
TRPM	Transient receptor potential-melastatin
TRPV	Transient receptor potential-vanilloid
VOCC	Voltage-operated calcium channel
WHO	World Health Organisation
α-receptors	Alpha-receptor
β-receptors	Beta-receptor

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Chapter 1: Literature review and introduction

1.1 Cardiovascular disease

Cardiovascular diseases (CVDs) are disorders of the heart and blood vessels which include coronary heart disease, cerebrovascular disease, rheumatic heart disease and other conditions. According to World health organisation (WHO, 2018) reports, CVDs take the lives of 17.9 million people every year, which makes up 31% of all global deaths. In Europe, CVDs remain the leading cause of mortality and a major cause of morbidity. Each year CVDs cause 3.9 million deaths in Europe and over 1.8 million deaths in the European Union (Wilkins *et al.,* 2017). While in Malaysia, the number 1 killer in terms of diseases and health-related problem is coronary heart disease. In 2010, according to the WHO the total number of deaths in Malaysia resulted from coronary heart disease was 22,701. This constitutes to about 22.18% of the total deaths in the country. Since 70s the rate of death from cardiovascular diseases has declined markedly in several high-income countries, owing to reductions in risk factors and improved management of cardiovascular disease. But the incidence of cardiovascular disease has been increasing in some low-income and middle-income countries, with 80% of the global burden estimated to occur in these countries (Yusuf *et al.*, 2014). The high burden of risk factors in high-income countries may have been mitigated by better control of risk factors and more frequent use of proven pharmacologic therapies and revascularization, whilst in low and middle-income countries the risk-factor burden is higher due to more frequent newly merging CVD risk factors like low birth weight, folate deficiency and infections. Thus explaining the statistical difference between the communities and fast increase of cardiovascular mortality in the low- and middle-income countries.

CVDs can be referred to a number of conditions which affect the heart and blood vessels with direct effects on the heart (acute coronary syndromes, angina, arrhythmia, cardiomyopathy, congenital heart disease, coronary heart disease, heart failure, inflammatory heart disease, ischaemic heart disease, rheumatic heart disease and valvular disease), brain (cerebrovascular disease/ stroke) and circulatory system (deep vein thrombosis, hypertensive heart disease , peripheral artery disease and pulmonary embolism) (WHO, 2018). CVDs are usually associated with a build-up of fatty deposits inside the arteries (atherosclerosis) and increased risk of blood clots. They can also be associated with damage to arteries in organ such as the brain, heart, kidneys and eyes.

There are several risk factors associated with CVDs such as genetic (inherited profile), behavioural (tobacco use, inadequate physical activities, unhealthy diet e,g diets that are high in fat combined with carbohydrates and alcohol use), metabolic (increase in blood pressure, blood sugar, and blood lipids, overweight and obesity), socioeconomic (age, gender, economic and educational status) and psychological conditions (stress, depression and lack of sleep), environment (radiation, chronic obstructive pulmonary disorder (COPD) and reduced lung function). However, the most common risk factors for CVDs appear to be atherosclerosis and hypertension (Nordqvist, 2018). This thesis will be centred on the latter as the focus of this research.

1.2 Hypertension

Hypertension is defined as increase in blood pressure (BP). Generally BP is denoted as two numbers, e.g 120/80 mm Hg. The top number is known as systolic pressure measuring the arterial pressure when the heart beats while the bottom indicates the diastolic pressure measuring the arterial pressure when the heart rests between beats.

Typically, most people with high BP (hypertension) do not show any signs or symptoms, even when the blood pressure readings reach dangerously high levels. Some people may have headaches, shortness of breath or nosebleeds, but these signs and symptoms aren't specific and usually don't occur until high blood pressure has reached a severe or life-threatening stage. Uncontrolled high blood pressure increases the risk of serious health problems, including heart attack and stroke. Worldwide, high blood pressure is estimated to cause 7.5 million deaths, about 12.8% of the total of all deaths (WHO, 2018). Therefore, it is crucial to treating systolic blood pressure and diastolic blood pressure until they are less than 140/90 mmHg to reduce cardiovascular complications.

1.2.1 Regulation of normal blood pressure

In order to understand the pathogenesis of hypertension, the knowledge of blood pressure regulation is essential. Blood pressure is the pressure exerted by blood on the walls of a blood vessel that helps to push blood through the body. The optimal systolic blood pressure is 120 mmHg and the optimal diastolic blood pressure is 80 mmHg. The circulatory system is the continuous system of tubes through which the blood is pumped around the body. It supplies the tissues with their nutritional requirements and removes waste products. It can be divided into two circulation systems, systemic and pulmonary circulation. The systemic circulatory system circulates oxygenated blood from the heart around the body into the tissues before returning deoxygenated blood to the heart. In contrast, the pulmonary circulatory system circulates deoxygenated blood from the heart to the lungs via the pulmonary artery and returns it to the heart via the pulmonary vein. There are there 3 keys factors that influence the blood circulation, which involve the blood resistance, pressure and flow (Ross and Pawlina, 2011).

1.2.1.1 Resistance

Resistance to flow must be overcome for blood circulation. When the resistance increases, either the pressure increases to maintain the flow, or the flow rate is reduced to maintain the pressure. Numerous factors can alter resistance, but the three most important are vessel length, vessel radius, and blood viscosity. With increasing length, increasing viscosity, and decreasing radius, resistance is increased. The arterioles and capillary networks are the main regions of the circulatory system that generate resistance, due the small calibre of their lumen. However, arterioles are able to rapidly alter resistance by altering their radius through vasodilation or vasoconstriction. The resistance offered by peripheral circulation is known as systemic vascular resistance (SVR), while the resistance offered by the vasculature of the lungs is known as pulmonary vascular resistance (PVR) (Ross and Pawlina, 2011).

1.2.1.2 Pressure

The pressure originates in the contraction of the heart, which forces blood out of the heart and into the blood vessels. If the flow is impaired through increased resistance then the blood pressure must increase to meet the blood supply of the tissues, therefore blood pressure is often used as a test for circulatory health. Blood pressure can be modulated through vascular reactivity like vasoconstriction, or vasodilation (Ross and Pawlina, 2011).

1.2.1.3 Flow

Flow is the movement of the blood around the circulatory system. A relatively constant flow is required by the body's tissues, so that the pressure and resistance are altered to maintain this consistency. A too-high flow can damage blood vessels and tissue, while a too-low flow may not serve the tissues with sufficient oxygen to function.

1.2.2 Renin-angiotensin pathway

The control of blood pressure is complex and time-dependent which involves the integration of numerous physiological factors that contribute to short-and long –term regulations. Past research studies show that the kidney play a major role in the long term regulation of arterial pressure and in the pathogenesis of hypertension. A pivotal part of the renal-blood feedback control system for long-term volume and pressure regulation is the renin-angiotensin-aldosterone system (RAAS).

As the name suggests, there are three crucial components to this system; renin, angiotensin and aldosterone. Stimulated by low blood pressure or certain nerve impulses (e.g. in stressful situations), the kidneys release an enzyme called renin. Renin stimulates the formation of angiotensin in blood and tissues, which in turn stimulates the release of aldosterone from the adrenal cortex. As renin is released into the blood, it acts upon angiotensinogen a circulating substrate and undergoes proteolytic cleavage to form decapeptide, angiotensin 1. Then the enzyme, angiotensin converting enzyme (ACE) mostly found in the vascular endothelium in the lungs, cleaves off to amino acids to form the octapeptide, angiotensin II (ANG II). Other tissues in the body such as heart, brain and vascular also produce ANG II. ANG II induce contraction through specifically activating phospholipase C (PLC)-mediated breakdown of phosphatidylinositol (4,5)-bisphosphate (PIP_2) to inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG), then increases intracellular calcium $[Ca^{2+}]_i$, by releasing from the endoplasmic reticulum and activates protein kinase C (PKC), respectively (Mashiko *et al.*, 1995). The PKC stimulates Ca^{2+}-ATPase activity in the sarcolemmal reticulum activation through phosphorylation and Ca^{2+} uptake into the vesicles (Fukuda *et al.,* 1990). ANG II not only causes blood vessels to narrow (vasoconstriction), it also simultaneously stimulates the secretion of the water-retaining hormone vasopressin in the pituitary gland (hypophysis) as well as the release of adrenaline, noradrenaline and aldosterone in the adrenal gland. Adrenaline and noradrenaline enhance vasoconstriction, while aldosterone influences the filtration function of the kidneys. The kidneys retain more sodium and water in the body and excrete more potassium. The vasopressin from the pituitary gland prevents the excretion of water without affecting the electrolytes sodium and potassium.

Angiotensin, aldosterone and vasopressin can also have a direct effect on the heart. Particularly in certain remodelling processes, for example after a heart attack, these hormones are involved in the abnormal enlargement of the heart or development of scar tissue, which can ultimately lead to heart failure (Goldsmith, 2006).

1.2.3 Autonomic nervous system

While short-term changes in blood pressure are regulated by sympathetic nervous system (SNS) and renin–angiotensin-aldosterone system (RAAS), the long-term blood pressure control is controlled by the kidney. High pressure baroreceptors (stretch sensing receptors) in the carotid sinus and aortic arch respond to elevations in systemic blood pressure by causing a reflex vagal bradycardia that is mediated through the parasympathetic systems and inhibition of sympathetic output from the central nervous system (CNS). Low pressure cardio pulmonary receptors in the atria and ventricles likewise respond to increases in atrial filling by causing tachycardia through inhibition of cardiac SNS, increasing atrial natriuretic peptide (ANP) release and inhibiting vasopressin release.

Besides baroreceptors, chemoreceptors are also located in the aortic carotic and aortic bodies. The play a role in monitoring the blood oxygen, carbon dioxide and pH. Impulses sent from the chemoreceptors are conducted to the control centres for the heart and the blood vessel via the gasopharyngeal and vagus nerves. Chemoreceptors in the medulla oblongata monitor blood carbon dioxide and pH. When there is decrease in oxygen (hypoxemia), increase in carbon dioxide (hypercapnia), or decrease in pH (acidosis), the sympathetic stimulation of the heart increases, while decreasing the parasympathetic stimulation of the heart. These changes stimulate chemoreceptor activity which leads to enhanced sympathetic outflow to the heart and vasculature via activation of the rostral ventrolateral medulla. Cerebral ischemia activates central chemoreceptors in a manner that produces simultaneous activation of sympathetic and vagal nerves to the cardiovascular system (Klabunde, 2012).

1.2.4 Endothelial derived factors

The endothelial derived factors like nitric oxide (NO) and endothelin are also known to regulate the blood pressure. NO is also called endothelium-derived relaxing factor (EDRF), is a free radical gas with a very short half-life. It is released in response to blood flow-induced shear stress and by activation of a number of receptors to cause vasodilatation. NO also has anti-proliferative, anti-thrombotic, leukocyte adhesion inhibition effects, and influences myocardial contractility (Chopra *et al.,* 2011). Endothelin on the other hand causes vasoconstriction. They are produced in the vascular endothelial cells. There are two different types of endothelin receptors which have been cloned, ET_A and ET_B. ET_A receptor activation leads to increased arterial pressure and sodium retention via increased sympathetic activity, positive inotropy of the heart, increased catecholamine release and, systemic vasoconstriction (Emoto *et al.,* 1995). Whilst, ET_B receptor activation leads to decreased arterial pressure and natriuresis through effects on adrenal gland, heart (negative inotropy), decreasing sympathetic activity and systemic vasodilatation.

1.2.5 Hormones

There are also various hormones involved in the blood pressure regulation mechanism such as the natriuretic peptides, vasopresin system, vasodilator peptides e.g adrenomedulin, Substance P, and calcitonin gene related peptide, tissue Kallikrein-Kinin system, phosducin, (stress-related hypertension), adipose tissue and adipokines, and leptin (linked to obesity) (Chopra *et al.,* 2011).

1.2.6 Other factors

Genetic factors likely play some role in high blood pressure, heart disease, and other related conditions. The risk for high blood pressure is higher when heredity combines with unhealthy lifestyle habits, such as smoking cigarettes and eating an unhealthy diet. Some characteristics like the age, race, or ethnicity can also be contributing risk factor for high blood pressure. The risk for high blood pressure increases with age. Women are about as likely as men to develop high blood pressure at some point during their lives. African origins are most likely to develop high blood pressure than Europeans, Hispanics, Asians, Pacific Islanders, American Indians, or Alaska Natives (Go *et al.,* 2014).

1.3 Pathogenesis of hypertension

Hypertension is a complex phenotype that can be caused by genetic, environmental, behavioural and social issues. Any derangement in the pathophysiological mechanisms of maintaining a normal blood pressure may play a part in the development of hypertension. The pathophysiological mechanisms involved are baroreflex and chemoreflex, renin-angiotensin-aldosterone system, autonomic nervous system and the involvement of other body-produced vasoactive substances that control the sodium transport and vascular tone, such as bradykinin, endothelin and atrial natriuretic peptide, endothelial dysfunction, genetics, salt intake, obesity and insulin resistance (Ross and Pawlina, 2011).

The maintenance of a normal blood pressure is dependent on the balance between the cardiac output and peripheral vascular resistance. Most patients with hypertension have a normal cardiac output but a raised peripheral resistance. Peripheral resistance is determined not by large arteries or the capillaries but by small arterioles, the walls of which contain smooth muscle cells (Beevers *et al.,*2001). Hypertensive patients experience an increase in vasoconstriction due to a greater sensitivity to vasoconstrictors, as compared to healthy subjects. Vasoconstriction of smooth muscle cells is mostly dependent on intracellular calcium concentration. Prolonged smooth muscle constriction is thought to induce structural changes with thickening of the arteriolar vessel walls possibly mediated by angiotensin, leading to an irreversible rise in peripheral resistance (Beevers *et al.,*2001). Vascular remodelling also happens when there is interaction between the autonomic nervous system and the renin-angiotensin system, together with other factors, including sodium, circulating volume, and some of the more recently described hormones (**section 1.2.5**).

Hypertension is often associated with metabolic abnormalities such as diabetes and dyslipidemia. It is estimated that 30-60% of diabetic patients have associated hypertension (Baradaran *et al.,*2014). It is estimated that at least 50% of hypertensive patients are insulin resistant (Manrique *et al.,* 2009). Insulin exerts important biological effects on cardiovascular tissue as well conventional tissues such as skeletal muscle and adipose tissue. Insulin and its homologous autocrine/paracrine peptide Ins-like growth factor (IGF-1) induce vasorelaxation by mechanisms that include stimulation of nitric oxide (NO) production and reductions in vascular smooth muscle cell (VSMC) intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and Ca^{2+}-myosin light chain (MLC) sensitization (Sowers, 2004). IGF-1, is produced by the vascular smooth muscle cells and cardiomyocytes in response to ANG II and other growth factors and mechanical forces such as stretch. The effect of insulin and IGF-1 are mediated by activation of the phosphatidylinositol 3-kinase (PI3K) and downstream signalling pathways, including

protein kinase B (Akt). The activation of PI3K/Akt signalling lead to vasorelaxation mediated by endothelial NO (Walsh *et al.,* 1996) and reduces VSMC intracellular $[Ca^{2+}]_i$ and Ca^{2+}-MLC sensitization (Sandu *et al.,* 2001). However, genetic predisposition leads to insulin resistance and hypertension. Furthermore, ANG II inhibits insulin and IGF-1 signalling through the PI3K/Akt pathway resulting in inhibition of mechanisms involved in the vasodilator and glucose transport properties of insulin and IGF-1 (Begum *et al.,* 2000). The mechanisms mediated by ANG II include increase in the generation of reactive oxygen species (ROS) and the activation of low-molecular-weight G proteins such as RhoA (Berry *et al.,* 2000). The consequent generation of ROS and RhoA inhibit actions mediated through PI3K/Akt signaling including activation of endothelial NO synthase activity, Na^+ pump activation, and Ca^{2+}-MLC desensitization thus leading to high blood pressure.

On the other hand, the prevalence of the co-existence of hypertension and dyslipidemia is in the range of 15-31% (Muthusamy, 2016). At which the gradual increase in blood pressure is associated with increase in blood lipid level (Otsuka *et al.,* 2016). The possible explanation for this relationship is that hypertension and dyslipidemia share common pathophysiological etiologies (McGill *et al.,* 2009). There are possibly several pathophysiological mechanisms involved in the association between dyslipidemia and increased risk of hypertension. First, dyslipidemia tend to impair endothelial function which may consequently disrupt production of NO. Second, dyslipidemia may also predispose individuals to development of hypertension by reducing the baroreflex sensitivity which in turn activates the parasympathetic nervous system to counteract any changes in blood pressure. Third, dyslipidemia decreases the distensibility of large elastic arteries and also adversely affects the functional and structural arterial properties, promoting raised blood pressure and eventually atherosclerosis. Finally, physical inactivity and a high-fat diet promote obesity and dyslipidemia. In obese individuals, adipose tissue excessively secretes adipocytokines, e.g leptin, thereby inducing insulin resistance and subsequent activation of the sympathetic nervous system and the renin-angiotensin system thus leading to hypertension (McGill *et al.,* 2009).

It has been hypothesized that oxidative stress is a key player in the pathogenesis of hypertension (Montezano *et al.,* 2012). Oxidative stress occurs when there is an imbalance between the generation of reactive oxygen species (ROS) and the antioxidant defence systems. Therefore it is important to maintain the balance between formation and the removal of ROS. Physiologically, ROS regulate vascular function through redox-sensitive signalling pathways. Hypertensive stimuli, such as high salt and ANG II promote the production of ROS in the brain, the kidney, and the vasculature and that each of these sites contributes either to hypertension or to the sequelae of this disease (Harrison and Gongora, 2009). The main

source of ROS is nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Several other enzymes including NO synthase, xanthine oxidase, and mitochondrial enzymes may also contribute to ROS generation. NADPH promote the formation of oxidant superoxide anion ($.O^{2-}$) that later reacts with nitric oxide (NO) in forming oxidant peroxynitrite ($ONOO^{-}$) leading to impaired endothelium dependent vasodilation, vascular lesion and remodelling, thrombosis and inflammation (Baradaran *et al.*,2014). ANG II, not only act as a potent activator of NADPH oxidase, but it also upregulates the activity of superoxide dismutase, possibly to compensate the increased levels of ROS. Due to detrimental effects of effect of long term ANG II infusion, angiotensin converting enzyme inhibitors (ACEIs) and angiotensin II receptor blockers (ARBs) have become the main therapeutic target of hypertension.

Inflammation is well-documented to contribute to vascular remodelling and consequent hypertension (Vazquez-Prieto *et al.,* 2011). Nuclear Factor kappa B (NF-κB), a transcription factor, is known to partake in the pathology of hypertension. It induces endothelial cell dysfunction, oxidative stress, and inflammation (Kang *et al.,* 2009) through the release of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) (Kang *et al.,* 2009) and ANG II.

It has long been known that genetic factors play a major role in determining an individual's propensity to hypertension. However, the genetics of hypertension is complex with no known single gene playing a major role, but rather many genes each with mild effects reacting to different environmental stimuli contribute to blood pressure. In recent years, the field of molecular genetics has revolutionized the study of hypertension by identifying single gene syndromes or Mendelian forms and several candidates of genes that lead to the alteration of blood pressure. The heritable component of blood pressure has been documented in familial and twin studies suggesting that 30%-50% of the variance of blood pressure readings are attributable to genetic heritability and about 50% to environmental factors (Butler, 2010). Albeit 30 new blood pressure or hypertension-associated genetic variants have been identified in the general population, the variants in RBM47, COL21A1 and RRAS have larger effects (>1.5 mm Hg/allele) than the other common variants (Surendran *et al.,* 2016). RBM47 is a gene that encodes for a protein responsible for modifying RNA while the RRAS is involved in cell cycle processes. The gene COL21A1 is involved in collagen formation in many tissues, including the heart and aorta. Both COL21A1 and RRAS warrant particular interest since both are involved in blood vessel remodelling, with relevance to hypertension. Besides that, another gene named ENPEP have also been identified. This gene codes for an enzyme that is a key molecule involved in regulating blood pressure through the dilation and constriction of blood vessels, therefore mutation in the key molecule ENPEP tend to affect the blood pressure

(Surendran *et al.,* 2016). In regard to this, large scale genetic studies should continue, to expand the number of genes that may contribute to the development of cardiovascular diseases, identifying risk factors of high blood pressure and to possibly provide valuable clues for new drug targets.

Hypertension may be categorized as either essential or secondary. Primary (essential) hypertension is diagnosed in the absence of an identifiable etiology / secondary cause. Approximately 90-95% of adults with hypertension have primary hypertension whereas secondary hypertension accounts for around 5-10% of the cases (Hajjar *et al.,* 2003). Secondary hypertension is less common and can be caused by conditions that affect the kidney, arteries, heart and endocrine system like diabetic nephropathy, polycystic kidney disease, glomerular disease, renovascular hypertension, Cushing syndrome, aldosteronism, pheochromocytoma, thyroid problems, obesity, sleep apnea and pregnancy (Poulter *et al.,* 2015). Secondary forms of hypertension, account for 20% of resistant hypertension (hypertension in which BP is >140/90 mm Hg despite the use of medications from 3 or more drug classes).

1.4 Antihypertensive therapy

The aim of antihypertensive drug therapy is to reduce the patients' blood pressure below 140/90 mmHg. However, a target blood pressure below 130/85 mmHg should be considered for those with established atherosclerotic cardiovascular disease, diabetes or chronic renal failure. Even though the goal blood pressure of 130/85 mm Hg is relatively arbitrary, it is also recognized by other organizations, such as the Canadian Hypertension Society, as a reasonable goal to maximally preserve function and reduce CVD morbidity and mortality (Sowers *et al.,* 2001).

There are several categories of drugs to treat high blood pressure in the market such as angiotensin-converting enzymes inhibitors (ACEIs), angiotensin II receptor blockers (ARBs), thiazide, calcium channel blockers, beta-blockers, and renin inhibitors. The category of medication prescribed depends on the blood pressure measurements and other medical problems and if targeted blood pressure is not achieved then additional drugs are added with the combinations mentioned above. For instance, alpha-blockers, alpha-beta blockers, central-acting agents, vasodilators, and aldosterone antagonists (NICE guidance, 2011).

Antihypertensive drug treatment is mainly offered to the following; 1) people aged under 80 years with stage 1 hypertension who have one or more of the conditions like target organ damage, established CVD, renal disease, diabetes, and a 10-year cardiovascular risk equivalent to 20% or greater. 2) people of any age with stage 2 hypertension 3) people under 40 years with stage 1 hypertension and no evidence of target organ damage, cardiovascular disease, renal disease or diabetes, however they are encouraged to seek specialist evaluation of secondary causes of hypertension e.g. lifestyle interventions and a more detailed assessment of potential target organ damage first, before initiating treatments (NICE guidance, 2011).

A single antihypertensive is often inadequate in the management of hypertension, therefore an additional antihypertensive drugs are usually added in a step-wise manner until control is achieved. The choice of medication depends on the age and the ethnicity background of the patients as these factors affect the response to drug treatment. For instance, the ACEIs and ARBs are more effective in younger Caucasians, whilst calcium channel blockers work better in Afro-Caribbean patients and patients above 55 years old. The initial treatment for essential hypertension, based on the recommendation by NICE guidance (2011) is summarised in a chart below (see **Figure 1.1**). The summary of antihypertensive drug classes used in treating hypertension is provided in the chart below (see **Figure 1.2**) and the details of each antihypertensive classes is discussed further in the subtopics below.

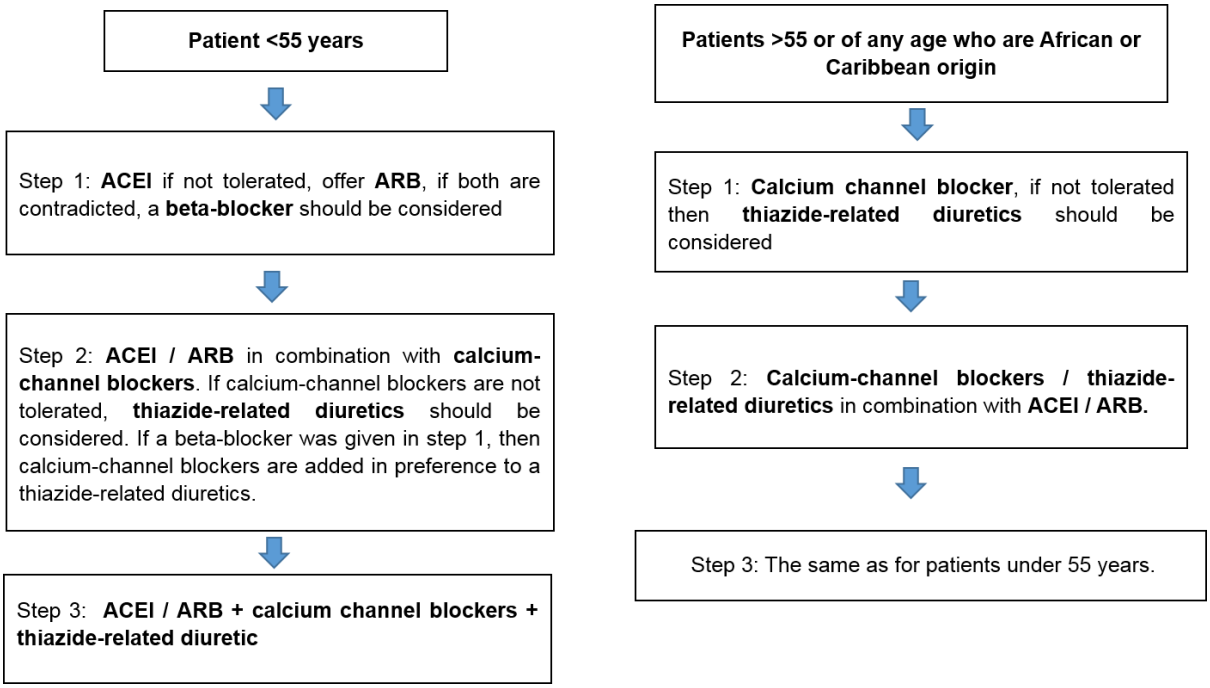


Figure 1.1: Summary of drug treatment steps for patients under 55 years and over 55 years/ African Caribbean origin [Joint Formulary Committee (2018, p 109)]

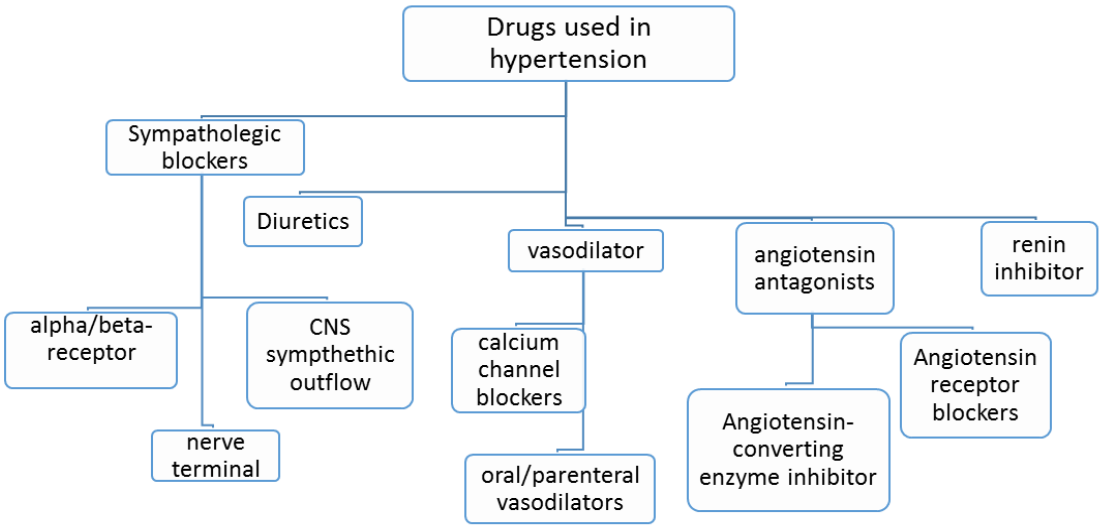


Figure 1.2: Summary of drug classes used and their role in the management of hypertension (Joint Formulary Committee, 2018).

1.4.1 Angiotensin-converting enzyme inhibitor (ACEI) and Angiotensin receptor blockers (ARB)

The ACEIs reduce blood pressure by blocking the renin-angiotensin system by preventing conversion of angiotensin I to the blood pressure raising hormone angiotensin II (ANG II). They also increase availability of the vasodilator bradykinin by blocking its breakdown. Excess bradykinin cause dry cough as a side effect of this medication. Some examples of ACEI are captopril, enalapril, fosinopril, imidapril, lisinopril, moexpril, perindopril, quinapril, ramipril, and trandolapril. Besides lowering blood pressure these medications can also slow the progression of kidney disease and atherosclerosis.

ARBs, like ACEIs, antagonize the renin-angiotensin system. They reduce blood pressure by blocking the action of hormones ANG II on angiotensin-1 receptors type 1 (AT-1), thus preventing the vasoconstrictor effects of the hormones on blood vessels. These drugs have the same benefits on cardiovascular and renal outcomes as ACEIs. However, ACEI and ARBs should not be given together as each of these drug is beneficial in its own way in patients with kidney disease, but in combination they may have significant adverse effects on kidney functions. Some examples of ARBs include azilsartan, candesartan, eprosartan, irbesartan, losartan, olmesartan, telmisartan and valsartan.

Despite these two classes of antihypertensive agents acting on renin-angiotensin aldosterone system (RAAS), most guidelines for the management of patients with cardiovascular disease recommend ACEIs as first choice therapy, whereas ARBs are merely considered an alternative for ACEIs-intolerant patients. This is because the results of past clinical trials and meta-analyses indicated that the treatment with ACE inhibition, but not with ARBs, leads to a statistically significant further reduction in mortality in hypertensive patients (Ruschitzka and Taddei, 2012). However, in a recent study by (Messerli *et al.,* 2018) have shown that ARBs and ACEIs have equal outcome efficacy but lower adverse events with ARBs than with ACEIs.

1.4.2 Calcium channel blockers (CCB)

In last 20 years, the CCBs have been one of the most widely used classes of antihypertensive agents, based on their effectiveness in reducing BP levels, good tolerability, and abundant evidence on reducing cardiovascular and renal consequences of hypertension (Tocci *et al.,* 2014). CCBs inhibit the flow of extracellular calcium through voltage-gated L-type calcium channels, which are responsible for the constriction of blood vessel smooth muscle and cardiac muscles and aldosterone secretion from the adrenal cortex in humans. When calcium influx is inhibited, the vascular smooth muscle relax, resulting in vasodilation and blood pressure reduction. CCBs also reduce the contractility of the cardiac muscles and decelerate sinus pacing and atrioventricular conduction (Abernethy and Schwartz, 1999). There are two major subtypes of CCBs that bind to separate sites on the L-type calcium channel: dihydropyridines (e.g., nifedipine and amlodipine) and nondihydropyridines (e.g., diltiazem and verapamil) (Materson, 1995). All CCBs are vasodilators that exert BP-lowering effects across all patient groups, regardless of sex, ethnicity, age, and dietary sodium intake *(*Lin *et al.,* 2017). Second-generation CCBs, including manidipine, felodipine, and nicardipine, and third-generation CCBs, including lacidipine, lercanidipine, barnidipine, and amlodipine, show more selective interaction at vascular calcium channels with favourable pharmacokinetic and pharmacodynamic profile (Tocci *et al.*, 2014).

1.4.3 Diuretics

Diuretics are the second most commonly prescribed class of antihypertensive medication. There are four classes of diuretics, which include thiazides, thiazides-related, potassium sparing and loop diuretics. Among all, the thiazide-related diuretics have increased at a rate greater than that of antihypertensive medications as a whole (Roush and Sica, 2016). These agents work by reducing plasma volume by increasing excretion of sodium and water. Initially, this effect reduces blood pressure by decreasing carbon monoxide (CO). With time, CO and extracellular fluid volume return toward normal values, but the hypotensive effect persists because of a reduction in peripheral resistance which accounts for the vasodilation.

Some examples of most commonly used thiazide-related diuretics are chlorthalidone and indapamide. The main side effects of these drugs are metabolic (hypokalemia, hyperglycemia, and hyperuricemia). The likelihood of these problems can be reduced by using low doses. In large-scale clinical studies, the ability to reduce cardiovascular events is well documented for chlorthalidone, indapamide, hydrochlorothiazide, amiloride and triamterene (potassium-sparing diuretics). In the context of congestive heart failure and end-stage renal disease, spironolactone seems to be a better option (Roush and Sica, 2016). Besides that, loop diuretics, such as furosemide, bumetanide and torsemide are also used as antihypertensive drugs. In general, loop diuretics are less effective than thiazide-type drugs in reducing BP in the nonedematous patient, however it is more likely to be prescribed to patients with chronic kidney disease transitions from stage 3-5 (Musini *et al.,* 2015) and as adjunctive agents in refractory hypertension. Diuretics are effective in reducing blood pressure when combined with ACEIs or ARBs, or with CCBs.

1.4.4 Beta-blockers

Beta-blockers are also known as β-adrenergic receptor blockers. They act by inhibiting the activity of the β-adrenergic receptor and causing vascular relaxation. They also reduce cardiac output and decrease the release of renin from the kidney. Some recent guidelines consider beta-blocker as an inferior option for first-step treatment of hypertension due to its inefficacy and undesirable side effects. Beta-blockers appear to be to be best suited for patients with sympathetically driven, neurogenic, hypertension, in patients with labile hypertension or as an add-on drug in patients with resistant hypertension (Mann, 2017).

The principal adrenoceptors present in the human cardiovascular system are the β_1, β_2, and α_1-receptors (Bakris *et al.,* 2010). A variety of beta-blockers are prescribed according to the physiological conditions, for example drug tolerance and other health complexities in patients. These include atenolol, metoprolol, propranolol, bisoprolol, nebivolol and carvedilol. Beta-blockers vary in their β_1 / β_2-adrenoceptor selectivity and vasodilatory properties, and this diversity has given rise to their classification into first, second, and third generation. First-generation beta-blockers exercise identical affinity for β_1 and β_2 receptors and are thus classified as non-selective beta-blockers (e.g. propranolol). Second-generation beta-blockers are more attracted to β_1 than β_2 receptors, and are thus termed selective beta-blockers (e.g. atenolol, metoprolol, bisoprolol). The third-generation of beta-blockers are known for their intrinsic vasodilatory properties (e.g. nebivolol and carvedilol) (Wiysonge *et al.*, 2017). Beta-blockers should not be prescribed to patients who are at risk for diabetes as this drug has adverse effects on glucose metabolism especially in combination with diuretics. However, newer generation beta-blockers like carvedilol and nebivolol have been shown not only to be better tolerated than other beta-blockers but also these agents do not increase the risk of diabetes mellitus (DM), atherogenic dyslipidaemia or weight gain. Moreover, carvedilol has the most evidence for reducing morbidity and mortality in patients with heart failure and those who have experienced an acute myocardial infarction (DiNicolantonio *et al.,* 2015).

1.4.5 Other antihypertensive agents

In addition to the commonly prescribed hypertensive drugs mentioned above, other hypertensive drug classes such as α-adreneoceptor blockers, vasodilators and centrally acting agents are also prescribed. The α-adrenoceptor blockers reduce blood pressure by blocking arterial α -adrenoceptors, thus preventing the vasoconstrictor actions of these receptors. These drugs are less widely used as first-step agents than other classes because clinical outcome benefits have not been as well established as with other agents. However, they can be useful in treating resistant hypertension when used in combination with agents such as diuretics, beta-blockers, and ACEIs. Some examples of prescribed α-blockers are doxazosin, indoramin, prazosin and terazosin (British Hypertension Society, 2011). Some α-blockers are used in the short-term management of hypertension episodes in phaeochromocytoma. For instance, phenoxybenzamine, a powerful α-adrenergic blocker is effective in the management of phaeochromocytoma. Pheochromocytoma is a rare tumor of adrenal gland tissue. It results in the release of excessive phenylephrine and norepinephrine, hormones that control the heart rate, metabolism, and blood pressure. On the other hand, vasodilators like hydralazine, sodium nitroprusside (SNP), and minoxidil are often reserved for the treatment of severe hypertension resistant to other drugs. They are normally prescribed in combination with a beta-

blocker and a thiazide due to the unpleasant side effects like tachycardia and fluid retention when they're used alone (British Joint Formulary, 2018). Some centrally acting antihypertensive drugs are also licensed to treat moderate to essential hypertension when other drugs have failed to control blood pressure. Some examples include, methyldopa, clonide, and monoxidine. Besides these drugs, there is also a renin inhibiting drug that can directly inhibit renin. Aliskiren is licensed for the treatment of hypertension, either alone, or in combination with other antihypertensives but combination treatment with ACEIs or ARBs is not recommended.

1.5 Resistant hypertension

Resistant hypertension (RH) is defined as blood pressure that remains above goal (≥140/90 mm Hg), despite concurrent use of three antihypertensive agents of different classes, one of which should be a diuretic (Carey *et al.,* 2018). Patients whose blood pressure is controlled with four or more medications are considered to have resistant hypertension. Despite improvements in hypertension awareness and treatment, 30%–60% of hypertensive patients do not achieve BP targets and subsequently remain at risk for target organ damage (Braam *et al.,* 2017). The diagnosis of RH requires a systemic approach. First, the assurance of antihypertensive medication adherence and exclusion of the "white-coat effect" (office BP above goal but out-of-office BP at or below target) should be confirmed and validated. The next step would be to assess the adherence of RH patients to dietary and lifestyle modifications. The patients are also evaluated for potential secondary causes of hypertension such as chronic kidney disease, sleep apnea, tumours or disease in the adrenal gland, pregnancy, alcohol addiction and use of birth control pills.

The exact cause of RH is not very clear. Current evidence from a systematic review and meta-analysis of 24 studies estimates the prevalence of resistant hypertension to be between 14% and 16% of all patients with hypertension, equalling 140-160 million people globally (Achelrod *et al.,* 2015). There are believed to be many contributing factors to RH, such as age and sex, poor drug adherence, poor lifestyle and dietary habits, technical and systematic error in measuring blood pressure, affordability of drugs and lack of awareness. Those who are aged more than 70 years are more likely to develop incident resistant hypertension than those aged 65-69 years. Generally, men are more likely to develop RH than women (Achelrod *et al.,* 2015). The non-adherence to antihypertensive medication is very common among patients with RH. However, non-adherence or poor adherence to antihypertensive medication should be considered as a cause of pseudoresistance rather than a risk factor for RH (Judd *et al.,* 2014). Moreover, RH is also characterised by multiple side effects subsequent to intake of

many drugs and sometimes the cost of its treatment may be prohibitive, especially in economically deprived countries. Therefore, the patients may become less and less compliant over time. Consequently, leading to reduce non-adherence and uncontrolled blood pressure. There is evidence that RH prevalence is higher among patients with diabetes, obese patients, those with renal insufficiency, excessive intake of dietary salt and smoking (Nansseu *et al.*, 2016). Besides that, clinicians and physicians especially in developing countries lack regional guidelines and policies, therefore leading to the diagnosis of apparent-RH rather than true-RH.

Early recognition of RH using a standardized definition and adoption of a consistent approach to evaluation and management may increase the chance of successful implementation of therapeutic approaches that combine medical treatment and lifestyle changes to prevent adverse outcomes. Therefore, clinicians and researchers must bound to rely on international guidelines in conducting ambulatory blood pressure measurement (ABPM) to exclude pseudo-RH, harmonise and standardise the definition of RH for a better reporting and pooling of its related patterns. Future research focusing on improving understanding of the intrinsic (physiological and psychological factors) and extrinsic (environmental stressors) mechanisms that contribute to a lack of response to blood-pressure-lowering drugs in adherent patients is warranted.

1.6 Drug discovery

As mentioned in the above section (**section 1.5**), the identification of broader mechanisms of treatment resistance is lacking, particularly attempts to elucidate potential genetic causes of resistant hypertension. Therefore, this necessitate us to expand our understanding on the causes of resistant hypertension and thereby potentially allowing for more effective prevention and/or discovery new pharmacological treatments to improve the long-term clinical management of this disorder.

The pipeline of discovery takes about 10-15 years to accomplish. It begins from early drug discovery which involves the target identification to 'HIT' compound, then to lead compound, then preclinical development, Investigational New Drug (IND) filing and finally clinical development, New Drug Application (NDA) filing and eventually the Food and Drug Administration (FDA) filing (Hughes *et al.,*2011) (see **Figure 1.3**). In each phase, numerous evaluations are undertaken to confirm the therapeutic activity and safety of the potential drug.

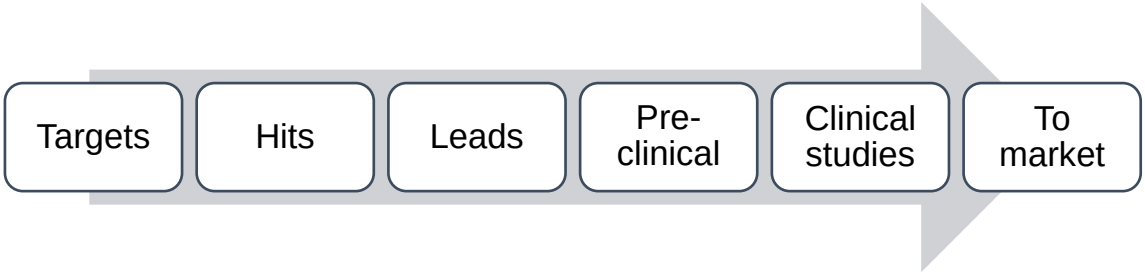


Figure 1.3: Streamline of drug discovery process from target identification to target validation and pre-clinical drug development.

Initially, thousands of compounds are typically examined and they come from all over, from natural compounds that already exist, to synthetic compounds synthesised in the labs, to bioengineering. This process is called the discovery phase. A few essential steps such as the target identification and validation, compound screening, secondary assays and finally the *in-vivo* analysis are undertaken. Through a long and difficult process of elimination process which could take as long as 6 years, to obtain a single best and promising candidate for further development ('hit' compound). The 'hit' compound is defined as the compound with desired and confirmed activity after compound screening and retesting (reviewed by Hughes *et al.,* 2011). There are some common compound screening assays, including high-throughput screening, focussed screening, fragment screening, virtual screening, physiological screening and nuclear magnetic resonance (NMR) screening. Before testing the 'hit' compound in people, researchers must find out toxicity profile of the newly discovered drug through preclinical research such as *in-vitro* and *in-vivo* studies using animal models. These steps are crucial before proceeding with the preclinical development phase to identify the following;

- Pharmacokinetics and pharmacodynamics of the drug
- The absorption, distribution, metabolisation, and excretion (ACME) profile of the drug.
- Its potential benefits in groups of people (e.g, by gender, race or ethnicity), mechanisms of action and suitable dosage
- The best way for administration of the drug (such as by mouth or injection).
- Side effects or adverse events that can often be referred to as toxicity.
- The effectiveness of the new drug as compared with similar drugs

The next phase is called the pre-clinical trials. Usually, the pre-clinical studies are not very large. However, these studies must provide detailed information on dosing and toxicity levels. After the preclinical testing, researchers review their findings and decide whether the drug should be tested in people. While preclinical research answers basic questions about a drug's safety in animal model, clinical research / trials are done in humans. This is important in understanding the ways of the drug interact in the human body. Before the clinical research begins, a developer have to consider developing a specific objective and outcome they want to accomplish for each of the different Clinical Research Phases and begin the Investigational New Drug Process (IND). The clinical trials follow a typical series from early, small-scale, Phase 1 studies to late-stage, large scale, Phase 3 studies. After the trial ends, researchers must submit study reports. This process continues until the developer decides to end clinical trials or files a marketing application. Before filing a marketing application, a developer must have adequate data from two large controlled clinical trials.

Despite the global burden of cardiovascular disease, investment in cardiovascular drug development has stagnated over the past 2 decades, with relative underinvestment compared with other therapeutic areas (Fordyce *et al.,* 2015). The growing rate of obesity and diabetes, and an aging population has led to increased demand for new antihypertensive compounds. Moreover, there are no drugs as of yet to treat oxidative stress and genetic factors that cause hypertension. Therefore, it is essential to develop new therapies or specific biomarkers to treat hypertension.

1.7 Natural products as therapeutic agents

History of medication is as old as human civilization (Lahlou, 2013), dating back to 4000 years. The use of natural products as medication has been proven by ancient written documents originated from India, North Africa and China (Phillipson, 2001). Natural products are derived from plants, animals, microbial and marine sources for treatment of several diseases. Today, our modern medicine has been developed on the basis of scientific knowledge and observational attempts by scientists, which has been established majorly from ancient knowledge of our ancestors (Brahmachari, 2012).

Nature is a valuable reservoir of novel bioactive entities. According to an estimate, about 50% of the medications validated from 1981-2010 have natural origins, for instance, 28% semi-synthetic, 17% mimics of natural compounds and 5% natural entities. Besides that, natural products hold paramount potential in the discipline of cancer therapeutics. From 1940-2010, 175 anti-cancer drugs have been developed. Out of these, 48.6% drugs have either natural origins or derived from natural products (Gurnani *et al.,* 2014).

Metabolism is divided into two main categories in living organisms: primary and secondary metabolism. Primary metabolites include biological molecules like nucleic acids, fats, carbohydrates and proteins, essential for the survival and well-being of the organism. Secondary metabolism on the other hand involves biosynthesis of secondary metabolites (natural products). Generally, the extracts prepared from natural sources contain secondary metabolites. The major difference between primary and secondary metabolites lies in their biological effects. Primary metabolites exert their biological effect on cell or body of the organism. Whereas, secondary metabolites exert biological effect within the organism and also on other organisms (Jabeen *et al.,* 2014). These products can be harvested directly from microbial fermentation, tissue samples of plants and marine organisms or synthesized on an industrial scale (Lahlou, 2013). Approximately more than 300,000 secondary metabolites exist in nature (Gurnani *et al.,* 2014). The process of drug development from natural products is based on two strategies, chemically and biologically driven method. Chemically driven method is the assessment of biological activities of pure compounds while the biologically driven method is the bioassay-guided process which involves working with crude extracts.

1.7.1 Plant-based products as therapeutic agents

Plants species have been reported to have medicinal use since ancient times with better patient tolerance and acceptance. According to WHO, 80% of the world's population is committed to folk medicine derived from plants for primary health care (Mushtaq *et al.,* 2018). There are many famous plant-derived drugs in the market to treat various conditions such as morphine, apomorphine, aspirin, digitoxin, quinine, silymarin, artemisinin, and pilocarpine. The first natural and pure plant-derived medicine was morphine isolated from *Papaver somniferum* in 1803. In the 1870s, morphine in crude form was boiled in acetic anhydride, producing heroine which was readily converted into codeine, used as a painkiller. Apomorphine derived from morphine acts as an agonist of dopamine receptor (D_1 and D_2) which has been utilized for the treatment of Parkinson's disease. Another famous drug is aspirin, an anti-inflammatory agent isolated from the bark of *Salix alba*, introduced by Bayer in 1899.

Digitoxin, a potent cardiotonic glycoside was isolated from *Digitalis purpurea* and is used for the treatment of congestive heart failure. An antimalarial drug named as Quinine was obtained from *Cinchona succirubra* bark. Quinine is a drug of choice for numerous ailments including malaria, dyspepsia, fever, mouth and throat diseases, and cancer (Deleu *et al.,* 2004; Dias *et al.,* 2012). Silymarin isolated from *Silybum marianum*, is used for the treatment of liver disorders. Silymarin is also reported to exhibit anticancer, antiviral, anti-fibrotic, antioxidant, immunomodulatory and anti-inflammatory potential (Abbasi *et al.,* 2016). Artemisinin produced from *Artemisia Annua* is a treatment option for multidrug-resistant malaria. Traditionally, artemisinin has also been used as an antiseptic, antidiabetic, antispasmodic, digestive, choleretic, diuretic, trematocidal, anti-helminthic and depurative agent (Ali *et al.,* 2017). Another important drug, pilocarpine isolated from *Pilocarpus jaborandi* has been used to treat glaucoma and in the management of xerostomia and Sjogren's syndrome (Dias *et al.*, 2012).

1.7.2 Plant-based products to treat hypertension

Side-effects from current antihypertensive drugs have motivated researchers to find new medicines in metabolites or extracts from medicinal plants to control hypertension and that cause fewer side effects (Popovi *et al.,*2016). There are number of different plants that have been reported to exhibit antihypertensive properties, some have clinical and scientific evidence of their mechanism of action but some do not. The pharmacopeia includes aspirin (from *Salix alba*), lovastatin (from *Monascus purpureus*), reserpine (from *Rauwolfia serpentina*), and taxol (from *Taxus brevifolia*) (Frishman *et al.,* 2009; Al Disi *et al.,* 2016). Reserpine (which depletes adrenergic neurotransmitters) still remains an effective treatment for hypertension (Weber *et al.,* 2014). Many other bioactive metabolites from plants also have been reported such as inhibition of ACE by *Passiflora edulis* (Rawat *et al.,*2016), blockade of receptor operated and voltage dependent calcium channels by *Achillea wilhelmsii* (Niazmand *et al.,*2014), activation of β_2-adrenoceptors by *Crocus Sativu* (Llorens *et al.,* 2015), and inhibition of α_1-adrenoceptors by *Viscum álbum* (Poruthukaren *et al.,*2014).

As aforementioned in **section 1.7**, the secondary metabolites possess sufficient structural complexity and exhibit a broad spectrum of bioactivities including antihypertensive activity. Secondary metabolites are mostly small organic molecules produced in plants which are not essential for its growth, development or reproduction. They can be classified based on the pathway by which they are synthesized. A simple classification includes three main groups, terpenoids (polymeric isoprene derivatives and biosynthesized from acetate via the mevalonic acid pathway), phenolics (biosynthesized from shikimate pathways, containing one or more hydroxylated aromatic rings) and the extremely diverse alkaloids (non-protein, nitrogen-containing compounds, biosynthesized from amino acids such as tyrosine, with a long history in medication (Seca and Pinto, 2018). There are number of medicinal plants whose antihypertensive / vasorelaxant effects have been scientifically validated using *in-vitro* method. They belong to a broad spectrum of plant families used in folk medicine worldwide where phenols are pointed as the major constituents followed by alkaloids and terpenes (Maione *et al.,* 2013).

The alkaloids can be divided into several groups based on their chemical structures. Examples of plant alkaloids include phenylalkylamine, piperadines, pyrrolidines, pyridines, tropane, quinoline, indole, purine, tropolone, isoprenoid, and diterpenoid. The alkaloids groups are used as therapeutic agents and lead compounds due to their unique properties. The alkaloid molecule can act, depending on a type of amine functionality present in alkaloids, as either hydrogen-acceptor or hydrogen-donor for hydrogen bonding which is imperative for the interaction (binding) between targets e.g. enzymes, proteins and receptors and drugs (ligands). The mechanism of action of the alkaloids molecule include modulation of potassium channels, calcium channel inhibition, inhibition of ACE, and α-adrenoceptors inhibition (Maione *et al.,* 2013). Some examples of alkaloid identified with scientific evidences include *Annona cherimolia*, *Cassytha filiformis*, *Corydalis racemosa Pers*, *Evodia rutaecarpa*, *Fritillaria usuriensis*, *Illigera luzonensis*, *Ligusticum wallichii*, *Lindera megaphylla*, *Magnolia fargesii*, *Mahonia aquifolium*, *Platycapnos spicatus*, *Stephania glabra*, *Stephania tetrandra, Uncaria rhynchophylla*, and *Verbesina caracasana* (Maione *et al.,* 2013).

1.7.3 Plant-based products advantages and disadvantages

Usage of botanical sources as starting point in the drug development program is associated with advantages and also disadvantages. Some of the advantages are, selection of a candidate species for investigations are mostly based on long-term use of plants / herbs by humans (ethnomedicine). This is due to an assumption that the active compounds isolated from such plants are likely to be safer than those derived from plant species with no history of human use. For instance, drugs like reserpine and digitoxin are developed from *Rauwolfia serpentine* and *Digitalis purpurea*, respectively, which have been commonly used in the past to treat hypertension. However, such approaches sometimes lead to un-selectivity of active compound at the targets, unwanted side-effects and toxicity. For instance, reserpine causes adverse side effects like depression, Parkinson's disease, dizziness, nightmare, nasal congestion, headache, diarrhoea, abdominal pain, inattention and suicidal thoughts, that reserpine have been discontinued in some foreign countries. Such limitations could be overcome to a great extent by semi-synthesis or synthesis of synthetic compounds that has similar pharmacophore to the original compounds. For example, podophyllin derived from *Podophyllum hexandrum* was faced with dose-limiting toxicities and camptothecin (originally isolated from *Camptotheca* species and subsequently from *Mappia* species led to development of novel anticancer molecules like topotecan and irinotecan (Katiyar *et al.,*2012). All in all, natural resources as starting point has a bilateral promise in providing lead compounds and foundation for alternative/synthetic drug development due to its increased chemical diversity.

Beside a lot of advantages, drug development from natural resources is also associated with certain disadvantages. Drug discovery and eventual commercialisation would pressurize the resource substantially and might lead to undesirable environmental concerns. As mentioned earlier, not every molecule from the plants are amenable for complete synthesis, therefore the degree of dependence on the lead resource would continue to grow for further modifications or new synthesis. For instance, anticancer molecules like etoposide, paclitaxel, docetaxel, topotecan, and irinotecan continue to depend upon highly vulnerable plant resources for obtaining the starting material for a complete synthesis (Katiyar, *et al.,*2012). Sometimes, the full potential of natural plant compounds cannot be exploited due to their complexity and difficult to synthesise, some are found in low quantities and rare plants, and their synthesis is influenced by weather conditions. Moreover, the discovery and development of natural products is perceived as a slow process. The length of time required to obtain pure and active compounds from crude extract is longer. The factors like instability of compounds, difficult separations, and unreliability of bioassays may also impact the speed. Taken together,

although the natural compounds are mostly valuable lead compounds, seldom can they be directly used in clinical applications. Structural modifications are necessary. The aim of structural modification is to increase potency and selectivity, to improve physico-chemical, biochemical, and pharmacokinetic properties, to eliminate or reduce adverse effects, to simplify the structural complexity, including removal of redundant atoms and chirality while retaining activities, and to generate patentable and therapeutically beneficial compounds.

1.8 A local Fig plant: *Ficus Schwarzii*

In this study, *Ficus Schwarzii* is the plant of interest. The plant genus *Ficus*, consist of a group of about 900 species of trees, shrubs, and vines, commonly called figs. They are distributed throughout the world's tropical regions, particularly in tropical areas of East Asia (Thailand, Burma, Peninsular Malaysia, Sumatera, Borneo and Sulawesi). All *Ficus* species are members of the mulberry family known as the Moraceae. They can be found at altitudes up to 1900 m along rivers, streams, on hillside, on sandy to clay soil, and also on limestone. *Ficus schwarzii* is a fig tree that can grow up to 20 metres tall, though is usually much shorter with leafy twigs and lamina that are partly brownish, with the bole of approximately 29 cm in diameter and globose fig fruits in clusters up to 8 cm long along the stem (Berg and Culmsee, 2011), refer **Figure 1.4** a better view.

Figure 1.4: *Ficus Schwarzii* plant with leafy twigs and globous figs along the stem. Picture taken from (Amar Fitri, 2014, Ficus Schwarzii Koord, FRIM Flora Database).

The *Ficus* species are harvested in wild to be used as food source and medicines. Traditionally, the latex of *Ficus schwarzii* is used externally against ringworm infection (Asian Tropical Plant, 2014). To date only 3 species of *Ficus* are reported to produce alkaloids *Ficus hispida, Ficus fistulosa* and *Ficus septica*. Since there are 99 *Ficus* species in Malaysia, *Ficus* was regarded as a promising source of biologically useful alkaloids. Through continuous collection of *Ficus* plants for screening, our team have identified several other *Ficus* plants that contain alkaloids, amongst them is *Ficus schwarzii.*

In a recent study, alkaloids from *Ficus* species have been shown to possses anti-proliferative effect on human lung (A549), brain (U87MG) and colon (HT-29) cancer cell lines and have been proposed as future chemotherapeutic agents against cancers (Abubakar *et al*.,2015). Some *Ficus* species like *Ficus deltoidea* leaves extract are known to have good therapeutic properties such as antioxidant, anti-inflammatory and anti-diabetic (Abu Bakar *et al.,* 2018). The leaves extract of *Ficus schwarzii* yielded two novel tri-nor-sesquilignan alkaloids, schwarzinicines A and B. However, the therapeutic effects of *Ficus schwarzii* are yet to be discovered. According to a recent study, Schwarzinicine A (Sch A) causes relaxation of rat aorta possibly by blocking calcium channel entry, hence there is a potential to develop Schwarzinicine A as a new antihypertensive therapy (Loong *et al.,*2016 unpublished data).

1.8.1 Shortcomings and synthesis of synthetic novel compounds with similar pharmacophore as Schwarzinicine A

Due to low isolation yield from natural source, with only 0.0005% (50 mg from 10 kg of dried leaves), it was hard to conduct further experiments to postulate the exact mechanism of action of Sch A. Therefore, it prompted our team to synthesise synthetic compounds with similar pharmacophore to Sch A to overcome the problem and serve as an alternative compound to investigate the mechanism of action of the novel compound. N-phenethyl-1-phenyl-pentan-3-amine, (FK70) is a novel compound synthesised based on the main pharmacophore of the phenylethylamine structure (Lee Fong Kai, School of Pharmacy of University of Nottingham, UNM).

1.8.2 Similarities and differences between FK70 and Schwarzinicine A

As aforementioned in (**section 1.7.3**), structural modifications to novel compounds are necessary. Schwarzinicine A (Sch A) is a novel phenylethylamine alkaloid isolated from the leaves of *Ficus schwarzii* plant. The scientific name of this plant compound is N-(3,4-dimethoxyphenethyl)-1,4-bis(3,4-dimethoxyphenyl)butan-2-amine. The structural complexity of Sch A has been simplified by removal of redundant atoms and chirality while retaining activities and to increase potency and selectivity, which has led to the synthesis of new compound FK70 (see **Figure 1.5**). The structural similarities and differences between FK70 and Sch A are listed in detail in **Table 1.1**.

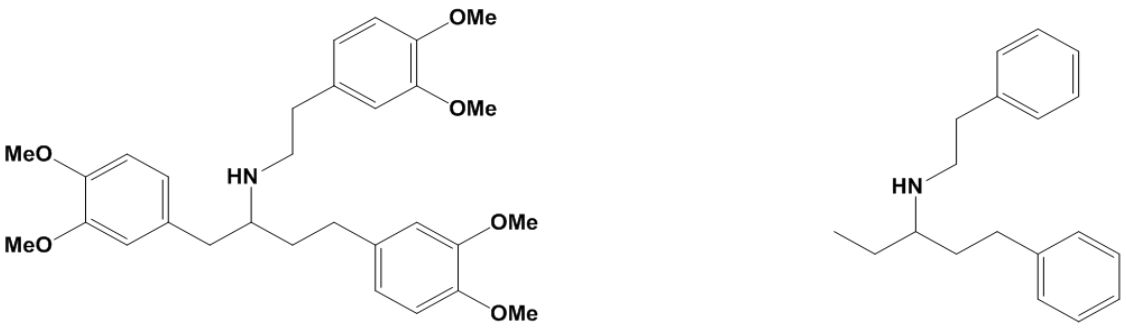
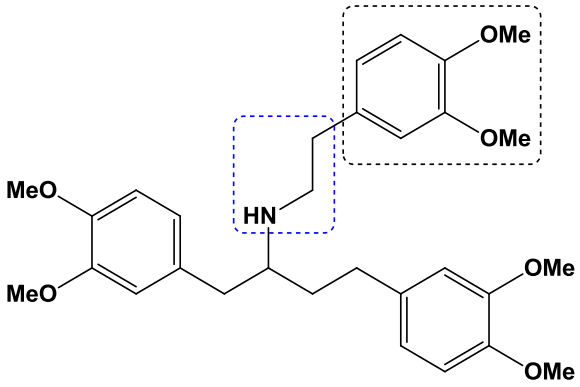
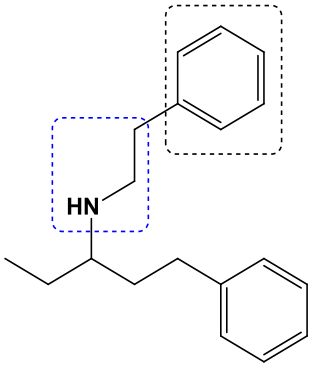


Figure 1.5: Chemical structures of Schwarzinicine A (left) and FK70 (right)

Table 1.1: List of similarities and differences between chemical structures of Sch A and FK70.



Schwarzinicine A



FK70

Similarities

- Both Sch A and FK70 are phenylethylamine. The blue box shows the ethylamine side chain (secondary amine) with dimethoxy groups for (Sch A) and and phenyl ring for FK70, hence they are called phenylethylamine.
- Both Sch A and FK70 are secondary amines.
- They both have aromatic phenyl rings.

Differences

Sch A	FK70
Sch A has 3 aromatic rings	FK70 has two aromatic rings
Presence of dimethoxy substituent at position 3 and 4 on phenyl rings.	Absence of dimethoxy substituent at position 3 and 4 on phenyl rings.
The molecular weight is 509.63	The molecular weight is 267.41

1.8.3 Pharmacological screening of FK70 as potential drug compound

As discussed in **section 1.6**, a typical drug discovery process include several steps from target identification to the licensing of the drug as therapeutics. Through basic scientific research we have identified that Sch A cause relaxation in the aortic smooth, however due to insufficiency of Sch A source, there was a need to synthesise compounds similar to Sch A with modified and improved features. After a primary screening (*in-vitro* studies), FK70 (best HIT compound) among all other hit compounds synthesised, showed better relaxation response in aortic smooth muscle. Furthermore, two common screening assays have been employed on FK70, functional assay and toxicity assay. The functional assay screening on FK70 was performed to determine the estimated values of IC_{50} (half maximal inhibitory concentration), EC_{50} (half maximal effective concentration) or K_i (inhibitory constant). These estimated values determine the potency of a drug. Whilst the toxicity assay determine the toxicity of the compound before it can developed as a lead compound. The two common screening employed will be discussed in detail in **section 1.8.3.1** and **1.8.3.2.** The selection of screening assays of FK70 was based on their pharmacological relevance, reproducibility, costs and quality. FK70 was dissolved in organic solvent, dimethyl sulfoxide, (DMSO).

1.8.3.1 Organ bath technique and functional studies of smooth muscle tissues

In-vitro isolated tissue functional assays have played an invaluable role in basic research and drug discovery for over 100 years. Organ bath assay measures concentration-dependent changes to isometric contraction and the efficacy and potency of contractile agonists (by manipulating concentrations of antagonists or inhibitors). This study allow the *in-vitro* study of contractile and relaxation of muscle in the target tissue and neural responses e.g. electrical stimulation and release of neurotransmitters can also be obtained. Besides that, it is also useful in measuring isometric tension development and changes in intracellular calcium (Tykocki *et al.,* 2013). Tissues or organs commonly used for this technique include smooth muscle [e.g vascular (arterial/aortic rings), ileum, Vas deferens, uterine tissue, and colon], skeletal muscle (e.g. abdominus rectus, biventer cervis, and diaphragm), and cardiac muscle (e.g atrium and ventricle).

The design of the organ bath apparatus is to replicate the physiological condition in an external environment. They are also more readily instrumented and can be easily subjected to controlled changes in perfusate, oxygen availability, and drug administration. The basic experimental design can also be extensively modified to allow for the recording of additional parameters or the introduction of other external stimuli. For instance, addition of electrodes allow for electric field stimulation of innervating nerves, addition of thermal or pH probes allow

the effects of temperature and pH on contractile responses to be measured and also the oxygen can be substituted partially or in full with nitrogen (N_2) to evaluate hypoxia induced effects (Jespersen *et al.,* 2015). Typically, tissue-organ baths are used for *in-vitro* dose response experiments, to investigate the physiology and pharmacology of tissue preparations (ring / vessel or strip) from various animal models such as rats, mice, pigs, toads, rabbits and guinea pigs.

The organ bath apparatus consists of a series of double layered glass baths that are connected to a water bath to maintain the glass baths temperature at 37 °C. The physiological salt solution, Krebs-Ringer bicarbonate solution is then filled into the bath and gassed with 95 % O_2 and 5% CO_2. The tissues of interest are mounted to a fixed rod and connected to an isometric force transducer (Loong *et al.,* 2015). Different mounting devices are used depending on the nature of tissues used, whether they are in the form of vessels or strips and the purpose of experiment. For neuropharmacology study, the tissues will be attached to an electrode rod to stimulate the connective nervous activity of the tissues. Recordings of their generated isometric forces are translated and interpreted using suitable computer software. The overview of an organ bath setup is illustrated in **Figure 1.6.**

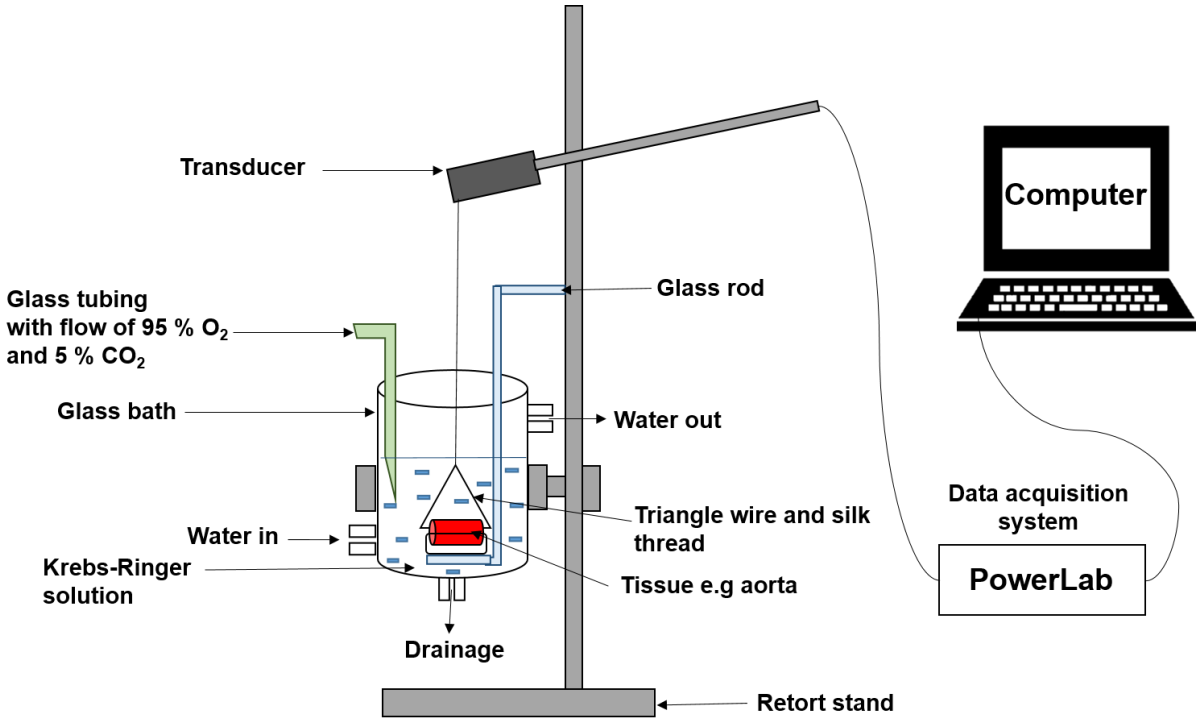


Figure 1.6: Schematic illustration of organ bath set up that is connected to an isometric transducer to a data acquisition system and to a computer.

One of the primary advantages of organ bath experiments is its straight forward, which allows us to observe the experiment as it unfolds and make rapid conclusions and plan the next necessary steps, as well as troubleshoot during an experiment. Furthermore, the water jacketed organ bath provides a stable and readily adjustable means of temperature control and constant oxygen delivery, which serve as the basic requirements for any isolated tissue preparation.

Generally, muscle contraction occurs when the tension-generating sites within muscle fibres are activated. It occurs as a result of sliding of two contractile protein filaments actin and myosin. The type of muscle contraction can be divided into three types, isotonic, isometric and isokinetic. Organ baths have been designed to accurately record isometric and isotonic contraction. The isotonic contractions cause the muscle to change in length as it contracts and can be further divided into two types; concentric and eccentric. Isometric contraction on the other hand, occur when there is no change in the length of the contracting muscle. The amount of force a muscle is able to produce during an isometric contraction depends on the length of the muscle at the point of contraction. That said, organ bath is a great pharmacological method to investigate the contractile/ relaxant ability of smooth muscle tissues, such as vascular, airway, bladder, gastrointestinal and intestinal smooth muscles. Vascular smooth muscle is of the main focus of this study, therefore a brief knowledge of the contractile and relaxation mechanisms is essential prior to conducting organ bath experiments. Layers of smooth muscle cells line the walls of various organs and vessels in the body, and the contractile function of smooth muscle is involuntary. Contractile activity in smooth muscle is initiated by calcium-calmodulin interaction to stimulate phosphorylation of the light chain of myosin. Calcium sensitization of the contractile proteins is signalled by the RhoA/Rho kinase pathway to inhibit the dephosphorylation of the light chain by myosin phosphatase, thus leading to contraction. Removal of calcium from the cytosol and stimulation of myosin phosphatase initiate the process of smooth muscle relaxation. The level of intracellular calcium depends on extracellular calcium entry and internal calcium store. These two pathways are discussed further under **section 1.9.**

During organ bath experiment, an initial tension is applied to the mounted tissues of interest depending on the length of cut tissues. The tension applied to a smooth muscle preparation at the start of an isometric in-vitro experiment (resting tension) is an important factor in determining subsequent responsiveness since this tension determines tissue length and the development of active force which increases with length until an optimum maximal response is achieved (Hulsmann and De Jongste, 1993). In order to study the relaxant activity of the tissues, the tissues must first be contracted by a contractile stimulus to establish a submaximal tension, followed by a concentration-response curve of the relaxant.

Depending on the design of experiments and animal models, there are some commonly used vasoconstrictors and vasodilators to stimulate the contraction and relaxation of vascular smooth muscles respectively. Some examples of contractile agents are norepinephrine, phenylephrine, endothelin-1 (ET-1), and U46619. Norepinephrine and phenylephrine are α_1-adrenoceptors agonists which act on G_q-coupled protein receptor to stimulate IP_3 and DAG signal transduction pathway to cause contraction. Endothelin-1 binds to endothelin receptors, of which there are two subtypes: ET_A and ET_B. These receptors are also coupled to G_q-protein, which leads to the stimulation of inositol triphosphate signal transduction pathway (Klabunde,2012). U46619 is a potent thromboxane A_2 (TXA_2) agonist. It acts on TXA_2 receptors which are coupled to G_q and $G_{12/13}$ proteins to stimulate vascular contraction via IP_3 signalling pathway or Rho-kinase-mediated pathway (Brass *et al.,* 2013). The relaxation of muscle is stimulated by various vasodilators such as isoprenaline and dobutamine (β-adrenoceptor agonists), forskolin (activates adenylyl cyclase pathway), sodium nitroprusside (nitric oxide donor), and acetylcholine (cholinergic receptor agonist). These vasodilators cause relaxation by involving cyclic nucleotides; cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) downstream pathway upon activation of their respective receptors. The detailed mechanism will be discussed in **section 1.9.2.**

All in all, besides being a commonly applied assay in cardiovascular research, organ bath assay is an excellent tool for lead optimization and elucidation of the mechanism of action of compounds.

1.8.3.2 Toxicity assay using *Caenorhabditis Elegans*

Toxicity assay or testing is developed to provide an early indication of the toxicity characteristics of drug candidates. Traditionally, drug screening extensively relied on expensive and low throughput animal models for the validation of toxicity. Recently, scientist have explored the use of *Caenorhabditis Elegans* (*C. elegans*) as a viable tool for automated high-throughput drug screening (O'Reilly *et al.,* 2014). *C. elegans* is a small about 1 mm in length, soil worm or nematode (member of phylum Nematoda) and it shares a common ancestor with humans that lived in the pre-Cambrian era, 500-600 million years ago. The *C. elegans,* is either a male or hermaphrodite (have both male and female reproductive organs). The life cycle of *C. elegans* is comprised of the embryonic stage, four larval stage (L1-L4) and adulthood. An adult *C.elegans* contains 959 somatic cell, 302 neurones and 81 muscle cells (Schafer, 2005).

The experiments carried using this model are relatively simple, more affordable, and rapid. *C. elegans* are easily cultured in the laboratory, usually grown on agar plates seeded with bacteria and have a short (3-day) generation time. The short life cycle of *C. elegans* allows for low-dose lifespan and multi-generation testing in short period of time rather than years. They have extensive homology to human / mammals at the genetic level and many key cellular metabolic, signalling pathways and neuronal functions are well conserved in this model, which makes it a great tool in understanding specific roles of genes related to humans.

Unlike toxicity testing using cell cultures, *C. elegans* toxicity assays provide data from a whole animal with intact and metabolically active digestive, reproductive, endocrine, sensory and neuromuscular systems (Hunt, 2017). The toxicity ranking screens in *C. elegans* have repeatedly been shown to be as predictive of rat and mouse ranking (Hunt, 2017). The toxicity ranking in *C. elegans* adult models can be determined by screening the larval growth, reproduction and life span assay (Anderson *et al.,* 2004; Dhawan *et al.,* 1999; Boyd *et al.,* 2010). In this study, lifespan assay have been employed to determine the toxicity of FK70. Lifespan measurement is a direct method that determines the effect of the novel compound on the lifespan of the worms. Lifespan assays are either performed using solid or liquid culture system. The number of worms in each plate are synchronised and the live and dead worms are counted at a regular interval (e.g. every other day or every 2 days). The dead worms will be discarded during counting to prevent confusion while performing subsequent counting. Lifespan curves are then generated to illustrate the percentages of live worms in populations over time in the presence of chemical, and finally the data are statistically analysed (see **Figure 1.7**).

The *C. elegans* can also be used as oral toxicity model as they feed by pulling liquid phase components from their environment into their pharynx by pumping and peristalsis (Hunt, 2017). Furthermore, their alimentary system are comparable to that of mammals which includes an acidified lumen, microvilli that form a brush border, secretion of digestive enzymes, uptake of digested components and peristalsis (Hall and Altun, 2008; Chauhan *et al.,* 2013; Stutz *et al.,* 2015).

Current standards have emphasised on 3Rs replacement, reduction, and refinement the use of animals in regulatory testing. Therefore, the focus is mainly on high-throughput *in-vitro* testing. Although *in-vitro* assay can reveal molecular and cellular mechanisms of toxicity, it cannot currently provide information such as the effects of exposure route, metabolism by and transport through multiple tissues, and communication among interacting tissues (Tice *et al.,* 2013). *C. elegans* bridges the gap between *in-vitro* assays and mammalian toxicity testing where there is a combination of *in-vitro* handling techniques and cost ratios with toxicity test data from an intact organism (*in-vivo*). In addition, *C. elegans* also acts as a best 'replacement' model as described in the 3Rs principle in reducing the usage of other animals in this study (rats and pigs).

Numerous studies have demonstrated the versatility of *C. elegans* in compound screening, drug target identification and in elucidating mechanisms of drug action. For example, the discovery of nemadipine-A, a drug which resembles a widely prescribed anti-hypertension drugs called the 1,4-dihydropyridines (DHPs) that blocks the of L-type calcium channels (Kwok *et al.,* 2006), *C. elegans* model has also facilitated the high throughput screening of small molecules and extracts to produce new antibacterial agents (Zhou *et al.,* 2011). It also has been regarded as an excellent model to study dystrophinopathy in order to find curative treatment for Duchenne muscular dystrophy (Gaud *et al.*, 2004). Besides that, this model has also been developed to discover the pharmacological therapies for disorders caused by misfolded and aggregation-prone proteins (Gosai *et al.,* 2010), and aided in the identification of target receptor and toxicity studies of an antipsychotic drug, clozapine (Saur *et al.,* 2013).

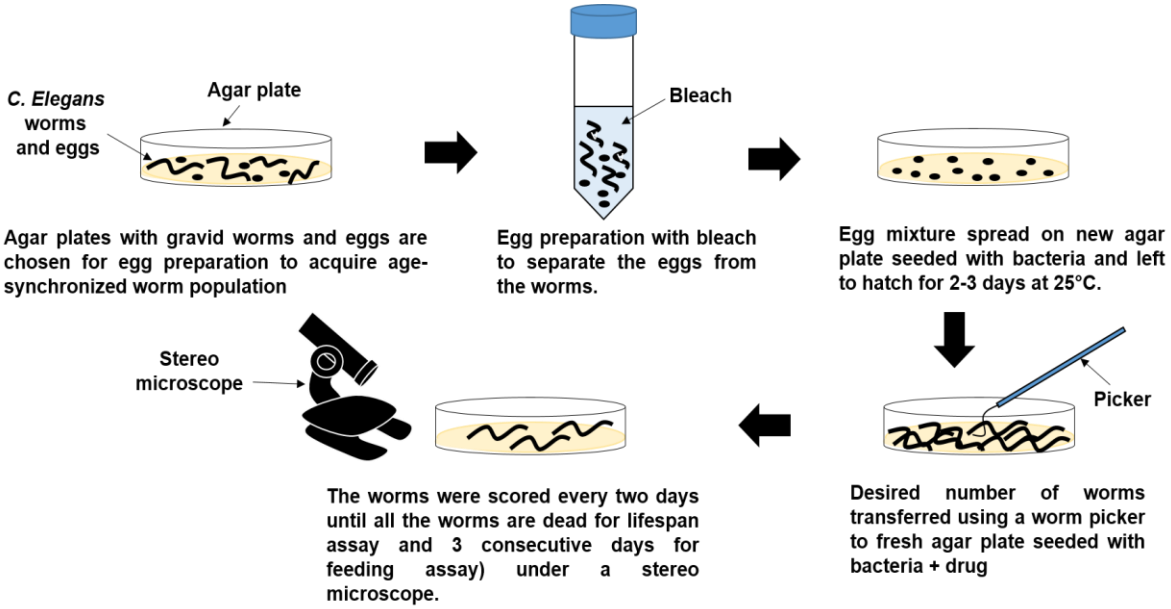


Figure 1.7: Schematic illustration of *C. elegans* lifespan and feeding assay using solid culture system

1.9 Blood vessels and vascular reactivity

Blood vessels are part of the circulatory system, and microcirculation that transports blood throughout the body. The blood vessels are made up of three layers namely; tunica adventitia is the outer layer which consist of thick and tough wall of connective tissue, tunica media is the middle layer that has smooth muscle cells and elastic fibre, which enable them to contract and relax to regulate blood flow and pressure, and tunica intima is the inner layer lined with thin single sheet of endothelium cells. The amounts of connective tissue and smooth muscle in the vessel wall vary according to the vessel's diameter and function, but the endothelial lining is always present (Alberts *et al.,* 2002).

The vascular reactivity is defined as the responsiveness of a blood vessel to a specific stimulus. It is regulated by both the intimal (endothelial) and medial (vascular smooth muscle) layers as well as through interlayer interactions. The most commonly noted responses are vasodilation and vasoconstriction (see **Figure 1.8**) for a brief overview of contraction and relaxation mechanism in the vascular smooth muscle. The mechanism of vascular contraction and relaxation will be discussed in depth in **section 1.9.1** and **1.9.2**, respectively.

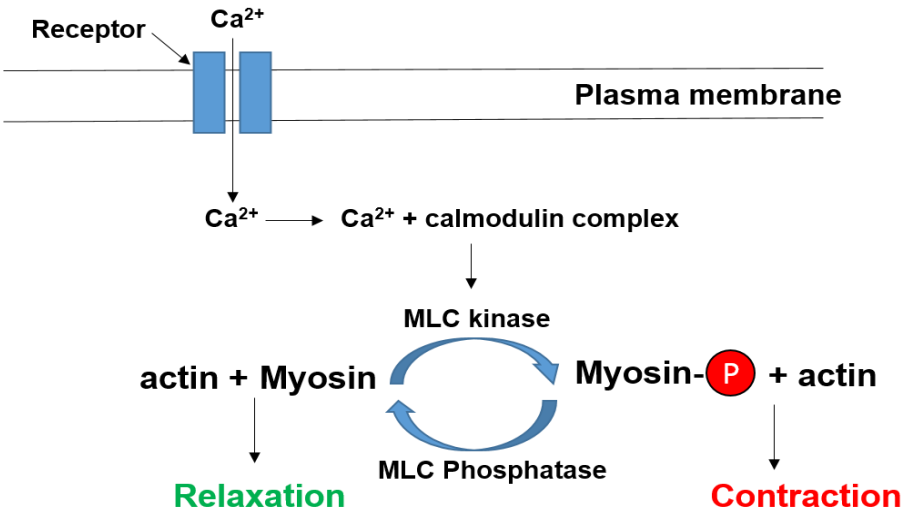


Figure 1.8: Schematic illustration of mechanism of contraction and relaxation in the vascular smooth muscle.

The free calcium binds to a calcium binding protein called calmodulin to form calcium-calmodulin complex. The complex activates myosin light chain kinase (MLCK), an enzyme that is capable of phosphorylating myosin light chains (MLC) in the presence of ATP. MLC phosphorylation leads to cross-bridge formation between the myosin heads and the actin filaments, and hence, smooth muscle contraction. Relaxation occurs when there is reduced phosphorylation of MLC by myosin light chain phosphatase (MLCP), phosphatase-activated MLC dephosphorylation.

1.9.1 Vascular contraction

The mechanism of vascular contraction involves different signal transduction pathways, all of which converge to increase intracellular calcium $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ complexes with calmodulin to form calcium-calmodulin complex, which mediates phosphorylation of MLC and actin–myosin cross-bridge cycling which generates force and shortening, consequently vascular contraction. Recent studies have shown that vascular smooth muscle contraction is also regulated by calcium-independent mechanisms involving RhoA-Rho kinase, protein kinase C and mitogen-activated protein kinase signalling, reactive oxygen species and reorganization of the actin cytoskeleton (Touyz *et al.,* 2018). Despite several factors causing vascular contraction, the $[Ca^{2+}]_i$ level act as the common denominator at the cellular level. The $[Ca^{2+}]_i$ level is regulated by external calcium entry and calcium release from the internal calcium store.

The replete amount of calcium distributed inside the cytosol as free ionic calcium (Ca^{2+}) could be due to a combination of increased leakiness across plasma membrane through plasma membrane calcium-permeable channels [e.g voltage-operated calcium channels (VOCCs), receptor-operated calcium channels (ROCCs), second messenger-operated calcium channels (SMOCs), and sodium-calcium exchanger (NCX)], decreased effectiveness of the extrusion-mechanism, incomplete compensation by the calcium-dependent ATPase, reduced capacity of buffering cytosolic calcium, or increased calcium release by organelles such as the mitochondria, endoplasmic or sarcoplasmic reticulum (ER / SR) (Touyz *et al.*, 2018).

The excitation-contraction coupling in smooth muscle occur by two overlapping mechanisms, electromechanical and pharmacomechanical coupling. The electromechanical coupling process, involves the rise in $[Ca^{2+}]_i$ as a result of membrane depolarisation followed by an activation of VOCC. Agonists, neurotransmitters or hormones are involved in depolarization of the membrane. In contrast to excitation-contraction coupling, pharmacomechanical coupling does not depends on changes in membrane potential (although changes may occur) or, consequently, on calcium entry via VOCCs but the rise in $[Ca^{2+}]_i$ is brought about by a combination of calcium release from intracellular stores and calcium entry through non-voltage-operated channels, ROCCs and SOCCs (McFadzean and Gibson, 2002). Besides that, other factors like circulating non-cellular factors like cytokines, diffusible ROS (nitric oxide and hydrogen peroxide), and miRNAs and cellular-derived factors such as microparticles and endothelial progenitor cells can also stimulate membrane receptors or cross the plasma membrane to regulate pathways that control $[Ca^{2+}]_i$ (Touyz *et al.,* 2018).

There are five VOCCs found in different cell types, including L-, N-, T-, P- and Q-type VOCCs. The L-type subunits are known to be involved in cardiac and smooth muscle cells (Catterall, 2011). Ca^{2+} enter through L-type voltage dependent Ca^{2+} channel is the primary Ca^{2+} influx pathway in vascular smooth muscle cells which leads to membrane depolarisation and subsequent Ca^{2+} entry through these channels (Earley and Brayden, 2015).

The ROCCs, SMOCs, SOCCs work hand in hand in the mechanism of Ca^{2+} influx. The ROCCs mostly belong to G-protein coupled receptors (GPCR). They response to stimuli such as neurohumoral stimuli (acetylcholine, norepinephrine) and vasoactive peptides (angiotensin II and endothelin-1) to stimulate phospholipase C (PLC), which leads to the generation of second messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG). The IP_3 binds to inositol triphosphate receptor (IP_3Rs) on the ER/SR to cause release of Ca^{2+} into the cytosol while the DAG activates protein kinase C and Ca^{2+} influx through internal or external SMOCs (e.g transient receptor potential cation (TRP) channels. Meanwhile, store operated Ca^{2+} entry

through SOCCs occurs in response to depletion of intracellular Ca^{2+} stores, where it can be typically achieved by prolonged stimulation of cell surface receptors or blocking sarcoendocoplasmic reticulum calcium entry (SERCA) activity with pharmacological agents such as thapsigargin or cyclopiazonic acid. Ca^{2+} enters the ER/SR compartment, to refill the depleted intracellular Ca^{2+}.

Recent studies have reported that Ca^{2+} influx by the Na^{+}/Ca^{2+} exchanger (NCX), is a bi-directional regulator of cytosolic Ca^{2+}, capable of both Ca^{2+} influx and Ca^{2+} efflux (Tykocki *et al.* 2012) is the primary mechanism of calcium entry in purinergically stimulated rat aorta smooth muscle cells and requires functional coupling with TRPC6, non-selective cation channels (Lemos *et al.*, 2007). In forward mode, the NCX transports Ca^{2+} out of cells whilst in the reverse mode, NCX takes up extracellular Ca^{2+}. The movement of Ca^{2+} through the NCX depends on the net electrochemical gradients for Na^{+} and Ca^{2+} as the NCX does not hydrolyse ATP to provide energy for ion transport. For every 1 Ca^{2+}, 3 Na^{+} ions are exchanged (Jung *et al.,*2007).

1.9.2 Vascular relaxation

Relaxation of VSMCs occur when the contractile stimuli are removed, contraction mechanisms are inhibited by endothelium-derived relaxing factors (EDRFs, e.g nitric oxide, prostacyclin and EDHFs), activation of potassium channel, activation of beta-adrenergic receptor-mediated pathway, inhibition of calcium channels and downstream pathways: involving cyclic nucleotides [eg: cyclic guanine monophosphate (cGMP) or cyclic adenosine monophosphate (cAMP).

The vascular endothelium is known to synthesis various vasodilating and constricting mediators. There are three major vasodilating mediators produced by endothelial cells (Durand and Gutterman, 2013). The first major vasoactive mediator is nitric oxide (NO). NO gas is released from nitrosothiols in haemoglobin or from endothelial cells, diffuses into smooth muscle cells of blood vessels. Once inside the smooth muscle cell, NO binds to an enzyme, called guanylate cyclase (GC), resulting in GC activation. Activated GC is able to cleave two phosphate groups from the compound called guanosine triphosphate (GTP) and leads to the formation of cyclic guanosine monophosphate (cGMP) which phosphorylates the contractile myosin, thus leading to relaxation (Heilpern, 2008). The second major endothelium-dependent mediators that elicit relaxation are prostaglandins (e.g. prostacyclin). Prostaglandins are generated by the reaction of cyclooxygenase (COX) enzymes on arachidonic acid. Then they move to the vascular smooth muscle cell to activate adenylyl

cyclase (AC) to elicit relaxation through cyclic adenosine monophosphate (cAMP). The third mediator that cause relaxation are generally known as the endothelium-derived hyperpolarising factors (EDRFs). There are multiple EDRFs that have been identified, which include hydrogen peroxide (H_2O_2), epoxyeicosatrienoic acids (EETs), carbon monoxide (CO), hydrogen sulfide (H_2S), C-natriuretic peptide (CNP), anandamide, and potassium ion (K^+) (Feletou *et al*., 2009). The EDRFs cause relaxation by hyperpolarising the membrane through activation of K^+ channels or the sodium-potassium ATPase (Na^+/K^+- ATPase) (Durand and Gutterman, 2013).

The degree of MLC phosphorylation and the VSM contractile tone is regulated by G-protein-couple signal transduction pathways and by nitric oxide activation of GC and cGMP formation. The cAMP generation in most cells occurs through a classical GPCR transmembrane signalling, where it is most commonly initiated following the binding of specific ligands to GPCRs of the G_s-family (Billington *et al.,* 2013). As aforementioned in **section 1.9.1**, G_q-protein activation by compounds such as norepinephrine/ phenylephrine (α_1-adrenoceptor agonists), angiotensin II (AT-1 receptors), endothelin-1 (ET_A receptors) and vasopressin (V_1 receptors) stimulates SR release of calcium via second messengers IP_3 and DAG and activates Rho-kinase, which inhibits MLCP leading to contraction, meanwhile G_s-protein activation via β_2-adrenoceptors, A_{2-} purinergic receptors and IP_3 receptors increase cyclic adenosine monophosphate (cAMP), which inhibits MLCK thereby reducing MLC phosphorylation and cause relaxation. In contrast, the activation of G_i-protein elicits contraction by reducing cAMP, which increases the activity of MLCK. The cGMP on the hand, activates a family of serine/threonine protein kinases, the cGMP-dependent protein kinases (PKG), in smooth muscle cells, which leads to several different phosphorylation events in response to PKG activation that lead to relaxation (Lincoln, Dey and Sellak, 2001).

In most tissues, the concentrations and the actions of cyclic AMP and cyclic GMP are regulated by cyclic nucleotide phosphodiesterases (PDEs) (Klabunde, 2012). The PDEs catalyse the hydrolysis of cyclic AMP and cyclic GMP, thereby regulating the intracellular concentrations of these cyclic nucleotides. cAMP- or cGMP-activate protein kinases protein kinase A (PKA) or PKG, respectively. There are four major PDEs found in the VSMCs, namely PDE1, PDE3, PDE4, and PDE5 (Stangherlin and Zaccolo, 2012). PDE3 and PDE4 are shown to be involved in the hydrolysis of cAMP whilst PDE1 and PDE5 account for the hydrolysis of cGMP. The inhibitions of PDE, increases cAMP and cGMP that lead to vasorelaxation. PDE3 inhibitors were discovered to exhibit cardiotonic, inotropic, bronchodilatory and vasodilatory activities in several species (Maurice *et al.*, 2014).

The VSMCs express adrenergic receptors e.g α - and β -adrenoceptors on their surface, and they are innervated by the adrenergic nervous system. Activation of α-adrenoceptors leads to contraction while activation of β-adrenoceptors lead to relaxation of the VSMCs. There are three distinct subtypes of β-AR that have been identified in mammals, (β_1, β_2, and β_3). However, the β_2-adrenoceptors are highly expressed subtype in the VSMCs (William *et al.,*2012). These receptors are coupled to a G_s-protein, which stimulates the formation of cAMP which leads to the relaxation of the smooth muscle.

There are four major classes of K^+ channels that have been detected in vascular smooth muscle cells, Voltage-dependent K^+ (K_v) channels, Ca^{2+}-activated K^+ (BK_{Ca}) channels, ATP-sensitive K^+ (K_{ATP}) channels, and inward rectifier K^+ (K_{ir}) channels. The K_v channels open upon depolarization of the plasma membrane in vascular smooth muscle cells, subsequently leading to efflux of K^+ through the channels to induce repolarization to the resting membrane potential. Changes in the $[Ca^{2+}]_i$ concentration and membrane depolarization stimulate large-conductance of Ca^{2+} -activated K^+ (BK_{Ca}) channels, which are thought to play an important role in maintaining the membrane potential (Ko *et al.,* 2008). The K_{ATP} channels form as the crucial functional bond between cellular metabolism and membrane excitability. The blockade of K_{ATP} channel function results in vasoconstriction and depolarization in various types of VSMCs. The K_{ir} channels are expressed mostly in smooth muscle of the small-diameter arteries, which contribute to the resting membrane potential and basal tone. K_{ir} channel activation has been shown to raise the extracellular K^+ concentration to 10-15 mM, resulting in vasodilation.

1.9.3 Rational for new therapies

Despite the currently available panoply of antihypertensive drugs, the rate of cardiovascular risks among people suffering from hypertension is still alarming. That said, high blood pressure alone is not the sole factor for why hypertensive patients are so susceptible to coronary disease. As mentioned above, hypertension most commonly is a syndrome of risk factors, including abnormalities of lipids, glucose, and insulin metabolism. Besides that, intrinsic abnormalities of the structure and function of the left ventricle, arteries, and kidneys are also part of hypertension syndrome. However, beyond these obvious risk factors lies a possibility that changes in the arterial wall could also be a key characteristic of hypertension. Intrinsic changes in the arterial wall produce increase stiffness in the artery. Current antihypertensive therapy improves arterial stiffness mainly by reducing BP as a major determinant of diminished arterial compliance which comes at a price, risk of inappropriately decreasing diastolic blood

pressure (Cernes *et al.,* 2008). Therefore, this necessitates discovery of new drugs which have the ability to work directly at the arterial wall to improve arterial wall compliance.

The family of voltage gated calcium channel is divided into high-voltage activated channels (including L- and P/Q-type), and low-voltage activated channels (T-type). Calcium antagonists are widely used as pharmacological treatment for hypertension to cause vasodilation in the resistance vessels. For long, high-voltage activated, L-type VOCC has been considered to be the major source of calcium necessary for persistent vascular tone, and has been linked to hypertension and cerebrovascular disease (Pesic *et al.*,2004). However, despite the successful use of blockers of L-type VDCCs and antagonists of the renin–angiotensin system in the treatment of hypertension, some 20–30% of patients remain refractory to this therapy (Epstein, 2007). Generally in essential hypertension, the endothelial function is impaired. T-type voltage-gated Ca^{2+} channels are mostly expressed than L-type voltage-gated Ca^{2+} channels in the vascular endothelium (Oshima *et al.,* 2005). Although physiological studies have not supported the involvement of a classical low voltage activated, T-type channel in vascular function, evidence is accumulating that points to the involvement of T-type channel in hypertension. Upregulation of T-type Ca^{2+} channels cause endothelial dysfunction thus leading to refractory hypertension. The blockade of T-type channels was previously targeted with the commercial launch of mibefradil in 1997, however mibefradil was withdrawn from the market a year later due to adverse drug interactions in a small group of patients (Kuo *et al.,* 2011). Since then, the potential role of T-type channel antagonism in the treatment of patients with therapy-resistant hypertension was never assessed. Likewise, the contribution of P/Q type Ca^{2+} channels are also underexplored in hypertension. The P/Q-type channels are of functional importance for the depolarization-induced vasoconstriction (Hansen *et al.,*2011). This therefore justifies a more intensive effort to develop selective antagonists for these calcium channels.

The G-protein coupled receptors (GPCRs) represent the most important targets in modern pharmacology due to their various functions, especially within the brain and the peripheral nervous system. The heptahelical GPCR or also known as the (7TM) receptors constitute the largest single family of cell surface, receptors with 800 members in the human genome and it constitutes the largest family in vertebrates (Schulein *et al.,* 2012). The GPCRs are classified into 6 classes based on their sequence homology, namely Class A (rhodopsin-like), Class B (secretin receptor family), Class C (metabotropic glutamate), Class D (fungal mating pheromone receptors), Class E (cyclic AMP receptors) and Class F (frizzled / smoothened) (Alexander *et al.,* 2017). The classes D and E are not found in the vertebrates.

The GPCRs are not only found on the surface of the cell, but they exist in the endoplasmic reticulum, Golgi apparatus, nuclear membrane and nucleus (Re *et al.,* 2010). There are various amount of ligands (stimuli) that can activate the GPCRs, such as ibiogenic amines, neuropeptides, amino acids, ions, hormones, chemokines, lipid-derived mediators, proteases peptides, proteins, photons, protons (H^+) and ions (Ca^{2+}) (Ghanemi, 2015). However, according to (Gloriam *et al.,* 2007; Chung *et al*.,2008), there are 120 to 130 receptors of orphan GPCRs with non-identified ligands. The G protein has three subunits: Gα, Gβ and Gγ (Hazell *et al.,* 2012) with at least 16 Gα, 5 Gβ, and 12 Gγ subunits in the human genome (Robishaw and Berlot, 2004). The GPCRs have a functional selectivity and can activate, depending on the ligand nature, more than one class of G proteins (Zheng *et al*., 2010) which makes the area of pharmacodynamics more interesting. The GPCRs' signalling pathways can lead to a variety of cellular responses, for instance growth, death, movement, transcription and excitation (Gloriam *et al*., 2007).

Among all hormones and neurotransmitters, about 80% of them exert their effects via interacting with GPCRs. The main common mechanism of GPCR is as follow, firstly, it receives an external signal through binding to ligands which makes it as the first messenger, and then it converts the stimulus into a cellular response within the intracellular medium via a chain of biochemical reactions and molecular interactions. According to the existence or the non-existence of bound ligands to it, the GPCRs have two states, high and low affinity states, which correspond to G protein-coupled and uncoupled states (Ma *et al*., 2011). The ligand-receptor interactions involves intermolecular forces including ionic bonds, hydrogen bonds and van der Waal forces that, all together, induce a spatial conformational change in the tertiary structure of the GPCR (Ma *et al.,* 2002). Finally, it activates the downstream signalling cascades within the cell to induce any physiological processes. The signal amplification and modulation within the pathway through downstream signalling cascades makes this pathway more important in both cell physiology and pharmacology. Therefore, the balance between these pathways, which in a large part is dictated by the cellular environment, determines the outcome as a diseased or non-diseased state.

As discussed earlier, the angiotensin receptor (AT1R), is a GPCR, is the main receptor which involved in the effects of the renin-angiotensin system. The AT_1R plays an important role in the regulation of blood pressure and in the development of pathological conditions, such as hypertension, heart failure, cardiovascular remodelling, renal fibrosis, inflammation, and metabolic disorders. Recent studies have shown that the AT1R is a versatile GPCR, which has multiple unique faces with distinct conformations and signalling properties.

Furthermore, the pathogenesis of cardiac hypertrophy and hypertension involves dysfunctions of G-protein signalling (Wieland *et al.,*2007). The Rho and Rho kinase activation implicate the development of CVDs (Jalil *et al.,*2005). The GPCR ligands, e.g. angiotensin II and phenylephrine, activate GTPase Rho which activates Rho kinase which leads to VSM contraction, stress fibre formation and cell migration. Although conventional drugs e,g., ACEIs and ARBs targeting the receptors are already in the market, better understanding of mechanisms and structural aspects of AT1R activation or other GPCRs-mechanisms linked to CVDs could lead to the development of novel type of drugs with better therapeutic effects and minimal side effects for the treatment of CVDs. One-third and one-half of all marketed drugs act by binding to GPCRs, they are the targets for the majority of drugs in clinical usage, however only a minority of these receptors are exploited therapeutically. Based on the properties listed, the pharmacological importance of GPCRs with no doubt make the ideal therapeutic targets for hypertension.

Mammalian genomes encode 28 distinct TRP protein subunits. The genes are grouped into six subfamilies based on amino acid sequence homology. The subfamilies are known as canonical (TRPC), vanilloid (TRPV), melastatin (TRPM), ankyrin (TRPA), mucolipin (TRPML) and polycystin (TRPP). All TRP subunits are expressed as polypeptides of 553-2022 amino acids (64-230kDa) containing six transmembrane domains (S1-S6) and intracellular amino (NH_2) and (COOH) termini of variable length. Intracellular calcium ($[Ca^{2+}]i$) homeostasis is essential for vascular function and blood pressure regulation. TRP channels have unique roles in regulating intracellular Ca^{2+} and vascular function. They have emerged at the frontier of hypertension research. TRP channels can be divided into two functional subtypes, based on their role in vasculature function regulation, one that involves in vasodilation and one that involves in vasoconstriction (Liu *et al.*,2014). A functional imbalance of these two subtypes of TRP channels may interrupt intracellular calcium ($[Ca^{2+}]_i$) homeostasis leading to vascular dysfunction and development of hypertension. TRPC3 expression in vascular smooth muscle cells isolated from spontaneously hypertensive rats (SHR) was increased and is correlated with enhanced ANG II-induced Ca^{2+} influx that is independent of VOCC (Liu *et al*.,2006). To date there is no approved antihypertensive drug that targets these TRP channels. Thereby justifies an effort to explore the potential of these TRP channels and develop novel pharmacological targets for the treatment of hypertension.

Hypertension is a multifactorial disorder and typically using more than one drug or combined therapy is necessary for effective management of high blood pressure. Yet this combined therapies fail to control blood pressure effectively in in most individuals for a number of reasons such as poor compliance due to the adverse side effects of some anti-hypertensive medications, diminished drug effectiveness with time or age, life style issues, as well as drug efficacy. Taken together, the need is great for novel drugs that target other contributing factors of hypertension to reduce the accelerating burden of hypertension in our society.

1.9.4 Usefulness of animal models for mechanistic and translational studies

Animal have been used as research models for millennia in human history. The ancient humans had used animals mainly to advance the understanding of living animals, and the use of animal models have expanded rapidly in 18th and 19th centuries. The use of laboratory animals remains as a crucial step in the preclinical evaluation of pharmaceuticals, biologics, and biomedical devices. Most treatments are tested on animals for several reasons. Firstly, they provide us with better understanding of human anatomy, physiology, pathology and pharmacology which are rarely feasible in humans and contribute to finding solutions for many unsolved biological and biomedical questions. Secondly, animal modelling gives an overview of the target of the drug and save a lot resources, for instance, it may not be necessary to test new treatments on humans if preliminary testing on animals shows that they are not clinically useful, as animal studies provide unique insights into the pathophysiology and aetiology of disease, and often reveal novel targets for directed treatments. Besides that, regulatory authorities concerned with public protection require extensive animal testing to screen new treatments for toxicity and to establish safety before testing it on humans.

In order to be used as a model, the animal species must meet specific criteria in line with the aim of the research. Many species are used in biomedical research, such as insects (Drosophila), nematodes (*Caenorhabditis elegans*), fish (Danio rerio, or zebrafish), frogs (Xenopus) and many mammals, such as mice, rats, dogs, cats, pigs and monkeys, due to their phylogenetic proximity to humans (Andersen and Winter, 2017). Modern day research have led to the generation of more promising and clinically significant animal models through genetic and genomic tools and genetic engineering techniques. For example, researchers can now develop knockout or transgenic animals to create better model of human diseases in animals that do not fully recapitulate the desired phenotype. An ideal animal model should be similar to humans in term of efficacy, potency, and duration of action of drugs.

In hypertension research, different types of animal models are used. Initial experiments to investigate the pathophysiology of hypertension were conducted on dogs (Goldblatt *et al.,*1934). Subsequently other models like rats, rabbits, sheep, cats and pigs were developed. Pharmacological approaches using a nitric oxide synthase (NOS) inhibitor or by activating the RAAS, or introduction of environmental stresses, including stress, cold temperature and diet, have been performed to induce hypertension in these models (Lin *et al.,* 2016). Despite many animal models used in hypertension studies, the rats have been a popular and leading model with different inbred strains and characteristics, such as the spontaneously hypertensive rats (SHR), Dahl salt-sensitive rats, New Zealand and Milan strains (Yagil and Yagil, 2001). Each of these models has been designed to express different phenotypes, including spontaneous hypertension, salt sensitivity, stress sensitivity and susceptibility to end-organ damage. The possible explanation to this could be due to its 99 % genome sequence homology to humans. The pathogenesis of hypertension in rats and humans are largely similar in terms of arterial pressure development from childbirth, response to environmental stressors, haemodynamic factors (including vascular resistance), mechanisms regulating arteriolar and venous constriction, neural modulation (including sympathetic nerve activity) and renal vascular dynamics (including perfusion parameters), as well as humoral influences by RAAS and NOS (Trippodo and Frohlich, 1981). Moreover, rat models are cost efficient, easy to handle, maintain and breed.

Despite the advantages of animal models, the use of animal models in research is highly controversial. Modern era research demands a solid and rational moral foundation for animal experimentation. Arguably one of the most heated debates in science, is efforts to reduce the number of animals used in studies. There are numerous historical examples where the use of animals has been essential and successful in developing treatments for human disease (e.g., development of vaccines, chemotherapy for syphilis and penicillin). This shows that, although alternative scientific approaches are being sought, animal research is still necessary in biomedical research in predicting human responses. Some of the challenges can be overcome by careful planning, correct animal handling technique and most importantly, the aim and objectives of the research should be very clear to prevent animal wastage and to obtain a more accurate translational data. Besides that, animal welfare organisations must also make sure that every animal project is critically assessed, and no project is allowed to go ahead unless it can be scientifically justified and does not cause excessive suffering. In summary, respect, knowledge, and responsibility should underline any plan for using animals in research.

1.9.5 Aim of thesis

Hypertension is now so widely prevalent, affecting one billion people worldwide and directly responsible for more than 10 million deaths per year, that it has been declared a global public health crisis by the World Health Organization. About 140-160 million people globally suffer from resistant hypertension (Achelrod *et al*.,2015). Those with resistant hypertension have double the risk of cardiovascular events than those without resistant hypertension (Daugherty *et al.,* 2012). This necessitates new effective pharmacological treatment to improve the long-term clinical management of this disorder.

The search for new and biologically active alkaloids from plants of the genus *Ficus*, led to a discovery of alkaloidal composition from the leaves of a previously un-investigated species, e.g., *Ficus schwarzii*. The work had resulted in the isolation of a class of novel alkaloids (namely schwarzinicines A – H and schwarziphenines A and B). *Ficus schwarzii Koord*, N-(3,4-dimethoxyphenethyl)-1,4-bis(3,4-dimethoxyphenyl)butan-2-amine, schwarzinicines A (Sch A) was isolated by Premanand Krishnan from School of Pharmacy. Due to the limited source of the plant and smaller yield obtained, N-phenethyl-1-phenyl-pentan-3-amine (FK70) was synthesised as an alternative compound to study the mechanism of action of the novel compound. The main aim of this study is to elucidate the mechanism of actions of FK70 using different functional bioassay methodologies using rat isolated tissues, porcine coronary artery, guinea-pig ileum, spontaneously hypertensive rats (SHR) and *C. elegans*. The choice of these biological samples allows a diverse investigation of FK70 compound. In previous studies, Sch A is shown to relax the pre-contracted aortic smooth muscle, which are of similar magnitude to dobutamine, isoprenaline and verapamil in the rat aorta (Loong *et al*., 2016, unpublished data). Its ability to relax the blood vessel is much better than these known molecules. However the biological activity of FK70 is yet to be found. Therefore, preliminary testing is essential in order to identify the magnitude of response of FK70 in different tissues and animal species (the details of this study will be discussed further in the next chapter). In order to achieve the aim, several objectives have been carried out as listed below;

Step 1: Pharmacological investigation of FK70 in rat tissues and other animal tissues (Chapter 2)

Step 2: Investigation of mechanism of relaxation to FK70 in rat isolated aorta (Chapter 3)

Step 3: Role of calcium in contractile agents, phenylephrine (PE), 5-hydroxytrptamine (5-HT) and potassium chloride (KCl)-induced contraction on rat isolated aorta (Chapter 4)

Step 4: Role of FK70 in calcium-mediated vascular responses in normotensive rat aortae (Chapter 5), and spontaneously hypertensive rats (SHR) (Chapter 6).

Step 5: *In-vivo* evaluation of FK70 effect on pharyngeal pumping and toxicity assessment in *Caenorhabditis Elegans* (Chapter 7)

All experiments involving animal tissues were performed using organ bath technique (Chapter 2 to 6) to elucidate the mechanism of FK70. Pharyngeal pumping and toxicity study was performed on *C. elegans* using feeding assay and life span assay, respectively (Chapter 7). The details of all the experiments are discussed in depth in their respective chapters.

Chapter 2: Pharmacological investigation of N-phenylethyl-1-phenyl-pentan-3-amine (FK70) in rat tissues and other animal tissues

2.1 FK70: Origin and structure

FK70 is a novel synthetic compound synthesised by Lee Fong Kai from University of Nottingham Malaysia, based on the structure of Schwarzinicine A (Sch A). Schwarzinicine A is a novel phenylethylamine alkaloid isolated from the leaves of *Ficus schwarzii* plant. The scientific name of this plant compound is N-(3,4-dimethoxyphenethyl)-1,4-bis(3,4 dimethoxyphenyl)butan-2-amine. FK70 shares similar structural features to Sch A and is a phenylethylamine, however with less elaborated structure as compared to Sch A, with only two phenyl rings and no extra dimethoxy benzene rings (see **Figure 2.1**)**.**

The therapeutic uses of *Ficus* species are yet to be discovered. In a previous study done by (Loong *et al*., 2016, unpublished data), Sch A had shown to relax vascular smooth muscle tissues, however the exact mechanism remained elusive. Due to limited natural sources of the plant, a series of derivatives resembling Sch A were synthesised chemically as alternatives to elucidate the mechanism of actions of Sch A. In order to recognise a promising compound for further evaluations, Lipinski's rule of five have been applied to identify a potential compound with good oral absorption and/or permeation. FK70 was found to meet Lipinski's rule of five and displayed drug like properties with theoretical good physicochemical and pharmaceutical characteristics.

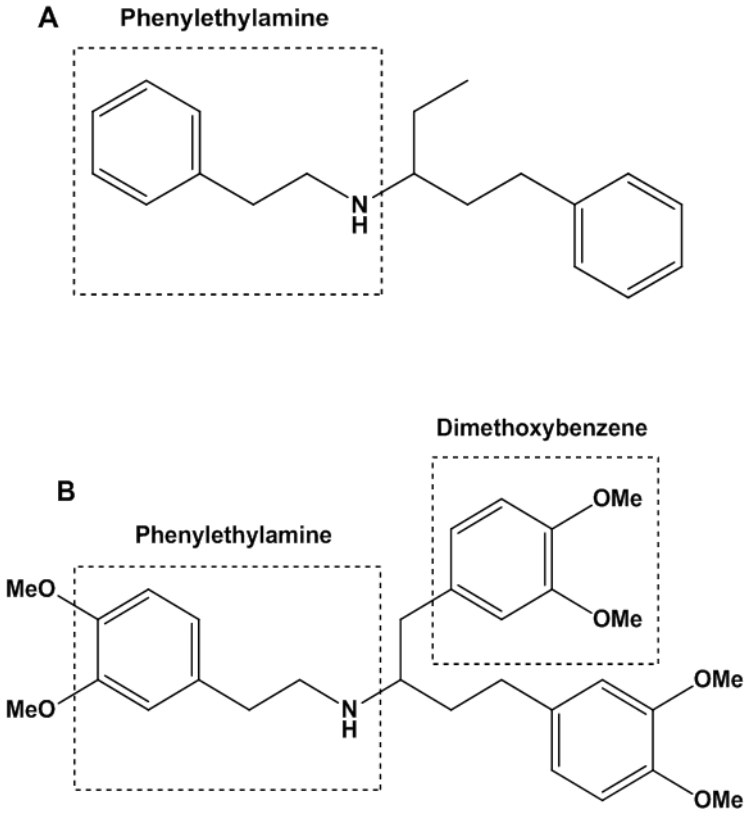
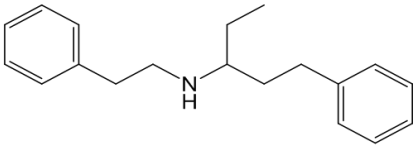
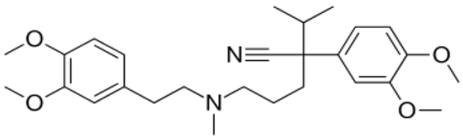


Figure 2.1: Chemical structures of (**A**) N-phenylethyl-1-phenyl-pentan-3-amine (FK70) and (**B**) N-(3,4-dimethoxyphenethyl)-1,4-bis(3,4 dimethoxyphenyl)butan-2-amine (Sch A)

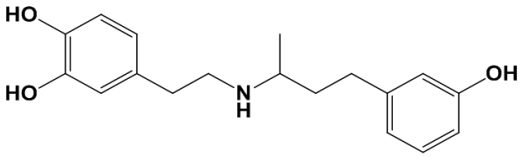
To date, neither the therapeutic effect nor the mechanism of action of any N-substituted phenylethylamine have been explored, therefore as a novel synthetic phenylethylamine compound, the biological activity of FK70 is unknown. In order to determine the putative bioactivity of FK70, dobutamine and verapamil structure-activity relationship were referred to as a guide as FK70 shares similar structure to these drugs (see **Figure 2.2**).



FK70



Verapamil



Dobutamine

Figure 2.2: Chemical structures of (from top anticlockwise); FK70, verapamil and dobutamine. (Chemical structures of verapamil and dobutamine were obtained from Pubchem database).

2.1.1 Lipinski rule of five (RoF)

Lipinski's Rule of Five (RoF), developed by Chris Lipinski and his colleagues at *Pfizer*, and is a comprehensive and straightforward strategy employed in selecting lead compounds. It was verified that about 89% of successful drugs fulfill the thresholds of the Ro5. Despite having thousands of hit compound evaluated rapidly using high throughput screening (HTS) to identify for promising leads, not many reach the market. A majority of compounds fail in the pre-clinical and clinical phases resulting in a massive wastage in cost and effort. As a way to solve the problem mentioned earlier, drug-like characteristics could be used as a parameter in the early stage of drug design to preliminarily screen and select more promising lead compounds from extensive drug libraries (Lipinski *et al*., 1997).

Four influential properties identified from *Pfizer's* library of compounds predicates Lipinski's RoF, namely, molecular weight (MW), log *P*, the number of hydrogen bond donors (HBD), and the number of hydrogen bond acceptors (HBA). Generally, the therapeutics have been small molecules that fall within the RoF. A compound with a molecular weight less than 500 Da, no more than 5 HBD, no more than 10 HBA, and an octanol-water partition coefficient log *P* not greater than 5 is far more likely to be orally absorbed (Lipinski *et al*.,1997; Lipinski *et al*., 2001). Compound that violate the boundaries of more than one of the four properties, which are, MW >500, log *P* > 5, number of HBD > 5 and HBA > 10 are considered compounds with poor absorption or permeability at the desired site of action. However, not all FDA-approved drugs meet the ROF; in fact, approximately 30% of the drugs violate the RoF

(Zhang and Wilkinson, 2007). Such violation is caused by the complexity in the oral absorption process, which does not solely rely on passive permeability as observed in the RoF but involves a complex interplay of factors, such as active transporters and first-pass metabolising enzymes.

The molecular weight describes the molecular size. Bigger molecules are lesser permeable through biological membranes and therefore have a low absorption rate (Navia and Chaturvedi, 1996). The capacity for intermolecular interactions, mainly with water molecules of a compound is directly correlated to the presence hydrogen bond donor and acceptor groups within the compound. The passage through cellular membranes becomes thermodynamically unfavourable with the increase of hydrogen bond groups because desolvation is needed to enter the lipid environment (Clark 1999; Veber *et al*., 2002). The log *P* value is one of the essential descriptors to evaluate oral bioavailability because it indicates the lipophilicity and hydrosolubility of a compound (Hansch *et al.*,1995). The higher the lipophilicity of a compound, the better the capacity of the compound to cross the lipid bilayer of the cellular membrane, and consequently, higher bioavailability.

Performing early evaluations of drug-likeness using Lipinski RoF profiling tool would be advantageous in identifying compounds with predicted poor biopharmaceutical properties. The drug-likeness evaluation of FK70 and Sch A based on Lipinski rule revealed that FK70 follows the all the rules while Sch A has surpassed both MW and log *P* rules having values more than > 500 and >5, respectively (see **Table 2.1**). Based on this simple filtering method, FK70 was chosen for further evaluation as it has shown to fulfill all the RoF criteria and serves as a promising compound with good oral bioavailability, permeability and better pharmaceutical characteristics.

Table 2.1: Calculated molecular properties of Sch A and FK70 based on the Lipinski rule of five.The descriptors molecular weight, HB donor and acceptor groups and log *P* were generated by the software ChemProp. The log *P* value was calculated using the method described by Broto's fragmentation method (Broto *et al*., 1984). *MW: Molecular Weight, HBD: hydrogen bond donors, HBA: hydrogen bond acceptors, log P:* estimation of logarithm of Partition Coefficient [n-Octanol / Water].

Compounds	Violation of Lipinski rule	MW	HBD (≤ 5)	HBA (≤ 10)	log *P*
Schwarzinicine A	**2**	**509.63**	**1**	**7**	**6.61**
FK70	**0**	**267.41**	**1**	**1**	**4.81**

2.1.2 Dobutamine and verapamil pharmacological actions

Dobutamine is known as the phenyethylamine drug with a cathecol moiety and 1-phenylbutan-3-amine as its R substituent, and it is of a synthetic origin. It was synthesised using isoprenaline as the parent chemical structure with a relative molecular mass of 301.38 g/mol. It is a direct-acting inotropic agent and acts primarily on β_1- adrenergic receptors, with little effect on β_2- or α-adrenergic receptors. It possesses a center of asymmetry and used clinically as a racemic mixture. The (-) isomer of dobutamine is a potent α_1-agonist while the (+)-dobutamine possess predominantly β_1 and β_2-adrenoceptor agonist activity, which can block the effects of (-)- dobutamine (Ruffolo and Messick, 1985).

Dobutamine has been long used to treat heart failures and cardiogenic shocks (Andersen *et al*., 2015). It stimulates the β-adrenegic receptors in the heart to produce comparatively mild chronotropic, hypertensive, arrhythmogenic, and vasodilative effects (Valet *et al*.,1991). Dobutamine stimulates β-adrenergic receptors coupled to G_s-protein to activate the cAMP pathway as its primary method of action. Recent studies have shown that dobutamine may also contribute to ionotropic activation via the reverse mode of NCX exchanger in cardiac muscle cells (Yan *et al.*, 2015). The catechol moiety in dobutamine makes it orally inactive and therefore administered intravenously.

Verapamil is a phenylalkylamine with a molecular mass of 454.6 g/mol and a first-generation synthetic calcium channel blocking agent introduced in 1962. It selectively inhibits the transmembrane influx of extracellular calcium ions (Ca^{2+}) into myocardial and vascular smooth muscle cells, causing dilatation of the main coronary and systemic arteries and decreasing myocardial contractility. The pharmacological effects of verapamil are primarily dependent on the autonomic nervous system. It also inhibits the drug efflux pump P-glycoprotein, which is overexpressed in some multi-drug resistant tumors and may improve the efficacy of some antineoplastic agents (Singh *et al*., 1978). The main therapeutic uses of verapamil are in the management of atrial tachyarrhythmias, angina, and hypertension. Verapamil binds to amino acids in the S6 segments in domains III and IV of the α_1 subunit of the voltage-gated calcium channels (VGCC). It gains access to its binding site *via* an intracellular route and shows preferential binding to the VGCC in the open state (Catterral *et al*.,2015), therefore displays frequency-dependence or use-dependence, where the frequent repetitive opening of VGCC favours its binding. This accounts for the efficacy of verapamil in the treatment of supraventricular arrhythmias, and the more pronounced cardiac effects of verapamil compared to other calcium channel inhibitors (Hughes, 2018).

These compounds have at least one structure partially or almost similar to the FK70 structure. The absence of phenolic OH groups in FK70 makes it less polar and has smaller molecular weight when compared to dobutamine and verapamil, which would increase its ability to cross the cell membrane far better than other cathechol containing drugs.

2.1.3 Smooth muscle activity in animal tissues

Smooth muscle (SM) tissues are found in blood vessels, respiratory and gastrointestinal tracts and urinary bladder. The activity of these smooth muscles can be accessed by exposing the tissues to a variety of contractile and relaxation agents. The medial SM lining enables the tissue to contract and relax as needed. Organ bath technique is effective in evaluating smooth muscle activity of animal and human tissues.

The SM contraction is controlled by signals from the autonomous nervous system, such as nerve impulses, hormones, and other chemicals released by specialized organs. The contraction is regulated by the changes in the concentration of free Ca^{2+} in the cytosol. An increase in free intracellular Ca^{2+} results from either 1) an increased flux of Ca^{2+} into the cell, e.g., through Ca^{2+} channels in the plasma membrane under the control of membrane depolarisation or upon agonist stimulation or 2) by the release of Ca^{2+} from internal stores, e.g., sarcoplasmic reticulum (SR), which is controlled by second messengers (Webb, 2003).

The structural arrangement of most smooth muscle-containing tissues is nearly similar. However, the layers are termed differently in different tissues. Discerning smooth muscles' structural arrangement, in general, allows for understanding the location of smooth muscles in different tissues. For instance, smooth muscles from the outer layer to the lumen and the ones found in airway tissues are termed as adventitia, submucosa, and mucosa, whilst the found in vascular tissue they are termed as tunica adventitia, tunica media and tunica intima (Herbert *et al.,* 2014; Bacakova *et al.,*2018). In the trachea, smooth muscles are found between the rings of c-shaped cartilage, and in the aorta, the smooth muscles are sandwiched between tunica adventitia and tunica intima (see **Figure 2.3)**. The airway smooth muscle expresses both M_2 and M_3 muscarinic receptors with the majority of the receptors being M_2 subtype (Roffel *et al.,*1990; Fryer and Jacoby 1998; Hirshman *et al.,*1999).

Activation of M_3 receptors, which couple to G_q-protein initiates contraction of airway smooth muscle while activation of M_2 receptors, which couple to G_i-protein, inhibits β-adrenoceptor-mediated relaxation (Hirshman *et al.,* 1999; Haga 2013) (see **Figure 2.4**). The α-adrenoceptors are largely responsible for constriction of the blood vessels (van Brummelen *et al.,* 1986; Tanoue *et al.,*2002). Typically, the α_1- ARs coupled to G_q-proteins, causes hydrolysis of phospholipase C to produce second messengers, IP_3 and DAG, resulting in muscle contraction whilst α_2-ARs mediate contraction via G_i-proteins to inhibit enzyme adenylyl cyclase (AC) that produces cAMP. Besides α-adrenoceptors, β-AR can also be found on vascular smooth muscle cells, where they mediate vasodilating effects of endogenous catecholamines (Chruscinski *et al.,*2001). The β-ARs mediate smooth muscle relaxation via G_s-proteins resulting in activation of enzyme AC to produce cAMP (Johnson,1998). The production of cAMP are involved in the relaxation of smooth muscle (see **Figure 2.4**).

The walls of the bladder are mainly formed of detrusor muscle (see **Figure 2.3**), which allows the bladder to contract to excrete urine or relax to hold urine (Fitz *et al.,*2017; Abelson *et al.,*2018). At the inferior end of the bladder, the detrusor muscle is continuous with the internal urethral sphincter. The detrusor muscle is under the control of autonomic nervous system and is composed of smooth muscle. The parasympathetic nervous system stimulates the muscarinic stretch receptors in the bladder through the pelvic nerve fibers to cause contraction of the detrusor muscle (Chess-Williams, 2002). The M_3-muscarinic receptors produce direct smooth muscle contraction while the M_2 muscarinic receptors which predominate in number appear to facilitate M_3 muscarinic-mediated contractions. In contrast the sympathetic nervous system activates the β_3- adrenoceptors through cAMP pathway in the bladder through the hypogastric nerve to cause relaxation of the detrusor muscle (Andersson, 2017).

The ileum on the other hand, consist of serosa, muscularis, submucosa and muscosa layers (Ross and Pawlina, 2011) (see **Figure 2.3**). The serosa is the outside layer of the small intestine and consists mesothelium and epithelium, which encircles the ileum. The muscularis consists of two smooth muscle layers, a thin outer longitudinal layer that shortens and elongates the gut, and a thicker inner circular layer of smooth muscle which causes constriction. Nerves lie between these two layers and allow these to muscle layers to work together to propagate food in a proximal to distal direction. The submucosa consists of a layer of connective tissue that contains the blood vessels, nerves, and lymphatics whilst the mucosa is the innermost layer with villi protruding into the lumen that increases the surface area for food absorption (Moore *et al.,*2013)**.** In various gastrointestinal smooth muscles, acetylcholine and its derivatives produce contractions by activating muscarinic receptors. It is generally assumed that the M_3-muscarinic receptor plays a key role in mediating this activity (Eglen *et al*., 1996). The ileum smooth muscle tissues also express M_2-receptors, which are generally more abundant than the co-expressed M_3-receptors (Unno *et al.,*2005; Takeuchi *et al.,*2007).

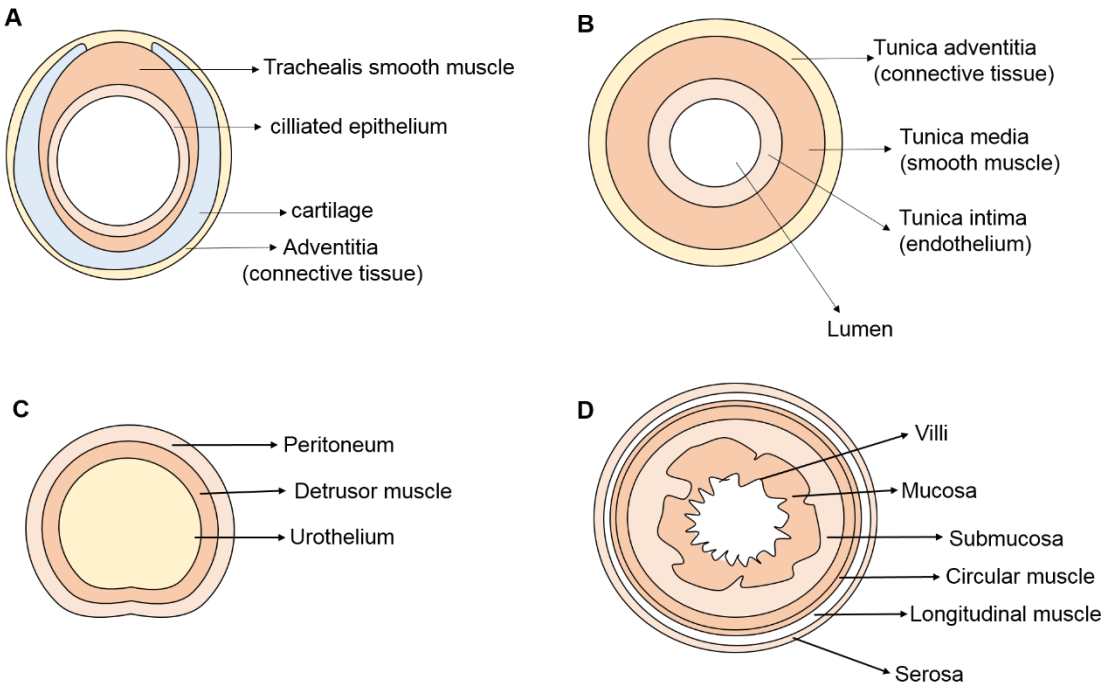


Figure 2.3: Schematic illustration of cross section of; from top left **A)** tracheal ring **B)** aortic ring **C)** bladder and **D)** ileum. (Picture adapted from A) Davies and Moores (2010). The Respiratory System (Chapter 25) *Second edition,* Churchill Livingstone B) Blausen (2014). Medical gallery of Blausen Medical, WikiJournal of Medicine. C) Patton K and Thibodeau G (2013). Anatomy and physiology (Chapter 31: Urinary system) *Eight edition,* Elsevier D) Ross M, Pawlina W (2011). *Histology: A Text and Atlas. Sixth edition*. Lippincott Williams & Wilkins)

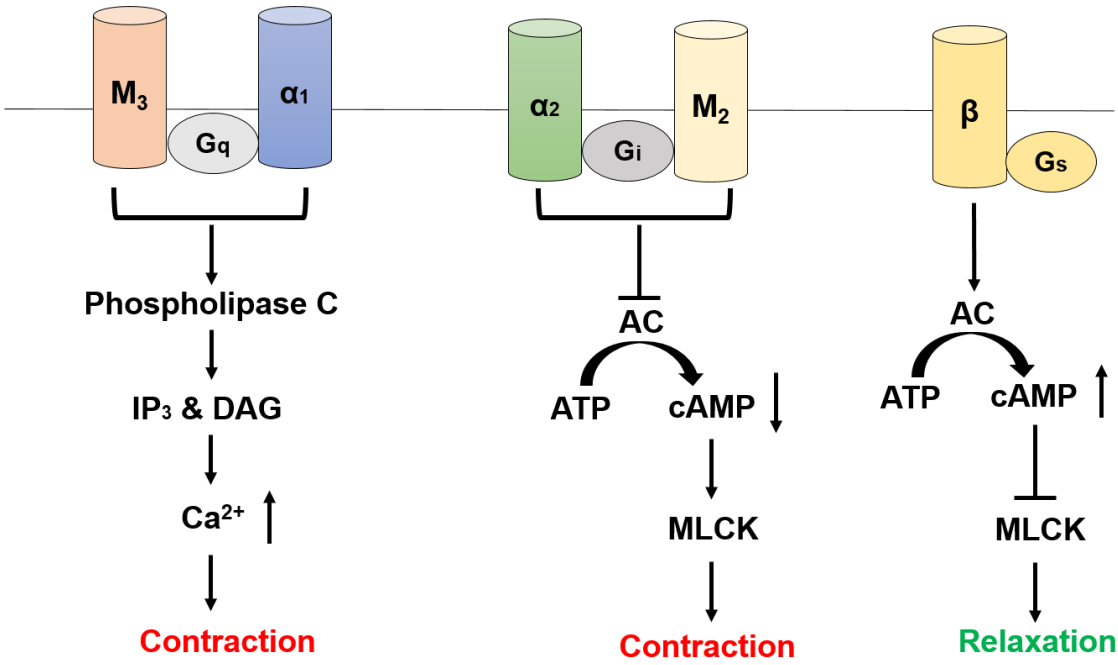


Figure 2.4: Schematic illustration of muscarinic (M_3 and M_2) and adrenoceptors (α and β) activation in smooth muscle. As a result of M_3, M_2, α_1- and α_2-adrenoceptors activation, the smooth muscle contract whilst β-adrenoceptors activation relax the smooth muscle cells. Activation of α_1-adrenoceptors or M_3 receptors lead to increased phospholipase C activity with a consequent release of IP_3 and DAG, increasing Ca^{2+} release from intracellular and extracellular milieu. Activation of α_2-adrenoceptors or M_2-receptors results in inhibition of AC activity with a resultant drop in cAMP levels. The β-adrenoceptors are coupled to G_s-proteins, which activate AC to form cAMP from ATP. Increase in cAMP leads to relaxation due to inhibition of MLCK. *AC: adenylyl cylase; cAMP: cyclic adenosine monophosphate; IP_3 : inositol 1,4,5-triphosphate; MLCK : myosin light chain kinase; ATP: adenosine triphosphate*

2.2 Study aim and objectives

FK70 shares similar pharmacophore with Sch A however the biological activity of FK70 is unknown due to its novelty. Therefore, the main aim of this chapter is to investigate the pharmacological effect of FK70 on vascular smooth muscle tissue using organ bath technique. In order to achieve the aim, several experiments were carried out on rat isolated aortae as following;

i) the pharmacological effect of FK70 and Sch A and known drugs e.g verapamil and dobutamine were compared

The pharmacological effect of FK70 was also tested on smooth muscle tissues from other biological systems using;

ii) rat isolated tracheal rings and bladder
- The effect of FK70 was tested on trachea and bladder pre-contracted with carbachol

iii) porcine coronary artery
- The effect of FK70 was examined on porcine coronary artery pre-contracted with KCl

iv) guinea-pig ileum isolated ileum segments
- The effect of FK70 was tested on guinea-pig ileum segments stimulated electrically.

2.3 Materials and methods

2.3.1 Drugs and Krebs-Ringer bicarbonate solution

(R)-(−)-phenylephrine hydrochloride (Sigma Aldrich, UK), carbachol (Nacalai Tesque, Japan), dobutamine (Santa Cruz Biotech USA), verapamil (Acros, UK), potassium chloride (KCl), Schwarzinicine A and FK70 were used. All drugs were dissolved dimethyl sulfoxide (DMSO) before subsequent dilutions in distilled water (final bath concentration of the solvent was <0.3 % (v/v)) to prepare 0.1M stock concentration except carbachol and phenylephrine which were dissolved in water. The drugs were first dissolved in 100% DMSO to achieve 0.1 M stock concentration, followed by ten-fold dilutions with descending DMSO content from 75% (v/v), 50 % (v/v), 25 % (v/v) to pure distilled water.

The Krebs-Ringer solution was freshly prepared daily, following the composition (in mM): NaCl [120], KCl [5.4], $MgSO_4.7H_2O$ [1.2], KH_2PO_4 [1.2], $NaHCO_3$ [25], glucose [11.7], $CaCl_2$ [1.26] and gassed with 95 % O_2, 5% CO_2. High calcium containing Krebs solution is made by doubling the amount of $CaCl_2$ used.

2.3.2 Tissue preparation

Ethical approval was obtained from the University of Nottingham's Animal Welfare and Ethics Review Body (UNMC#2kn and UNMC12). All applicable international, national and/or institutional guidelines for the care and use of animals were followed. Male Sprague Dawley rats (235-440 g; 2-3 months old) purchased from University Putra Malaysia, were euthanized using carbon dioxide and sacrificed on the day of experiment. The rat trachea, aorta and bladder were immediately excised and transferred into Krebs-Ringer carbonate solution. The tissues were cleaned and cut into strips or rings into desired length. The trachea was cut into 2 mm rings, aorta into 4 mm rings, and bladder into 5 x 2mm (length x width) strips. The methods used in this chapter were adopted from (Loong *et al.*, 2015).

Porcine hearts were obtained from the local abattoir in Nottingham, UK. The hearts were kept in Krebs-Henseleit solution and iced. No consideration for porcine gender and age were taken. The myocardium surrounding the left anterior descending coronary artery was harvested and immediately transferred into oxygenated Krebs solution. Tissues were stored in 4°C overnight and used in organ bath studies the day after. Remaining connective and fat tissues were removed from the adventitial layer and the artery was cut into 5mm-long ring segments on the day of experiment.

Male Dunkin-Hartley guinea pigs (855-998 g; 6-7 months old) were purchased from Monash University, Malaysia campus, were euthanized using carbon dioxide and sacrificed on the same day of experiment. The ileum were exteriorised and cleaned by flushing out the contents of ileum with Krebs-Ringer solution. The ileum was then divided into 5-6 cm segments and tied to electrode before inserting into high calcium containing Krebs-ringer solution.

2.3.3 Organ bath assembly

Rat tissues - The rat tracheal and aortic rings were suspended on metal wire triangles into the baths (Jespersen *et al*., 2015) (see **Figure 2.5A**). The bladder strips were mounted as (Kullmann *et al*.,2014) (**Figure 2.5B**).

PCA - The coronary artery was identified and isolated from the myocardium. The segments were mounted onto both triangular-shaped wire holder and mounting hook before placement in an organ bath. Any considerable stretch or force applied on the target tissue during the dissection was carefully avoided (Jespersen *et al*., 2015) (see **Figure 2.5A**).

Guinea-pig ileum- The ileum segment was tied to electrode on one end, while the other end was connected to force transducer via surgical suture thread (see **Figure 2.5C**).

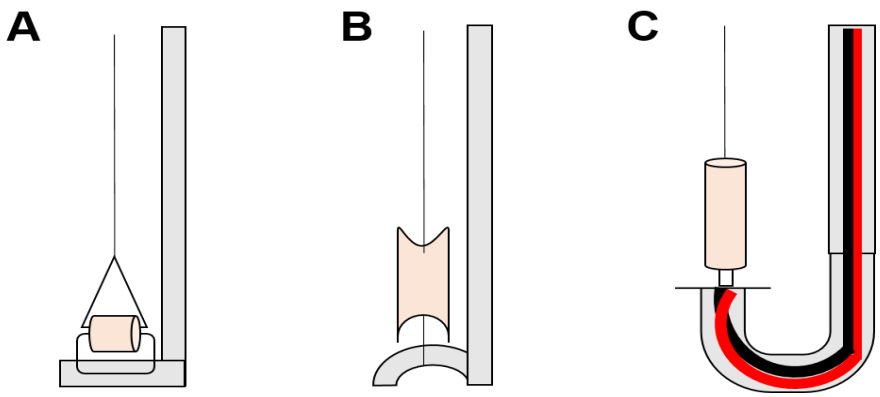


Figure 2.5: Schematic representative diagram of different tissues in the experiment ; (**A**) illustrates the horizontal mounting of aortic / tracheal rings / PCA to a glass rod and a triangle hook (**B**) illustrates the vertical mounting of bladder strip in which one end of strip is tied to metal road and the other end to a force transducer (**C**) illustrates the vertical mounting of ileum segment to an electric electrode for electrical stimulation, at which one end of segment is tied to an electrode rod while the other to a transducer via surgical suture thread.

The baths were filled with 10 ml Krebs-Ringer (rat isolated tissues and guinea pig ileum) / Hansleit solution (PCA) and maintained at 37°C and pH 7.4, with aeration of 95% O_2 and 5% CO_2.

These preparations were then connected to an isometric force transducer via a long surgical suture thread. Changes in muscle tension responses produced by tissue contraction were detected by a force transducer (MLTF050/ST, AD Instruments, US). Data were recorded by a PowerLab data acquisition system (LabChart v7.3.4) and a computer (Hewlett-Packard, US). Muscle tension was expressed in milliNewtons (mN). Tissues were left to equilibrate to bath

conditions for 30 minutes after the application of tension: 9.8 mN to trachea and bladder, 19.6 mN to aorta, 4.9 mN to guinea pig ileum and 98 mN to PCA. Following equilibration, all tissues except guinea pig ileum were exposed twice to 60 mM potassium chloride (KCl) to assess tissue viability and to provide reference contractions for subsequent data analysis. After the KCl was washed out and a stable basal tone was re-established, experiments were carried out as in **section 2.3.4**.

For guinea-pig ileum, the submaximal voltage suitable to be used for subsequent stimulation has been determined prior to conducting experiments (Rodriguez *et al*.,2009). The submaximal voltage was determined by doing the following,

1) First, the the frequency was set at 0.1 Hz for a duration of about 2 minutes
2) Then the stimulation was started at 5 volts, and then the voltage was increased by 5 volts until 20 volts.

The experiment in this study was done by stimulating the tissue electrically at 0.1 Hz, 10 volts.

2.3.4 Organ bath experimental protocols

2.3.4.1 Effect of FK70 on pre-contracted rat isolated aortae

The aorta was pre-constricted with phenylephrine (0.1 μM). The concentration of contractile agents used elicited at least 70% submaximal of contraction. Once a stable tone was established, FK70 concentration-response curves were constructed. The concentration range used was between (1 nM to 100 μM). Only one CRC was performed per tissue.

In order to compare the effect of FK70 with Sch A and other known drugs, Sch A, dobutamine and verapamil concentration response curves were performed. The aortae were pre-constricted with phenylephrine (0.1 μM) prior to addition of the drugs cumulatively. The concentration range used was between (1 nM to 100 μM). Only one CRC was performed per tissue.

2.3.4.2 Effects of FK70 on pre-contracted rat isolated trachea and bladder

The effect of FK70 on other tissue types apart from the aorta, trachea and bladder were then investigated. The tracheal rings and bladder strips were pre-constricted with carbachol (1 μM). The concentration of contractile agent used elicited at least 70% submaximal of contraction. Once a stable tone was established, FK70 concentration-response curve was constructed on each tissue types tested. The concentration range used was between (1 nM to 300 μM).

2.3.4.3 Effect of FK70 on pre-contracted porcine coronary artery (PCA)

To further confirm the vasorelaxant effect of FK70, another type of blood vessel from different animal model (PCA) was employed. The PCA was pre-constricted with 60 mM KCl prior to performing concentration response curve of FK70 (10 nM to 300 µM).

2.3.4.4 Effect of FK70 on guinea-pig ileum

After stable electrically-evoked contractions were obtained, FK70 concentration response curves were performed on fresh ileum segments. The concentration range used was between (1 nM to 300 µM). Only one CRC was performed per ileum segment.

2.3.5 Data analysis

Data were analysed and graphs were drawn using PRISM version 7.0 (GraphPad software). All data were expressed as mean ± standard error of mean (SEM) of *n* number of animals (rat tissues and PCA) while *n* for guinea pig ileum indicates number of segments. Maximum tissue contraction or relaxation response and concentration to produce 50 % response (pEC_{50}) was derived from nonlinear regression analysis of the obtained CRC. Statistical analyses were performed using Student's unpaired *t*-test. Unpaired *t*-test was used to compare the statistical differences from two experimental groups: one from a control condition while another from a treatment condition [PRISM Version 7 (Graph Pad Software)]. For comparing the statistical differences of more than two experimental groups (between control and different treatment conditions), one-way ANOVA followed by Dunnett's multiple comparisons test was applied (PRISM Version 6; Graph Pad Software). Results were considered statistically significant if $p < 0.05$.

2.4 Results

2.4.1 FK70 relaxed rat aorta at similar magnitude to Sch A and verapamil

The relaxation effect of FK70 in rat aorta was compared with the parent novel compound Sch A. FK70 relaxed the aortic rings with similar efficacy to Sch A (see **Figure 2.6A** and **Table 2.2**). Aortic rings treated with DMSO served as control. DMSO relaxed the aortic rings with a maximal value of (27.1 ± 6.0 %). The maximum concentration of DMSO added into the bath was 1%.

Interestingly, FK70 displayed similar efficacy to verapamil and showed better efficacy when compared to dobutamine in rat aorta (see **Figure 2.6B**).

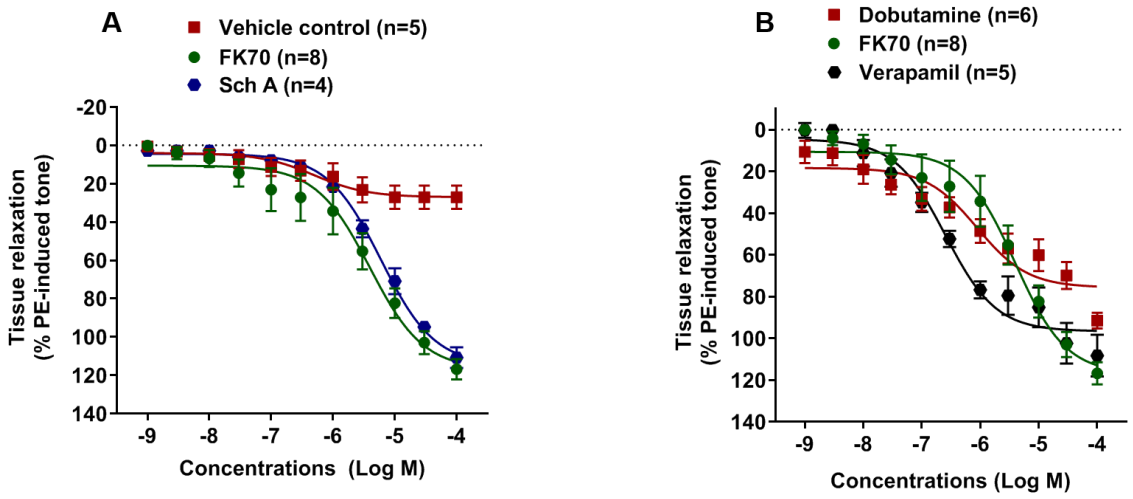


Figure 2.6: Effects of **A)** control, FK70 and Sch A **B)** dobutamine, FK70 and verapamil concentration-response curves in rat isolated aortic rings. Tissue responses have been expressed as PE-induced contraction and are shown as means ± SEM of 4 to 8 animals.

Table 2.2: Summary of effects of vehicle control, FK70, Sch A, dobutamine and verapamil in rat aortae. Tissue responses have been expressed as PE-induced contraction and are shown as means ± SEM of 4 to 8 animals. The one-way ANOVA followed by Dunnett's multiple comparison test compared the maximal relaxation effect between FK70, and all other treatment groups: control, Sch A, dobutamine and verapamil (*p<0.05, ****p<0.0001, a p value of >0.05 is considered no significant difference). NA not applicable.

Compound	Number of animal (n)	Maximal relaxation effect at (10^{-4} M) (%)	*p* value
Vehicle control	6	27.1 ± 6.0 ****	<0.0001
FK70	8	116.8 ± 5.2	
Sch A	4	110.7 ± 5.3	0.93
Dobutamine	6	91.4 ± 3.7*	0.02
Verapamil	5	108.2 ± 9.9	0.5

2.4.2 FK70 relaxed rat isolated trachea and bladder

FK70 also induced relaxant effects in other rat isolated tissues tested, in trachea and bladder (see **Figure 2.7**). Tracheal rings and bladder strips treated with DMSO served as control for this experiment. The vehicle control did not seem to have any significant effect on both the tissues. The relaxation response of FK70 is more rapid in trachea than that of bladder.

The response in bladder was disturbed due to development of spontaneous contractile oscillation after the addition of carbachol. FK70 reduced the spontaneous contractile oscillations in a concentration-dependent manner (see **Figure 2.8**).

No significance difference was observed in FK70 maximal relaxation response in aorta, trachea and bladder (one-way ANOVA, $p > 0.5$)(see **Table 2.3**).

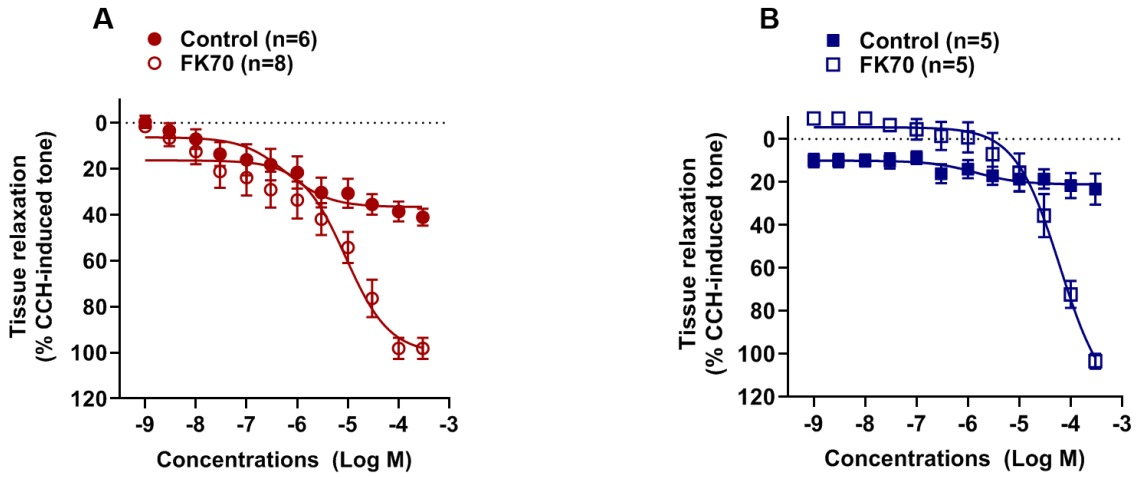


Figure 2.7: Effects of control and FK70 concentration-response curve in rat isolated **A)** trachea **B)** bladder. Tissue responses have been expressed as a percentage of carbachol-induced contraction and are shown as means ± SEM of 5 to 8 animals.

Table 2.3: Summary of relaxation effect of FK70 and vehicle control in aorta, trachea and bladder. Tissue responses have been expressed as agonists-induced contraction and are shown as means ± SEM of 5 to 8 animals. One-way ANOVA followed by Dunnett's multiple comparisons test comparing maximal relaxation value of FK70 in aorta with maximal relaxation values of trachea and bladder (***p*<0.01, a *p* value of >0.05 is considered no significant difference).

Tissue type	Number of animal (n)	Maximal relaxation effect of FK70 at (10^{-4} M) (%)	*p* value	Maximal relaxation effect of vehicle control (%)
Aorta	8	116.8 ± 5.2		27.1 ± 6.0
Trachea	6	98.1 ± 4.4	0.3	38.4 ± 2.4
Bladder	5	103.6 ± 3.4	0.007	23.2 ± 7.1

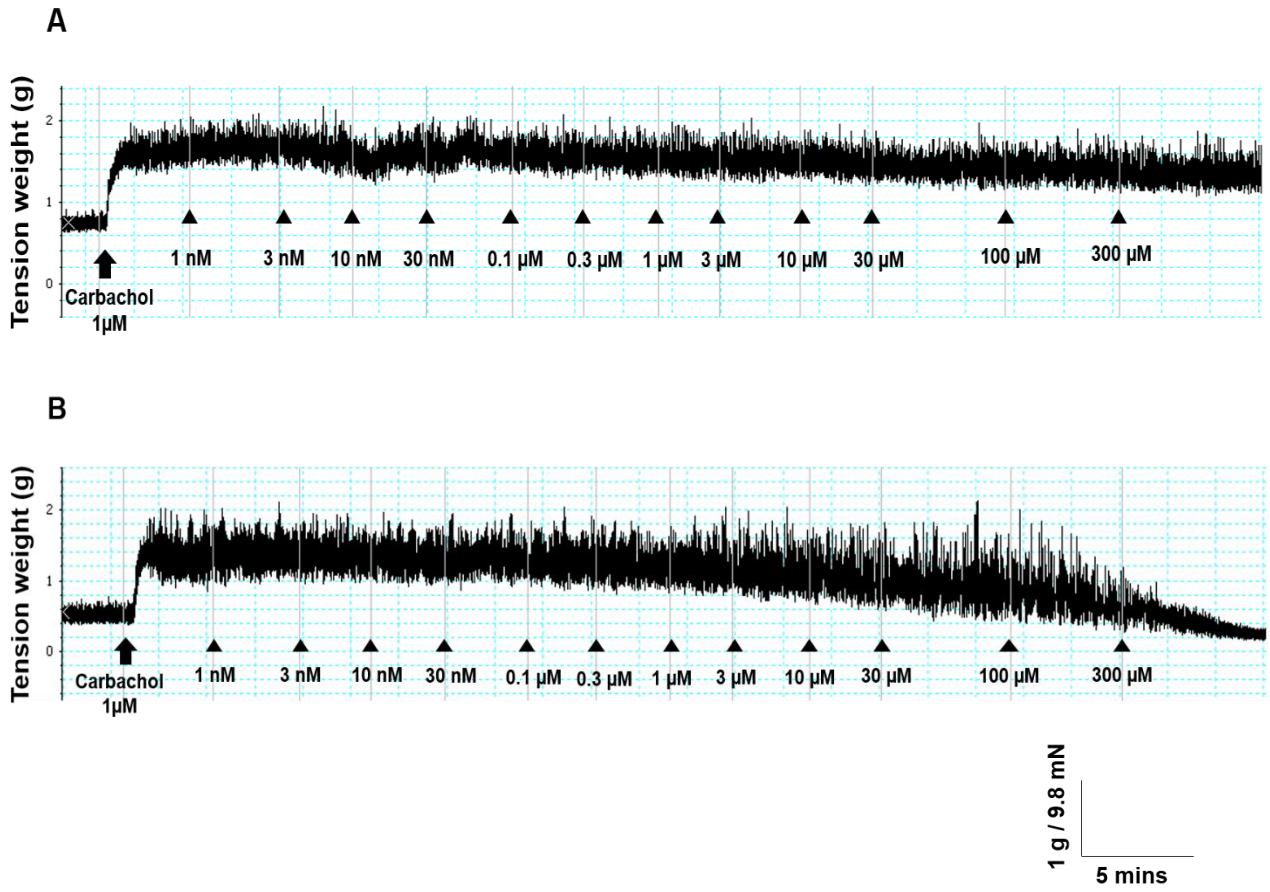


Figure 2.8: Representative trace recordings showing the concentration-dependent effects of **A)** vehicle control and **B)** FK70 in bladder strips pre-contracted with carbachol. The spontaneous contractile activity in bladder strips started to occur during contraction to carbachol. This effect was reduced by FK70 in a concentration dependent manner while treatment with vehicle control did not seem to affect the spontaneous contractile activity.

2.4.3 FK70 relaxed porcine coronary artery (PCA)

To further confirm the FK70-induced relaxation effect in blood vessel smooth muscle, PCA have been employed. FK70 significantly relaxed PCA (FK70 = 126.1 ± 10.8%, vehicle control = 42.5 ± 9.9%; unpaired *t*-test, *p*=0.0003) (see **Figure 2.9**).

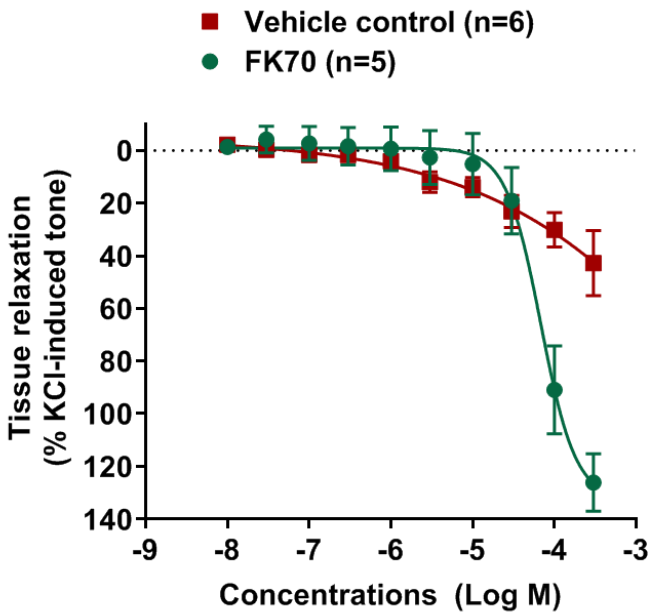


Figure 2.9: Effects of vehicle control and FK70 concentration-response curve in porcine coronary arteries. Tissue responses have been expressed as a percentage of KCl-induced tone and are shown as means ± SEM of 5 to 6 animals.

2.4.4 FK70 relaxed guinea pig ileum

In order to study the effect of FK70 on gastrointestinal smooth muscle, FK70 have been tested on guinea pig ileum segments. The vehicle control did not seem to inhibit electrically stimulated guinea pig ileum segments when compared to FK70 (vehicle control = -39.4 ± 14.6 %, FK70 = 105.7 ± 8.0 %; unpaired *t*-test, $p < 0.0001$) (see **Figure 2.10**). FK70 relaxed the guinea pig ileum segments in a concentration dependent manner, at which higher concentration of FK70 abolished the electrically evoked contraction (see **Figure 2.11**).

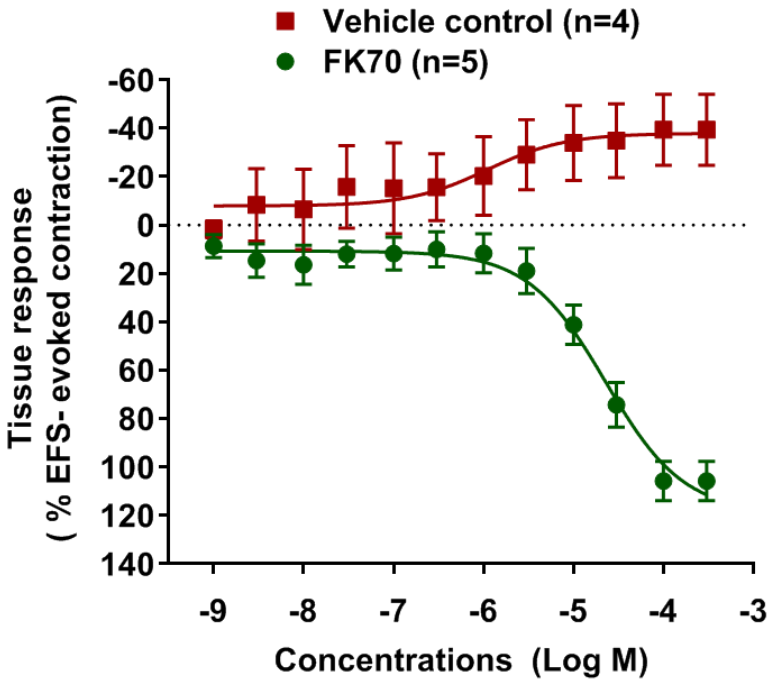


Figure 2.10: Effects of control and FK70 concentration-response curve in guinea pig ileum segment. Tissue responses have been expressed as a percentage inhibition of electrical field stimulated (EFS)-evoked contraction and are shown as means ± SEM of 4 to 5 segments

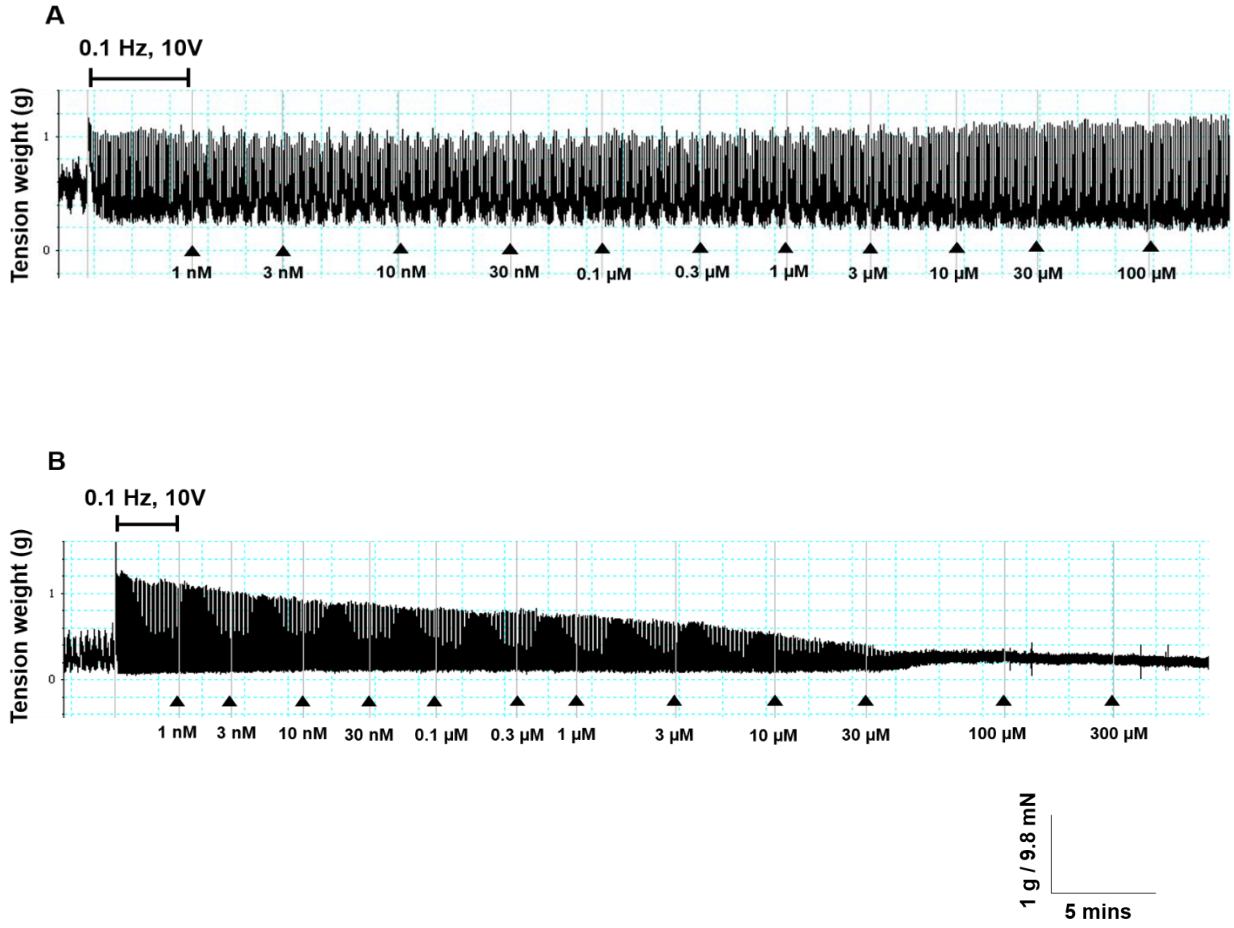


Figure 2.11: Representative trace recordings showing the concentration-dependent effects of **A)** vehicle control and **B)** FK70 in electrically evoked guinea pig ileum segments. Treatment with vehicle group did not seem to affect the contractile activity of the ileum segments while FK70 reduced the contraction in a concentration dependent manner.

2.5 Discussion

The present study demonstrated the effects of FK70 in different types of tissues and animals. The main reason for using different smooth muscle tissues from various animal species in this chapter was to evaluate FK70's effect on different biological systems. This study was useful in determining the general characteristics of FK70 in vascular smooth muscle and other smooth muscle tissues.

As aforementioned, FK70 was synthesised chemically based on Sch A, a novel and major compound isolated from *Ficus schwarzii*. Sch A was characterised as calcium antagonist in the blood vessel (Loong unpublished data, 2016). However, due to limited natural sources of the plant, FK70 was synthesised as means to elucidate the mechanism of action of these novel compounds continually. Based on Lipinski rule of five prediction, FK70 was shown to have better physiochemical properties or drug-like properties compared to Sch A. FK70 exhibited a similar degree of vasorelaxant effect as Sch A in the aorta. As such, it can be concluded that FK70 behaves like Sch A in the aorta and thus making it a reliable alternative compound for further characterisations.

Perusing the data from different types of smooth muscle studied in this chapter, FK70 showed higher relaxation response in the smooth muscle of blood vessels. FK70 managed to relax the pre-contracted and electrically evoked tissues. Generally, the relaxation response in smooth muscle occurs as a result of several factors. The first factor is the direct inhibition of either extracellular or intracellular Ca^{2+}. The second is through the activation of cyclic AMP or cGMP pathway mediated by GPCRs (e.g. α / β-adrenoceptors and muscarinic receptors) stimulation, thirdly via endothelium-derived relaxing factors (EDRF). The intracellular Ca^{2+} level is also influenced by calcium channel activated potassium channels and sodium-calcium exchanger (NCX). In this study, the rat aortae were pre-contracted with PE, which led to the activation of α-adrenoceptors while the muscarinic subtype 3 (M_3) in rat trachea and bladder were activated by carbachol. KCl evoked the PCA tissues, and the guinea pig ileum contractions were induced by electrical stimulation. These experiments, therefore, suggest that the contractile responses of all the tested tissues were as a result of extracellular calcium entry and intracellular calcium release.

FK70 resembles known phenylakylamine drugs dobutamine and verapamil, a calcium channel inhibitor; as such the relaxant effect of FK70 was compared with the known vasodilators. Based on the maximal response measured, the compounds relaxed the pre-constricted aorta in the order of FK70 > verapamil > dobutamine. Dobutamine is an ionotropic drug which acts as an agonist on both β and α-adrenoceptors (Vallet *et al.,*1991). It causes vasodilation by stimulating β_2-adrenoceptors in blood vessels. Generally, in vascular smooth muscle tissues, it has been suggested that the initial transient contraction induced by phenylephrine is the result of the release of intracellular calcium, while the sustained contraction is induced by calcium influx (Hamada *et al.,*1997; Lee *et al.,*2001). In this experiment, dobutamine had reduced the phenylephrine-induced contraction suggesting that the relaxation response is not only via β-adrenoceptors but might also involve α-adrenoceptors action. Verapamil, on the other hand, elicits relaxation effect in the aorta by inhibiting calcium entry via the voltage-gated calcium channels. As the vasorelaxant profile of FK70 resembled more that of verapamil, it can be hypothesised that FK70 possesses calcium inhibiting actions, which could play a substantial role in its dilator actions in the aorta. Concerning the structural activity relationship of amines, typically the primary and secondary amines in a compound have good adrenergic activity, whereas tertiary amines and quaternary ammonium salts do not. Besides that, the nature of amino substituent also affects the receptor selectivity of a compound. Considering all the points that have been discussed above, it is worth investigating the possible effect of FK70 (a secondary amine) on calcium channels and adrenoceptors.

The relaxation response of FK70 in aorta also suggest the involvement of α-adrenoceptors, as aorta appears to contain more α-adrenoceptors in the blood vessel smooth muscle as compared to in tracheal smooth muscle (Barnes and Basbaum, 1983). Moreover, FK70 reduced PE-induced contraction markedly in the aorta. The PE-induced contraction in rat aorta is caused by α-adrenoceptors, particularly via the activation of α_1-adrenoceptors (Iwanaga *et al*.,1998). Besides adrenoceptors, the role of the endothelium in the aorta should also be considered as a possibility. The endothelial cells are located on the intima, and they control vascular function by responding to various hormones, neurotransmitters, and vasoactive factors which affect vasomotion, thrombosis, platelet aggregation, and inflammation. The endothelium forms an integral part of the vasculature and is involved in promoting vasodilation via the actions of EDRFs. Some examples of EDRFs are factors such as nitric oxide (NO), prostacyclin (PGI_2) and endothelium-derived hyperpolarizing factors (EDHFs). These factors cause relaxation of vascular smooth muscle by activating cGMP, cAMP and altering membrane potential of smooth muscle to reduce intracellular Ca^{2+} level, respectively (Sandoo *et al*., 2010). Therefore, it is beneficial to investigate the role of the endothelium in FK70-induced relaxation further.

FK70 had also exhibited the relaxation response in trachea and bladder which suggests a possible muscarinic antagonist and β-adrenoceptors role of FK70 in mediating relaxation in both tissue types. However, the former hypothesis was not considered for further studies as activation of muscarinic receptors in different tissue types play a different role. Activation of muscarinic receptors in trachea and bladder cause contraction while in the aorta, it causes endothelium-dependent relaxation (Boulanger *et al*.,1994). Besides that, despite FK70 exhibited concentration-dependent relaxation effect on the bladder and tracheal smooth muscle, the relaxation response of FK70 in trachea is more rapid than in bladder.

The bladder's muscular wall is formed of smooth muscle cell, which comprises detrusor muscle. The detrusor muscle is the primary muscle component of the bladder wall. The most important receptors for activation of contraction are muscarinic (M_3) and purinergic receptors (P2X1). The contribution of these receptors to contraction may differ between species. In normal human detrusor and rat bladder, the muscarinic component predominates, and the main relaxant pathway is via the adenylyl cyclase (AC) / cAMP pathway. As a note, the adenylyl cyclase (AC) / cAMP pathway is activated by β_3-adrenoceptors (Andersson and Arner, 2004; Yamaguchi and Chapple 2007; Kullmann *et al*.,2009). Generally, the detrusor muscle exhibits spontaneous contractions, and it is associated with slow waves of depolarization. The frequency of the spontaneous action potentials is voltage-sensitive and is primarily modulated by intracellular Ca^{2+} level (Babu *et al.*,2001). The depolarisation of the muscle is generated by inward Ca^{2+} current while the repolarisation is generated by inactivation of Ca^{2+} and activation of outward K^+ current (Montgomery *et al*.,1992). In a previous study done on rat bladder strips, it was concluded that Ca^{2+} influx, Rho-kinase activation, and calcium-activated potassium channels deactivation might play essential roles in the spontaneous contractions (Wang *et al*.,2018). In this study, FK70 suppressed the spontaneous contraction at a higher concentration, suggesting non-selective inhibition of FK70 at the mentioned receptors and possess blocking effect on calcium-dependent contraction.

Both aorta and coronary arteries are known as the larger arteries and share the same vessel structural arrangement, consisting of tunica intima, tunica media, and tunica adventitia. In the present study, the maximal relaxation effect of FK70 was slightly higher in PCA than in rat aorta. The possible explanation for this observation could be due to the size difference between PCA and rat aortic tissues which would have affected the responses to FK70. Theoretically, the vascular response can be enhanced by the greater amount of smooth muscle cells within the layer. Besides that, the porcine coronary artery smooth muscle is also β-adrenoceptors dominant, and activation results with an increase in cAMP and reduction of cytosolic Ca^{2+} (Shogakiuchi *et al*.,1991), while the aortic smooth muscle is α-adrenoceptors dominant. This finding further recommends the evaluation of the effect of FK70 on α and β-adrenoceptors.

The ileum is a segment of the intestines between the pyloric sphincter and colon. Like the rest of the alimentary canal, the ileum consists of a tube of muscle and epithelial layers, innervated by bundles of fibres and highly vascularised. The muscle layers possess contractility that is modulated by the nervous inputs in the myenteric plexus and within the myenteric plexus are the ganglia linking pre and post-ganglionic neurons. Opioid, adrenoceptors and muscarinic receptors modulate the neurogenic contraction of the ileum. According to past studies, the contractile response of guinea-pig ileum to electrical stimulation involves the excitation of post-ganglionic cholinergic nerve endings and the release of acetylcholine (Ach) (Paton, 1955; Scriabine and Peklak, 1970; Jin *et al.*,1989). The neuronally released Ach either triggers the release of Ca^{2+} from an internal store via muscarinic receptors or causes membrane depolarisation via the opening of a set of voltage-independent Ca^{2+} channels (Cousins *et al*., 1993). The adrenaline and noradrenaline acting on (α / β-adrenoceptors) on the cholinergic nerve inhibit the Ach-induced contractions in guinea-pig ileum (Bauer, 1982). In this study, FK70 managed to reduce the electrically stimulated guinea-pig ileum at a significantly higher rate when compared to the control. Hence, a case to investigate further on adrenoceptors and calcium in FK70-induced relaxation can be established.

Overall, FK70 displayed relaxant activity on all the tissue tested with no evidence of a contractile effect. Further investigation of FK70-induced relaxation mechanisms was carried out on rat aorta to address the research problem while taking into consideration of cost, availability, and reproducibility. Other possible contributing factors to the mechanism of the FK70-induced relaxant effects were investigated and discussed in Chapter 3.

Chapter 3: Investigation of mechanism of relaxation to FK70 in rat aortae

3.1 Introduction

In the previous (**chapter 2**), the pharmacological screening of FK70 had revealed that FK70 elicited relaxation effect in all the tissues tested from various animal species, rats (aorta, trachea, and bladder), pig (coronary artery), and guinea pig (ileum). However, FK70 exhibited slightly higher relaxation effect on the vascular smooth muscle tissues. These exciting results prompted the investigation of the mechanism of action of FK70. This chapter discusses the possible mechanisms involved in the vasorelaxation effect of FK70 in rat aorta.

3.1.1 Vascular smooth muscle relaxation pathways

The vascular smooth muscle relaxation is determined by the level of intracellular Ca^{2+}, where the lowering of the intracellular Ca^{2+} concentration is a trigger for vascular smooth muscle relaxation. The intracellular calcium plays a central role in mediating the vascular activity by initiating the phosphorylation of myosin light chain (MLC) by enzyme myosin light chain kinase (MLCK) (Woodsome *et al.*,2006; Shen *et al.,*2010). Several modulators affect the concentration of intracellular Ca^{2+} via different signaling pathways. They include endothelium-derived relaxing factors (EDRFs, e.g., nitric oxide, prostacyclin, and EDHFs), activation of the potassium channel, activation of β-adrenoceptor-mediated pathway or blockade of α-adrenoceptors, and inhibition of calcium channels and downstream pathways: involving cyclic nucleotides (e.g., cGMP or cAMP).

The vascular endothelium has been known to synthesis various vasodilating mediators. The mediators include nitric oxide, EDHF (endothelium-derived hyperpolarising factors), and prostacyclin (Heilpern, 2008). Nitric oxide (NO) gas is released from nitrosothiols in hemoglobin or from endothelial cells, diffuses into smooth muscle cells that blood vessels. The NO binds to an enzyme, called guanylate cyclase (GC), resulting in GC activation. Activated GC cleaves two phosphate groups from another compound called guanosine triphosphate (GTP) and leads to the formation of cyclic guanosine monophosphate (cGMP) that is used to phosphorylate smooth muscle contractile protein called myosin resulting in dilation of the vessel that was initially exposed to NO (Heilpern, 2008).

The EDHF is a labile metabolite of arachidonic acid formed through the P-450 pathway (Vanhoutte *et al*., 1995). EDHF act on smooth muscle by opening potassium channels and on one of the few endogenous openers of these channels to cause hyperpolarisation of the smooth muscle, thus resulting in relaxation of the smooth muscle. The release of EDHF by the endothelial cell is controlled by the intracellular Ca^{2+} concentration (Feletau and Vanhoutte, 2006).

There are four major classes of K^+ channels that have been detected in vascular smooth muscle cells, voltage-dependent K^+ (K_v) channels, Ca^{2+}-activated K^+ (BK_{Ca}) channels, ATP-sensitive K+ (K_{ATP}) channels, and inward rectifier K^+ (K_{ir}) channels (Nelson and Quayle, 1995; Standen and Quayle, 1998; Ko *et al.*,2008). The K_v channels open upon depolarization of the plasma membrane in vascular smooth muscle cells, subsequently leading to the efflux of K^+ through the channels to induce repolarization to the resting membrane potential (Nelson and Quayle, 1995; Sobey 2001; Korovkina and England, 2002). Changes in the intracellular Ca^{2+} concentration and membrane depolarization stimulate large-conductance Ca^{2+} -activated K^+ (BK_{Ca}) channels, which are thought to play an essential role in maintaining the membrane potential (Ko *et al.*, 2008). The K_{ATP} channels form the crucial functional bond between cellular metabolism and membrane excitability. The blockade of K_{ATP} channel function results in vasoconstriction and depolarization in various types of vascular smooth muscle (Nelson *et al.,* 1990; Nakashima and Vanhoutte 1995; Quayle *et al.,* 1997; Teramoto, 2006). The K_{ir} channels, expressed mostly in the smooth muscle of the small-diameter arteries, contribute to the resting membrane potential and basal tone. K_{ir} channel activation has been shown to raise the extracellular K^+ concentration to 10-15 mM, resulting in vasodilation (Ko *et al.,*2008).

Prostacyclin on the other hand, causes relaxation of vascular smooth muscle by activating adenylate cyclase (AC) and increasing the production of cyclic 3',5'-adenosine monophosphate (cAMP) (Boie *et al.,* 1994) (see **Figure 3.1**).

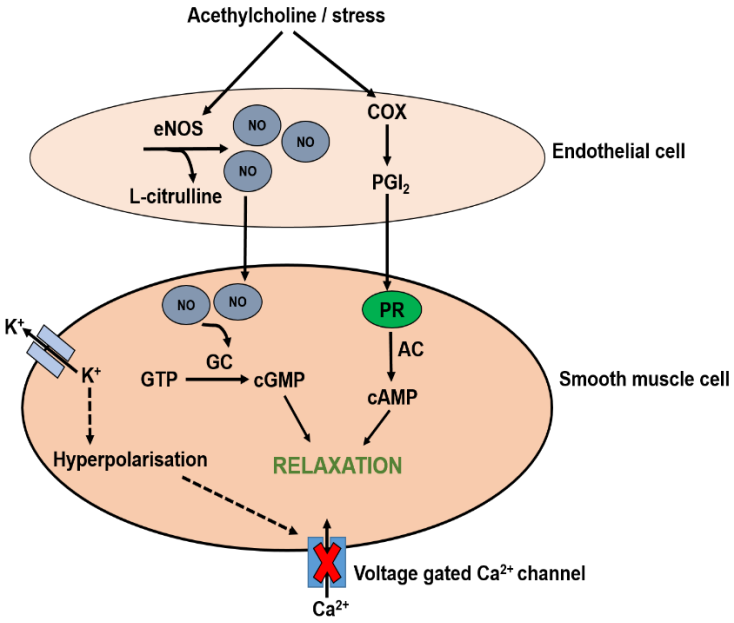


Figure 3.1: Schematic illustration of mechanism of endothelium mediated relaxation. Agonist e.g acetylcholine or shear stress increases the activity of endothelial NO synthase (eNOS) and cyclooxygenase (COX), resulting in nitric oxide (NO) and prostacyclin (PGI_2) mediated relaxation. NO activates cGMP, while PGI_2 activates cAMP. The cGMP activates MLCP, the enzyme that dephosphorylates myosin light chains, which leads to relaxation while cAMP inhibit MLCK to cause relaxation. Ca^{2+} influx further increases the calcium concentration and activates K channels to cause hyperpolarization and release of K^+ into the subendothelial space. The increase in K^+ in the interstitium activate K^+ channels on smooth muscle cells to cause hyperpolarization. Smooth muscle hyperpolarization reduce Ca^{2+} influx via closing of voltage-gated Ca^{2+} channels, subsequently leading to the relaxation of smooth muscle. (Picture adapted from Ozkor and Quyyumi 2011). *AC: adenylyl cylase ; GC: guanyl cyclase; cAMP: cyclic adenosine monophosphate; GTP: guanosine triphosphate; cGMP: cyclic guanosine monophosphate; NO: nitric oxide; MLCP : myosin light chain phosphatase; MLCK : myosin light chain kinase; K^+: potassium ion; COX: cyclooxygenase; PR: prostacyclin receptor; PGI: prostacyclin*

G-protein-couple signal transduction pathways also regulate the degree of MLC phosphorylation and the vascular smooth muscle contractile and relaxation tone. G-protein coupled receptors (GPCRs) are the most abundant transmembrane receptors in the cells (Rosenbaum *et al.,*2009). This receptor superfamily consists of some significant regulatory receptors in vascular activity, such as adrenoceptors, serotonin, thromboxane, and muscarinic receptors. These receptors can be classified based on their specific coupling to different G protein subtypes, including G_q, $G_{12/13}$, $G_{i/o}$ and G_s proteins, at which coupling via G_q or $G_{12/13}$ or $G_{i/o}$ proteins results in the vasoconstriction, whereas coupling via G_s-protein results in vasorelaxation (Wettschureck and Offermanns, 2005). G_q-protein activation by compounds such as norepinephrine (α_1-adrenoceptors), angiotensin II (AT1-receptors), endothelin-1 (ET_1-receptors) and vasopressin (V_1-receptors) stimulates sarcoplasmic reticulum (SR) release of calcium (IP_3 mediated) and activates Rho-kinase, which inhibits MLC phosphatase (MLCP) leading to contraction. In contrast, G_s-protein activation via β_2-adrenoceptors, A_2- purinergic receptors, and IP receptors increase cAMP, which inhibits MLCK, thereby reducing MLC

phosphorylation and cause relaxation. On the other hand, G_i-protein activation elicits contraction by reducing cAMP, which increases the activity of MLCK. In most tissues, the concentrations and the actions of cyclic AMP and cyclic GMP are regulated by cyclic nucleotide phosphodiesterases (PDEs) (Komas *et al.*, 1991). The PDEs are located in the SR of cardiac muscle (Kauffman *et al.*, 1987). They catalyse the hydrolysis of cAMP and cGMP, thereby regulating the intracellular concentrations of these cyclic nucleotides. cAMP- or cGMP-activate protein kinases protein kinase A (PKA) or PKG, respectively. Inhibitions of PDE increase cAMP and cGMP, thus leading to vasorelaxation. PDE3 inhibitors were discovered to exhibit cardiotonic, inotropic, bronchodilatory, and vasodilatory activities in several species (Maurice *et al.*, 2014). Taken together, the relaxation of vascular smooth muscle can be achieved by either activating or blocking the specific channels involved, pharmacologically (see **Figure 3.2**).

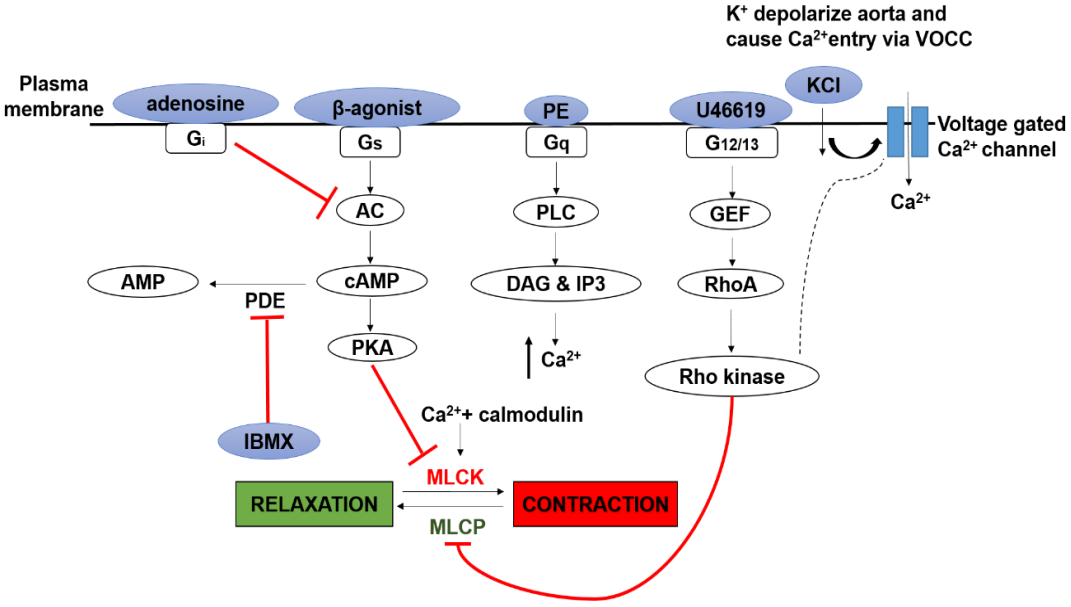


Figure 3.2: Schematic illustration of contraction and relaxation mechanisms in vascular tissues stimulated by various pharmacological agents. Vascular contraction can be triggered by the activation of either $G_{12/13}$ / G_q / G_i- protein-coupled receptors and the opening of calcium channels through different signalling pathways. KCl depolarises the membrane to allow calcium entry via the voltage-gated calcium channels to cause contraction. $G_{12/13}$ is typically stimulated by U46619, while G_q-protein by phenylephrine (PE) and G_i-protein by adenosine. The inhibition of these signalling pathways can be achieved by using the respective G-protein coupled receptor antagonists or calcium channel blockers, which would lead to the relaxation of vascular tissues. Activation of G_s-protein-coupled receptors by β-adrenoceptors agonists results in relaxation. These modulators elicit the downstream cyclic nucleotides- (cGMP or cAMP)-mediated signalling pathways. Phosphodiesterase (PDE) can convert cAMP / cGMP into their inactive form (AMP / GMP), hence causing contraction. This event can be reversed by PDE inhibitors e.g (IBMX). (Picture adapted from Wettschureck and Offermanns, 2005). *MLCP: myosin light chain phosphatase; PLC: phospholipase C; DAG: diacylglycerol; IP_3: inositol triphosphate; MLCK: myosin light chain kinase; AC: adenylyl cyclase; cAMP: cyclic adenosine monophosphate; PKA: protein kinase A; GC: guanylyl cyclase; cGMP: cyclic guanosine monophosphate; PKG: protein kinase G; PDE: phosphodiesterase; AMP: adenosine monophosphate; GMP: guanine monophosphate GEF: guanine nucleotide exchanger factors; PE: phenylephrine; KCl: potassium chloride*

3.2 Study aim and objectives

This study was designed to investigate the mechanism of relaxation to FK70 in rat aorta. In order to study the underlying mechanism of FK70, several objectives have been set and were carried out as follows;

i) to investigate the effects of FK70 in response to different contractile agents (phenylephrine, KCl and U46619).

ii) to study the effects of FK70 on calcium entry in calcium-free Krebs Ringer solution

iii) to investigate the role of endothelium and endothelium-derived factor, NO by endothelium removal, using a NO donor (SNP) and NO inhibitor (L-NAME).

iv) to investigate the role of potassium channels on FK70-induced relaxation by using specific potassium channel inhibitors, TEA (calcium-activated potassium channel inhibitor, K_{Ca},) and 4-AP (voltage-gated potassium channel inhibitor, K_v,).

v) to investigate the possible adrenergic effect of FK70 in α- and β-adrenoceptor pathways using relevant antagonists : for α-AR using (rauwolscine and prazosin); and for β-AR using (timolol and propranolol).

vi) to investigate the role of cAMP and cGMP on FK70-induced relaxation by using phosphodiesterase inhibitor, IBMX

3.3 Materials and methods

3.3.1 Drugs and Krebs-Ringer bicarbonate solution

FK70 was prepared as described in **Section 2.3.1** (Chapter 2). All dissolved drugs were made into stock concentration of 0.1 M. A serial of ten-fold dilution was made for all dissolved drug solutions. Details on the drugs, solvents used and stock concentration for each drug are summarised in **Table 3.1.**

Calcium-containing Krebs solution (or normal Krebs solution) was prepared as described in **Section 2.3.1.** Calcium free Krebs-Ringer solution was made in the similar composition as normal Krebs-Ringer solution but without the inclusion of $CaCl_2$ in the solution.

Table 3.1: Summary of drugs used, country manufactured, solvent and stock concentration used in the experiments.

Drugs	Brand / Country manufactured	Solvent	Stock concentration (M)
4-aminopyridine (4-AP)	Tocris Bioscience, UK	100 % DMSO	1
Phenylephrine hydrochloride	Sigma, USA	Distilled water	0.1
N^G-nitro-L- arginine methyl ester hydrochloride (L-NAME)	Tocris Bioscience, UK	Distilled water	0.1
Sodium nitroprusside (SNP)	Sigma, USA	Distilled water	0.1
U46619	Sigma, UK	100 % DMSO	0.1
Isobutylmethylxanthine (IBMX)	Tocris Bioscience, UK	100 % DMSO	0.1
Prazosin hydrochloride	Sigma, USA	100 % DMSO	0.1
Rauwolscine hydrochloride	Tocris Bioscience, UK	100 % DMSO	0.1
Propranolol hydrochloride	Santa Cruz, USA	100 % DMSO	0.1
Tetraethylammonium chloride (TEA)	Sigma, USA	100 % DMSO	1
Timolol maleate	Santa Cruz, USA	100 % DMSO	0.1

3.3.2 Tissue preparation and organ bath assembly

Refer to **section 2.3.2**

3.3.3 Organ bath experimental protocols

3.3.3.1 Effects of FK70 in response to different contractile agents

Three different contractile agents were used in this experiment, KCl, phenylephrine (PE) and U46619 to investigate the relaxant effect of FK70. The aortic rings were pre-constricted with either KCl (60 mM), PE (0.1 µM), or U46619 (0.1 µM). The concentrations of contractile agents used in this experiment were determined based on concentrations which elicited at least 70 % submaximal of contraction when concentration-response curve was performed on rat aortic rings. Once a stable tone was established, FK70 concentration-response curve was constructed for each contractile agents. The concentration range used was between (1 nM to 300 µM). Only one CRC was performed per tissue.

3.3.3.2 Effect of FK70 on $CaCl_2$-induced contraction in calcium- free Krebs solution

To investigate the concentration dependent effect of FK70 on $CaCl_2$-induced contraction, the tissues were equilibrated for 30 minutes in the calcium-free Krebs, then the tissues were pre-incubated with FK70 (10 µM or 30 µM) for 40 minutes. This was then followed by addition of 60mM KCl to confirm the viability of tissues in calcium-free Krebs solution. Once the KCl-induced contractile response plateaued, a concentration response curve to calcium chloride was constructed. The concentration range of $CaCl_2$ used was between (0.1 µM to 3 mM). The concentrations of FK70 for pre-incubation were chosen based on their cumulative concentration-dependent relaxant effect in pre-constricted aorta.

3.3.3.3 Effects of endothelium and nitric oxide on FK70- induced relaxation

To investigate the involvement of endothelium, FK70 was applied cumulatively on endothelium-denuded aortic tissues that were pre-constricted with PE. The denudation of aortic rings was achieved by surface abrasion method, where the endothelium is removed mechanically by gently rolling the lumen of the aortic rings using blunt tip of stainless steel forceps (Gokce and Arun 2014; Bello *et al.,* 2015). The endothelial integrity and functional removal was verified by the presence or absence of the relaxant response to carbachol (1 µM) on the PE (0.1 µM) contracted aortic rings. The concentrations of carbachol and PE were chosen on the basis of their ability to cause 80% relaxation (carbachol) and contraction (PE) which were determined from their respective concentration-response curves. The aortic rings were considered intact if the relaxation induced by carbachol was greater than 80 %, and the aortic rings were assumed denuded if relaxation were less than 10% (Arsyad, 2016).

In a separate experiment, in order to further confirm the involvement of endothelium-derived factors on FK70-induced relaxation L-NAME, a nitric oxide inhibitor and SNP, a nitric oxide donor were used. The endothelium denuded rings were incubated with L-NAME (100 µM), prior to performing FK70 concentration response curve in aortic rings pre-constricted with PE (0.1 µM). The concentration range of FK70 used was between (1 nM – 100 µM).

For SNP, the endothelium intact aortic rings were incubated with FK70 (30 µM) followed by cumulative addition of SNP in aortic rings pre-constricted with PE (0.1 µM). The concentration range of SNP used was between (0.01 nM – 1 µM).

3.3.3.4 Effects of potassium channels on FK70-induced relaxation

In order to investigate the involvement of potassium channels in the relaxation of FK70, specific potassium channel inhibitors, TEA (K_{Ca}, calcium-activated potassium channel inhibitor), and 4-AP (K_v, voltage-gated potassium channel inhibitor) were used. The aortic rings were incubated with TEA (3 mM) and 4-AP (1 mM) for 40 minutes followed by cumulative addition of FK70 in aortic rings pre-constricted with PE (0.1 µM). The concentration range of FK70 used was between (1 nM to 300 µM).

The concentrations of potassium channel inhibitors used in this experiment were referred from (Tammaro *et al.,*2004; Panthiya *et al*.,2019).

3.3.3.5 Effects of α- and β-adrenoceptor antagonists on FK70- induced relaxation

As aforementioned, α- and β-adrenoceptors are mainly involved in the contraction and relaxation of vascular smooth muscle. Therefore, the effects of these receptors on FK70 had been studied using α- and β-adrenoceptor antagonists. In order to investigate whether FK70-induced relaxation was caused due to blockade of α-adrenoceptors, the aortic rings were incubated with rauwolscine (10 µM), an α_2- selective adrenoceptor antagonist and prazosin (10 µM), an α_1- selective adrenoceptor antagonist for 40 minutes, prior to the cumulative addition of FK70 on aortic rings pre-constricted with 60 mM KCl. The concentration range of FK70 used was between (1 nM to 100 µM).

In order to investigate whether FK70-induced relaxant response is influenced by the activation of β-adrenoceptors, β-adrenoceptor antagonists, timolol and propranolol have been employed. Timolol (3 μM), a β_2- selective adrenoceptors antagonist and propranolol (3 μM), a non-selective β- adrenoceptors antagonist were incubated for 40 minutes before the cumulative addition of FK70 on aortic rings pre-constricted with PE (0.1 μM).

The concentrations of α- adrenoceptor antagonists and β- adrenoceptor antagonists used in this experiment were referred from (Macia *et al*.,1984; Mostaghim *et al.,*1986).

3.3.3.6 Effects of cAMP and cGMP on FK70-induced relaxation

In order to investigate the involvement of cGMP / cAMP in the relaxation of FK70, a non-selective phosphodiesterase (PDE) inhibitor, isobutylmethylxanthine (IBMX) was used. The aortic rings were incubated with IBMX (10 μM) for 40 minutes followed by cumulative addition of FK70 in aortic rings pre-constricted with PE (0.1 μM). The concentration range of FK70 used was between (1 nM to 300 μM).

3.3.4 Data analysis

Data were analysed and graphs were drawn using PRISM version 7.0 (GraphPad software). All data were expressed as mean ± standard error of mean (SEM) of *n* number of animals. Maximum tissue contraction or relaxation response was derived from nonlinear regression analysis of the obtained CRC. Statistical analyses were performed using Student's unpaired *t*-test. Unpaired *t*-test was used to compare the statistical differences from two experimental groups: one from a control condition while another from a treatment condition [PRISM Version 7 (Graph Pad Software)]. Results were considered statistically significant if $p < 0.05$. For comparing the statistical differences of more than two experimental groups (between control and different treatment conditions), one-way ANOVA followed by Dunnett's multiple comparisons test was applied (PRISM Version 6; Graph Pad Software). Results were considered statistically significant if $p < 0.05$.

3.4 Results

3.4.1 FK70 reduced contractile agents'-induced contraction

In order to investigate the effects of different pre-contractile agents on FK70, PE, KCl and U46619 were used. As shown in **Figure 3.3**, FK70 concentration-dependently relaxed aortic rings pre-contracted with PE and KCl at a similar magnitude. Whilst those pre-contracted with U46619 produced the lowest relaxation response. The cumulative addition of vehicle control (DMSO) did not seem to affect the tones of pre-contracted aortic agents, showing that the relaxation response measured in aortic rings treated with FK70 is due to FK70 but not due to DMSO (refer **Table 3.2**).

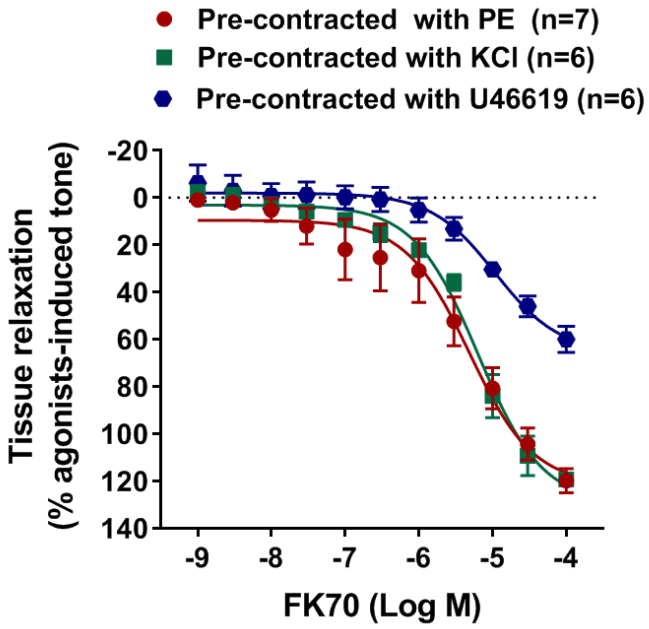


Figure 3.3: Effects of FK70 concentration-response curve in aorta pre-constricted with PE, KCl and U46619. Tissue responses have been expressed as a percentage of agonists-induced tone and are shown as means ± SEM of 6 to 7 animals.

Table 3.2: Summary of relaxation effect of FK70 and control induced relaxation in aorta pre-contracted with PE, KCl and U46619. The cumulative addition of vehicle control did not elicit significant relaxation on the aortae. Tissue responses have been expressed as a percentage of agonists-induced tone and are shown as means ± SEM of 5 to 7 animals.

Pre-contractile agent	**Number of animal (n)**	**Maximal relaxation effect of FK70 at (10^{-4} M) (%)**	**Number of animal (n)**	**Maximal relaxation effect of vehicle control (%)**
Phenylephrine	7	119.8 ± 4.9	5	27.1 ± 6.0
KCl	6	119.3 ± 2.3	5	20.1 ± 10.2
U46619	6	60 ± 5.5	5	24.9 ± 5.2

3.4.2 FK70 inhibited calcium-induced contraction

The next step of the experiment was to investigate the effect of FK70 on extracellular Ca^{2+}-induced contraction. Aortic tissues pre-treated with vehicle control (DMSO 0.22%) served as control. The contractile response to $CaCl_2$ following pre-treatment with FK70 (10 and 30 μM) was concentration dependent, with complete abolishment observed in the presence of FK70 (30 μM) (see **Figure 3.4** and refer **Table 3.3**). The contraction of aortic tissues to KCl reduced by > 50 % in calcium-free Krebs than in normal Krebs, however with no further alteration in the presence of FK70 (refer **Table 3.3**).

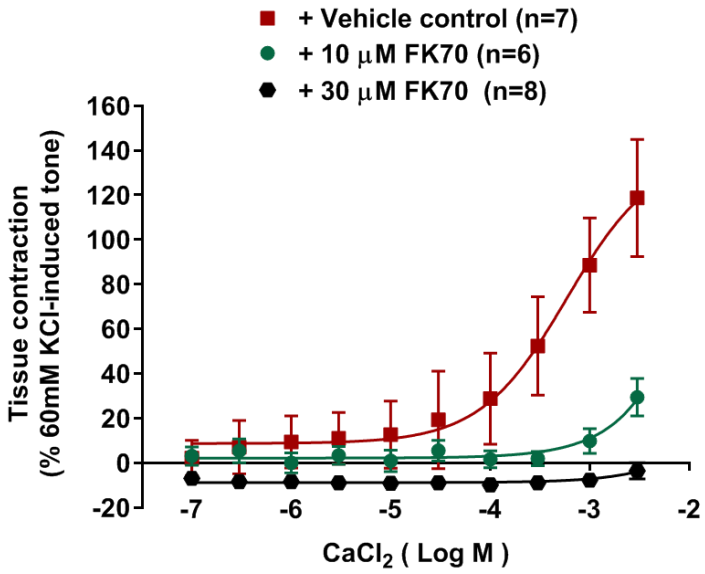


Figure 3.4: Effects of vehicle control, FK70 (10 µM) and FK70 (30 µM) on $CaCl_2$ concentration-response curves. Tissue responses have been expressed as a percentage of KCl-induced tone and are shown as means ± SEM of 6-8 animals.

Table 3.3: Summary of effects of vehicle control and FK70 (10 and 30 µM) on (i) KCl- and (ii) $CaCl_2$-induced maximum contraction, in calcium-free Krebs solution. The effect of FK70 on Ca^{2+}-induced contraction was concentration dependent. Highest concentration of FK70 abolished the Ca^{2+}-induced contraction.Tissue responses have been expressed as a percentage of KCl-induced tone and are shown as means ± SEM of 6-8 animals. A *p* value of >0.05 is considered no significant difference

Treatment	**n number**	**KCl-induced contraction in calcium free Krebs solution**		**$CaCl_2$-induced contraction**
		Maximal contraction at (3×10^{-3}M) (%)	***p* value**	**Maximal contraction at (3×10^{-3}M) (%)**
Control	7	37.1 ± 7.1	> 0.2	118.7 ± 9.9
+ 10 µM FK70	6	26.3 ± 4.9	0.25	29.5 ± 8.4
+ 30 µM FK70	8	50.4 ± 7.5	0.22	Abolished

3.4.3 FK70-induced relaxation is endothelium and nitric oxide independent

The endothelium intact (E+) aortic rings served as control for this experiment. The removal of endothelium did not seem to affect the maximal relaxation effect of FK70 (E- : 117.4 ± 12.7 %) when compared to endothelium intact aortic rings (E+: 119.8 ± 4.9 %). When in combination with L-NAME, endothelium-denuded aortic tissues relaxed the same as control to 106.4 ± 1.9 %) (see **Figure 3.5**).

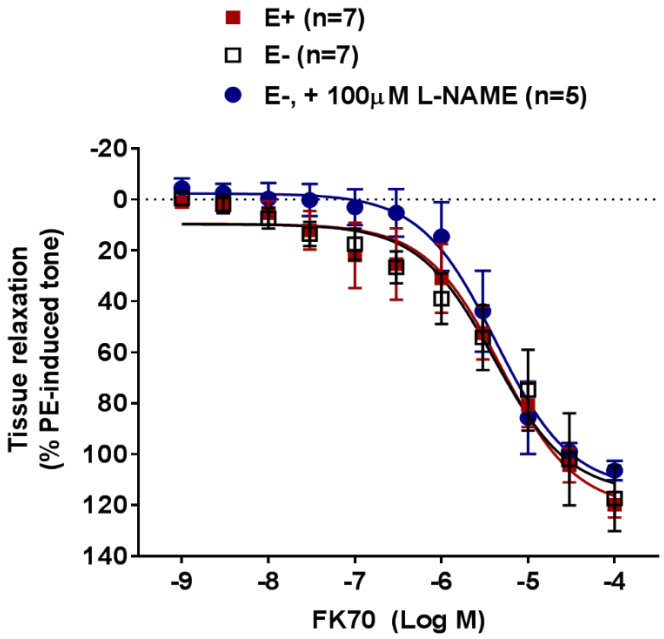


Figure 3.5: Effects of endothelium intact (E+), endothelium denuded (E-) and endothelium denuded aortic rings in combination with L-NAME (100 µM) on FK70-induced relaxation in aortic rings. Tissue responses have been expressed as a percentage of PE-induced tone and are shown as means ± SEM of 5 to 7 animals

To further investigate whether FK70 is involved in NO-mediated response, SNP was employed. Aortic tissues treated with (DMSO 0.22%) served as control for this experiment. Incubation with FK70 (30 µM) did not seem to affect SNP-induced relaxation in the aorta. The magnitude of response to SNP was almost similar between the control group (120.8 ± 4.5 %) and FK70 treated group (105.3 ± 5.6 %; unpaired *t*-test, p=0.06) (see **Figure 3.6**).

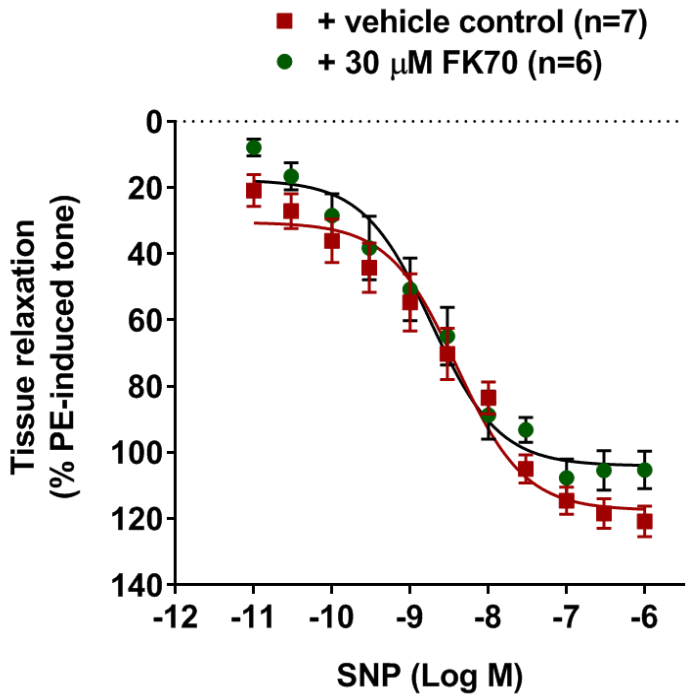


Figure 3.6: Effects of vehicle control and FK70 (30 μM) on SNP concentration-response curves. Tissue responses have been expressed as a percentage of PE-induced tone and are shown as means ± SEM of 6-7 animals.

3.4.4 FK70-induced relaxation does not involve potassium channels

This part of the experiment assessed whether potassium channels were involved in FK70-induced relaxation. The aortic rings were pre-incubated with specific potassium channel blockers: TEA and 4-AP before inducing contraction with phenylephrine. The rings pre-incubated with TEA were not influenced by FK70. Pre-incubation with 4-AP also did not affect the maximal response and potency of FK70 (see **Figure 3.7** and refer **Table 3.4**).

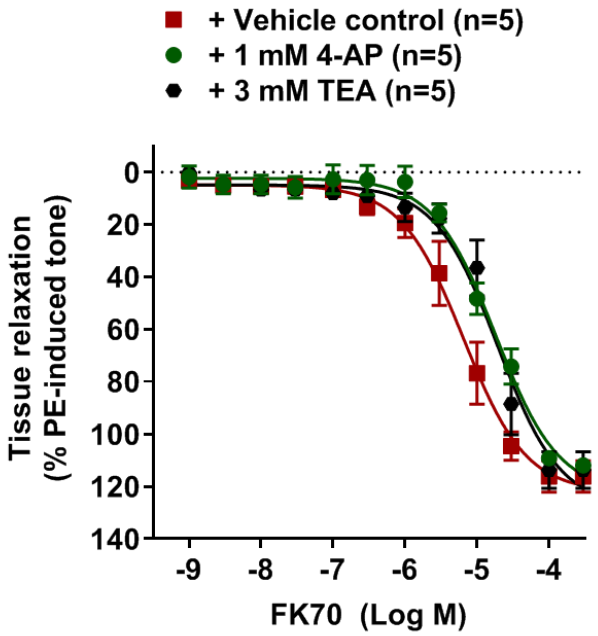


Figure 3.7: Effects of vehicle control, 4-AP (1 mM), and TEA (3 mM) on FK70-concentration-response curves. Tissue responses have been expressed as a percentage of PE-induced tone and are shown as means ± SEM of 5 animals.

Table 3.4: Summary of the effects of vehicle control, TEA (3 mM) and 4-AP (1 mM) on FK70-induced relaxation. Pre-incubation with TEA and 4-AP did not influence FK70-induced relaxation.Tissue responses have been expressed as a percentage of PE-induced tone and are shown as means ± SEM of 5 animals. A *p* value of >0.05 is considered no significant difference

Treatment	Number of animal (n)	Maximal relaxation effect of FK70 (%)	pEC_{50}	*p* value
+ Vehicle control	5	116.1 ± 5.8	5.1 ± 0.2	NA
+ 3 mM TEA	5	113.7 ± 6.8	4.7 ± 0.1	0.07
+ 1 mM 4-AP	5	111.9 ± 1.9	4.7 ± 0.1	0.06

3.4.5 FK70-induced relaxation is not via adrenoceptors

The effects of α and β-adrenoceptors antagonists on FK70-induced relaxation were tested. Aortic tissues without prior treatment with adrenergic receptor antagonists served as control. When treated with α-adrenoceptors antagonists, rauwolscine and prazosin, the relaxation response to FK70 was not affected when compared to control (see **Figure 3.8** and refer **Table 3.5**).

The beta-adrenoceptors antagonists, timolol and propranolol, also did not affect the relaxation to FK70 in response to PE-induced contraction (see **Figure 3.9** and refer **Table 3.6**).

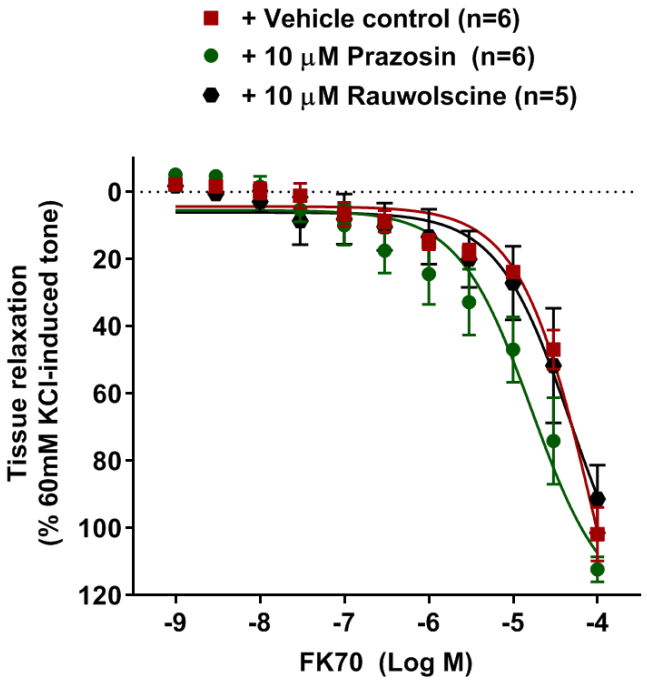


Figure 3.8: Effects of vehicle control, α-adrenoceptors antagonists, prazosin (10 μM) and rauwolscine (10 μM) on FK70 concentration-response curves. Tissue responses have been expressed as a percentage of KCl-induced tone and are shown as means ± SEM of 5-6 animals.

Table 3.5: Summary of the effects of control and α-adrenoceptors antagonists (prazosin and rauwolscine, 10μM) on FK70-induced relaxation Tissue responses have been expressed as a percentage of KCl-induced tone and are shown as means ± SEM of 5-6 animals.

Treatment	Number of animal (n)	Maximal relaxation effect of FK70 at (10^{-4} M) (%)
+ Vehicle control	6	101.8 ± 7.8
+ 10 μM Prazosin	6	112.2 ± 3.6
+ 10 μM Rauwolscine	5	91.3 ± 9.9

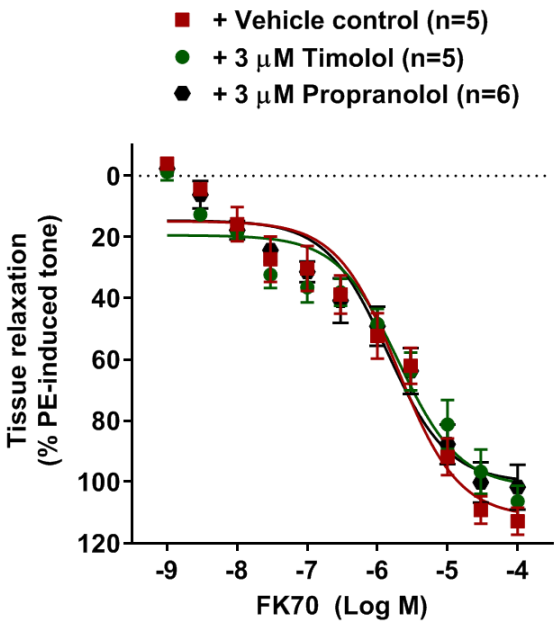


Figure 3.9: Effects of vehicle control, β-adrenoceptors antagonists, propranolol (3 μM) and timolol (3 μM) on FK70 concentration-response curve. Tissue responses have been expressed as a percentage of PE-induced tone and are shown as means ± SEM of 5-6 animals.

Table 3.6: Summary of the effects of vehicle control and β-adrenoceptors antagonists (propranolol and timolol, 3 μM) on FK70-induced relaxation Tissue responses have been expressed as a percentage of PE-induced tone and are shown as means ± SEM of 5-6 animals.

Treatment	Number of animal (n)	Maximal relaxation effect of FK70 at (10^{-4} M) (%)
+ Vehicle control	5	112.9 ± 4.3
+ 3 μM Propranolol	6	101.7 ± 7.3
+ 3 μM Timolol	5	106.3 ± 5.0

3.4.6 FK70-induced relaxation is not regulated via cAMP / cGMP pathway

In order to investigate the effects of phosphodiesterase inhibitors on FK70- induced relaxation, IBMX was used. Aortic rings without prior treatment with IBMX served as control. Pre-treatment with IBMX did not alter the relaxation curve of FK70 in aortic rings pre-contracted with phenylephrine, (control = 112.9 ± 4.3 %, IBMX = 100.2 ± 4.9 %; unpaired *t*-test, *p*=0.09) (see **Figure 3.10**).

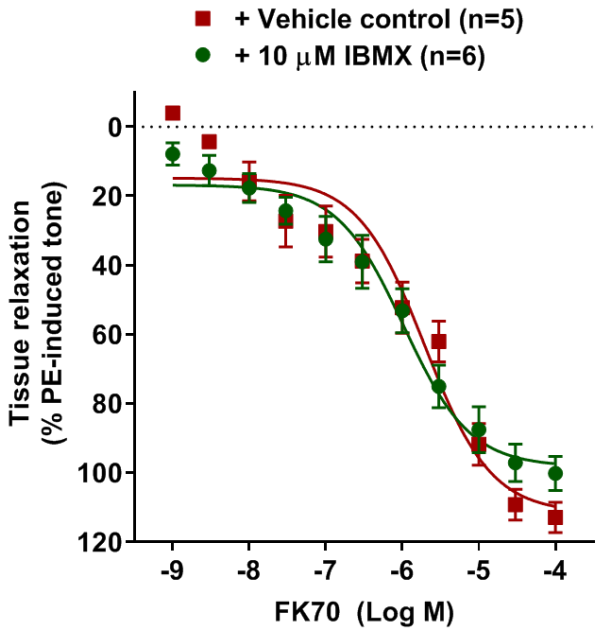


Figure 3.10: Effects of vehicle control and IBMX (10 μM) on FK70 concentration-response curves. Tissue responses have been expressed as a percentage of PE-induced tone and are shown as means ± SEM of 5-6 animals.

3.5 Discussion

Previous findings in **chapter 2** had demonstrated that the relaxation effect of FK70 was more potent and efficacious in the vascular smooth muscle. This exciting finding prompted further investigation focusing on the mechanism of action of FK70. Almost all drug discovery begins with the activity of molecules on a molecular target. Therefore the main aim of this study was to characterize the relaxant-induced mechanism of the novel compound, FK70, on rat isolated aortic smooth muscle. With only a rudimentary knowledge of FK70, a step-wise basic pharmacological approach was applied to provide a framework for further exploration and a better understanding of its vascular effect. The possible sites of action of drug studied in this study were based on the following categories (1) involvement of endothelium and endothelium-derived factors (2) receptors (α- and β-adrenoceptors) (3) second messengers (e.g. cyclic adenosine monophosphate and cyclic guanosine monophosphate) (4) enzyme that terminate the responses (e.g. phosphodiesterases).

FK70 exhibited different relaxation tones when pre-exposed to contractile agents, PE, KCl, and U46619. FK70 had produced the most potent and efficient relaxation response in the aortic rings pre-exposed to PE and KCl, while pre-constriction with U46619 exhibited the lowest maximal response to FK70. This suggests that FK70 has a lower effect on U46619-induced contraction, and the effect of FK70 is selective, and its effect is possibly related to blockade of the α-adrenoceptor pathway (induced by PE) or membrane depolarisation (induced by KCl) to cause relaxation. It is also noteworthy to consider the involvement of transient receptor potential (TRP) channels. The TRPC channels modulate membrane potential and intracellular Ca^{2+}. The TRP channels are not only activated by G-protein coupled receptors stimulus but also via KCl stimulus, which bypasses the G-protein-coupled receptors. KCl-induced contraction is due, in part, to Ca^{2+} entry through one or more of the TRP cation channel isoforms in rabbit femoral and renal arteries (Ratz and Berg, 2006). The sustained contraction to KCl may also be mediated by Ca^{2+} release from the sarcoplasmic reticulum, resulting in Ca^{2+} entry through store-operated Ca^{2+} channels (SOCC), e.g., TRP channels (Kobayashi *et al*., 1985; Ay *et al.,*2004). However, the involvement of TRP channels upon KCl stimulation in rat aorta is still unknown and needs further investigation. Although the mechanisms of KCl, PE, and U46619-induced contraction are not the same, FK70 relaxed the contraction induced by the contractile agents tested. Because PE, KCl, and U46619-induced contraction involve calcium modulation via voltage-operated Ca^{2+} channels (VOCC) and TRP channels, it seems appropriate to propose a hypothesis that FK70 may impose an effect on calcium-mediated mechanisms as these contractile agents play a significant role in Ca^{2+} signaling pathways in the aorta.

The vascular smooth muscle cell contraction predominantly controlled by the intracellular calcium concentration and calcium sensitization. A high concentration of K^+ induces vascular smooth cell contraction by depolarization of the cell membrane potential, thus leading to Ca^{2+} entry through the L-type Ca^{2+} channels. Pre-treatment of FK70 inhibited extracellular Ca^{2+}-induced contraction in a concentration-dependent manner. This suggests that FK70 affected Ca^{2+} sensitivity and reduced Ca^{2+} influx into rat aortic smooth muscle. This experiment further reiterates the effects of FK70 calcium modulation. However, the full underlying pathway is yet to be discovered.

The endothelium forms an integral part of the vasculature and releases various vasodilatory factors such as nitric oxide (NO), prostacyclin (PGI_2), and endothelium-derived hyperpolarizing factor (EDHF). The two most common agents used to test endothelial functions are ACh and SNP (Morris and Shore, 1996). SNP is NO donor, which induce vascular smooth muscle relaxation mainly through the activation of the soluble guanylate cyclase (sGC) and the subsequent increase in cGMP levels (Cogolludo *et al.,*1999). The cyclic nucleotide reduces the intracellular Ca^{2+} concentration by activating the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA), the plasma membrane Ca^{2+}-ATPase, the Na^+/K^+- and different potassium channels. (Barnes and Liu, 1995; Khan *et al.,* 1998). The L-NAME, on the other hand, inhibits the production of endothelium-derived NO and also exerts its effect directly on the rat aortic smooth muscle cells. With the usage of popular drugs, the present findings have demonstrated that FK70-induced relaxation is not endothelium or endothelium-derived factors dependent. Hence, rules out the possible influence of endothelium on FK70-induced relaxation in the aorta.

Besides the endothelium-dependent pathway, the vascular tone is also regulated by smooth muscle cells via an endothelium-independent pathway. Ion channels in the plasma membrane of vascular smooth muscle cells, such as potassium channels and calcium channels, play an important role in the regulation of the vascular tone. The activation of K^+ channels results in membrane hyperpolarization channel which leads to closure of L-type Ca^{2+} channels, and Ca^{2+} removal from the cytoplasm due to the activity of sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and plasmalemmal Ca^{2+}-ATPase (Jackson, 2000). Both large-conductance Ca^{2+} (K_{Ca}) and voltage-gated (K_V) K^+ channels could be activated under these conditions, thereby hyperpolarising the cell membrane and relaxing the blood vessel (Nelson and Quayle, 1995). Therefore, TEA and 4-AP which are K^+ channel blockers, were used to test for potential K^+ channel-related vasorelaxant effects of FK70-induced relaxation. Furthermore, stimulation of α_1-adrenergic had been shown previously, to involve K^+ conductance in rat aortic smooth muscle (Tammaro *et al.,*2004). In the present study,

treatment with TEA and 4-AP did not affect the maximal response of FK70-induced relaxation suggesting that FK70-induced relaxation in aorta is not mediated via the K^+ channels.

Adrenoceptors (α- and β-adrenoceptors) signaling pathway is one of the major pathways in mediating vascular activity. The α-adrenoceptors are responsible for vasoconstriction, while β-adrenoceptors are involved in vasodilation. Therefore, blockade of the α-adrenoceptors would lead to relaxation. As discussed in the previous chapter, the comparable relaxation efficacy and similar pharmacophore with a known drug, dobutamine, has raised the possible roles of FK70 in mediating α- and β-adrenoceptors signaling pathways. Furthermore, the relevance of α- and β-adrenoceptors investigation is also clearly supported by the current findings where FK70 reduced phenylephrine-induced contraction. However, this possibility was ruled out as no influence of α-adrenoceptors (rauwolscine and prazosin) and β-adrenoceptors (timolol and propranolol) antagonists on FK70-induced relaxation were observed. The results indicate that FK70 does not act directly on both α- and β-adrenoceptors. Moreover, the structure-activity relationships for beta-adrenoceptor agonists e.g isoprenaline, orciprenaline, procaterol show that a secondary amine in the phenylethanolamine side chain ending is essential for receptor stimulation (Labrid *et al*.,1989). The beta-adrenoceptor agonist activity is generally enhanced with increasing size of the substituent of the amine: this substituent can be an isopropyl group (isoprenaline, orciprenaline, procaterol) or a tert-butyl one (salbutamol, terbutaline, carbuterol, clenbuterol, pirbuterol), or a larger moiety due to introduction of an aromatic ring on the N-linked alkyl chain (ritodrine, zinterol, salmefamol, fenoterol). Practically, compounds with a big N substituent (including aromatic rings) display increased beta-adrenoceptor agonist potency and longer duration of action when compared to their N-isopropyl or N-tert-butyl analogues. Besides that, the phenolic or phenol equivalent groups in meta- and/or para-positions of the aromatic rings are necessary for β_2-adrenoceptor agonism (Labrid *et al.,*1989). The absence of phenolic groups in FK70 perhaps explains the non-involvement of FK70 in β-adrenoceptor agonism.

The secondary messengers, cyclic nucleotides (cAMP and cGMP), play a central role in mediating the relaxation of vascular tissues. These cyclic nucleotides are synthesised from enzyme adenylyl cyclase and guanylyl cyclase, respectively. Mediators, such as nitric oxide and β-adrenoceptors, could also activate the synthesis of these cyclic nucleotides. Therefore, further experiments were carried out to verify the possible role of secondary messengers in FK70-induced relaxation. These cyclic nucleotides are regulated by the enzyme phosphodiesterases (PDE). The enzyme hydrolyses cyclic nucleotides and thus plays a crucial role in regulating intracellular levels of cAMP and cGMP.

Inhibition of this enzyme would prevent hydrolyses of cyclic nucleotides and retain the amount of active cAMP or cGMP in mediating vascular relaxant response. However, in the present study, pre-treatment with the phosphodiesterase inhibitor, (IBMX) did not affect FK70- induced relaxation. The gathered evidence indicates that FK70-induced vascular relaxation is not mediated via cAMP- or cGMP-dependent pathways.

Taken together, the evidence from the present study suggest that the relaxation to FK70 was independent of endothelium and endothelium factor, NO, not mediated via α- and β-adrenoceptors and unlikely to involve in cGMP/cAMP-mediated pathways. While this study has not narrowed down the exact mechanism of action FK70, it has generated a possibility of the involvement of calcium in FK70-induced relaxation. The focus was thereafter diverted to investigate the role of FK70 on calcium-mediated mechanisms. The details of the experiments will be discussed in the subsequent chapters.

Chapter 4: Role of extracellular and stored calcium sources in mediating potassium chloride (KCl), 5-hydroxytryptamine (5-HT) and phenylephrine (PE) contractile response in rat aortae

4.1 Introduction

In the previous (Chapter 3), some significant pathways possibly involved in FK70-induced relaxation were discussed. The result of the investigation showed that FK70 attenuates pre-contractile agents-induced contraction and calcium-induced contraction, which raised an important question as to whether FK70-induced relaxation involved calcium dynamics. In order to explore the involvement of Ca^{2+} in FK70-induced relaxation, it is crucial to first understand the regulation of KCl and other agonists-induced Ca^{2+} oscillations in vascular smooth muscle. The relative contribution of Ca^{2+} to agonists-induced contraction has not been investigated in rat isolated aortic smooth muscle. Therefore, this study set out to assess the role of Ca^{2+} in potassium chloride (KCl), 5-hydroxytryptamine (5-HT), and phenylephrine (PE) induced contraction in rat aorta. A brief introduction about the regulation of calcium in smooth muscle and GPCR receptors in vascular tissues are stated in the context.

4.1.1 Regulation of calcium in smooth muscle

The smooth muscle (SM) contraction is controlled by signals from the autonomous nervous system, such as nerve impulses, hormones, and other chemicals released by specialized organs. The contraction is regulated by the changes in the concentration of free Ca^{2+} in the cytosol. An increase in free intracellular Ca^{2+} results from either 1) increased influx of Ca^{2+} into the cell e.g., through Ca^{2+} channels in the plasma membrane under the control of membrane depolarisation or upon agonist stimulation or 2) by release of Ca^{2+} from internal stores e.g. sarcoplasmic reticulum (SR), which is controlled by second messengers. The free Ca^{2+} in the cytoplasm binds to a calcium-binding protein called calmodulin to form a calcium-calmodulin complex (Robinson and Colbran, 2013). The calcium-calmodulin complex activates myosin light chain kinase (MLCK), an enzyme that is capable of phosphorylating myosin light chains (MLC). MLC phosphorylation leads to cross-bridge formation between the myosin and actin filaments, and thus is followed by smooth muscle contraction. In contrast, as the Ca^{2+} concentration begins to decline, Ca^{2+}-sensitization of the contractile proteins is signalled by the RhoA/Rho-kinase pathway to inhibit the dephosphorylation of MLC phosphatase (MLCP) to maintain the force generation (Feher, 2017). Removal of Ca^{2+} from the cytosol and stimulation of MLCP initiates the process of smooth muscle relaxation (Wynne *et al*., 2009). The degree of MLC phosphorylation and the vascular smooth muscle contractile tone is

regulated by G-protein-coupled signal transduction pathways and nitric oxide activation through guanylyl cyclase and cyclic guanine monophosphate (cGMP) formation (Klabunde, 2012).

Membrane depolarisation is believed to be an essential process for the activation of Ca^{2+} events. KCl causes contraction as K^+ depolarises the aorta, which stimulates an enhanced uptake of Ca^{2+} by activating voltage-dependent Ca^{2+} channels, thus leading to contraction (Ratz *et al.*, 2005). Besides voltage-dependent Ca^{2+} channels, KCl also activates additional Ca^{2+} entry pathways via transient receptor potential (TRP) channels to permit strong force-maintenance in tonic vascular smooth muscle (Ratz *et al*., 2006). The mechanism of the KCl caused Ca^{2+}-sensitization is a complex one, involving multiple cells signaling such as translocation of proteins from the cytoplasm to the plasma membrane, and activation of enzymes such as RhoA kinase and protein kinase C.

4.1.2 GPCRs in smooth muscle

GPCRs respond to various extracellular signals and transduce them to heterotrimeric G proteins, and play an essential role in various signaling pathways. Heterotrimeric G-proteins (Gα, Gβ/Gγ subunits) constitute one of the most critical components of the cell signaling cascade (Tuteja, 2009). Gα proteins are divided into four groups according to their structural and functional similarities; G_s, G_i, G_q, and G_{12}. The coupling of receptors to these G-protein subfamilies determines the decrease or increase in the SM tone. Typically, stimulation of G_s or G_i families causes relaxation of smooth muscle whist stimulation of G_q, and $G_{12/G13}$ subfamilies cause contraction of the smooth muscle (Wirth and Offermanns, 2012). The G_s and G_i families regulate adenylyl cyclase (AC) activity, while G_q and $G_{12/13}$ activate phospholipase Cβ and small guanine tri-phosphatases (GTP) Rho A, respectively. The classical pathway GPCRs increase SM contraction is via the G_q/G_{11} family.

The G_q family consists of four members: G_q, G_{11}, G_{14}, and $G_{15/16}$, and their respective α subunits are $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, and $G\alpha_{15/16}$ (Billington and Penn, 2003). Most of the specificity of signaling resides in the Gα subunit. In the most common signaling pathway, activation of a receptor coupled to G_q-protein results in the activation of β-isoforms of phospholipase C (PLC), which leads to the generation of inositol triphosphate (IP_3) and diacylglycerol (DAG), hence, releasing Ca^{2+} from intracellular and extracellular milieu, respectively. The second-messengers (IP_3 and DAG) play an important role in smooth muscle contraction by mobilising from Ca^{2+} multiple signaling pathways involved in agonist-induced contraction (Abdel-Latif, 2001).

The DAG activates non-selective cation channels (e.g., TRP channels), resulting in depolarization and opening of voltage-dependent Ca^{2+} channels. G_q signaling can also activate Rho/Rho-kinase (Rho/ROCK) signaling pathway via Rho-guanine nucleotide exchange factors (Rho-GEF) (Wirth and Offermanns, 2012). Activation of members of the Rho family is via GTP binding. The exchange of GDP for GTP on these proteins is controlled through guanine nucleotide exchange factors (GEF), which catalyse the exchange of GDP for GTP. RhoA activation, coupled to $G\alpha_{q/11}$, involves the intracellular release of Ca^{2+} involving the downstream activation of the two Rho-regulated protein kinases, ROCK and protein kinase C (PKC-related kinase) (Kamato *et al.*, 2015). ROCK and its substrate myosin phosphatase targeting subunit 1 (MYPT-1) are responsible for smooth muscle contraction by increasing calcium sensitivity (Loirand *et al*., 2006) (see **Figure 4.1**).

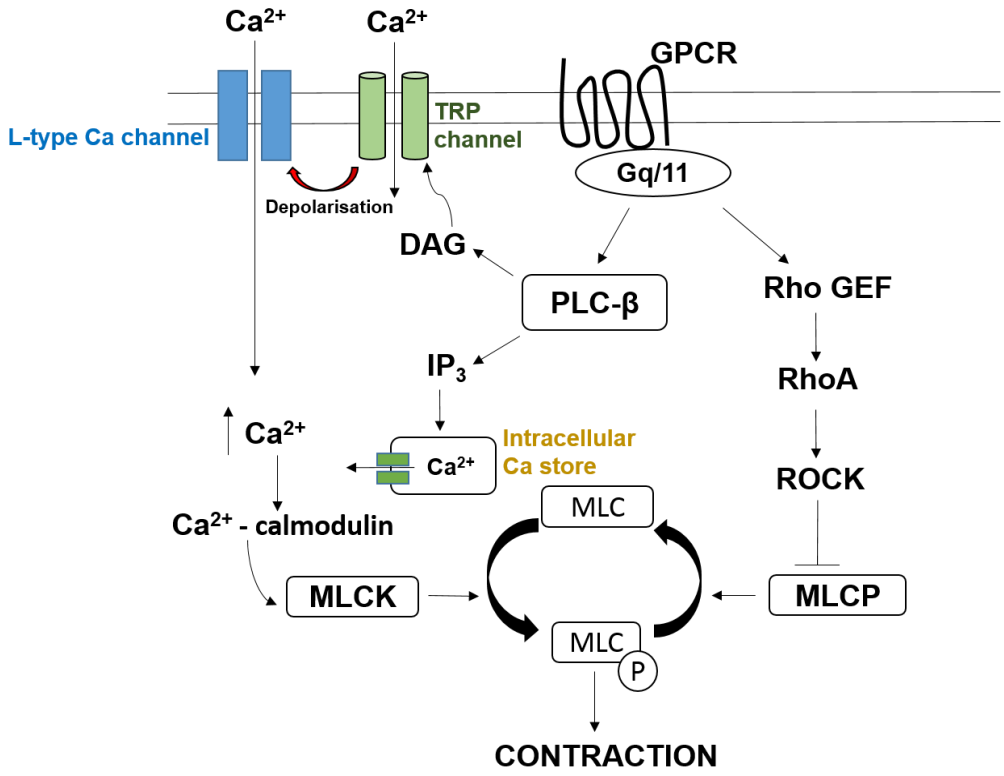


Figure 4.1: Schematic illustration of mechanisms of GPCR-mediated smooth muscle contraction coupled to G_q/G_{11} family. Activation of β-isoforms of phospholipase C (PLC-β) by G_q/ G_{11} results in inositol-1,4,5,-trisphosphate (IP_3) formation and release of intracellularly stored Ca^{2+}. Whilst the DAG activate non selective cation channels (e.g TRP channels) resulting in depolarization and opening of voltage-dependent Ca^{2+} channels (Earley and Brayden, 2015). Ca^{2+} together with calmodulin activates the myosin light chain kinase (MLCK) resulting in phosphorylation of the myosin light chain (MLC). It also activates Rho guanine nucleotide exchange factors (Rho-GEFs). The activated small GTPase RhoA via stimulation of Rho-kinase (ROCK) activity inhibits the myosin light chain phosphatase (MLCP) thus leading to contraction (Wirth and Offermanns, 2012).

The α_1-adrenoceptors and 5-HT receptors are GPCR linked specifically to G_q-proteins, which cause contraction upon stimulation of their respective agonists. In the rat aorta, there are two functional 5-HT receptors, 5-HT_{2A} and 5-HT_{2B}. However, the contractile activity is mainly mediated by 5-HT_{2A} receptors (Villazón *et al*., 2002), and these receptors are linked to G_q-coupled protein (Hoyer *et al*.,1994).

In the past, it was suggested that the extracellular Ca^{2+} plays a minor role, and virtually all the Ca^{2+} involved in contraction is derived from relatively large intracellular stores in the SR. This theory was supported by (Iino *et al.*,1994), who first reported that noradrenaline, an α_1-adrenoceptor agonist within the intact wall of the rat tail artery is maintained by asynchronous repetitive SR Ca^{2+} release rather than by sustained Ca^{2+} influx. However, (Lee *et al.*, 2001) had challenged the view by finding that, the initial phase of PE contraction in rabbit interior vena is initiated by Ca^{2+} release from the sarcoplasmic reticulum (SR), and the tonic phase is supported by sustained Ca^{2+} influx through L-type voltage-gated Ca^{2+} channels (L-type VGCCs) and/or receptor-operated Ca2+ channels (ROCCs). The 5-HT contraction, on the other hand, has been reported to depend on both extracellular and intracellular Ca^{2+} in the human pulmonary artery (Rodat *et al*.,2009), guinea-pig trachea (Watts *et al*.,1994) and intestinal smooth muscle (Burka *et al*.,1990). However, the contribution of intracellular and extracellular Ca^{2+} on PE- and 5-HT-induced contraction in rat aorta is still unclear, to date. Thus far, it is known that the contraction of smooth muscle to PE and 5-HT involves both intracellular and extracellular Ca^{2+}.

4.2 Study aim and objectives

KCl has long been used as a convenient stimulus to bypass G protein-coupled receptors (GPCR) and activate smooth muscle simple mechanism involving activation of VOCC, which leads to an increase in intracellular Ca^{2+}. The general notion of activation of receptors coupled to G_q-protein involves the mobilisation of stored and external Ca^{2+} which leads to smooth muscle tissue contraction. However, the contribution of internal and external Ca^{2+} on KCl, 5-HT, and PE induced-contraction in rat aorta has not been fully described. To date, it is known that the contraction of smooth muscle to PE and 5-HT involves both stored and external Ca^{2+}. Therefore this chapter aims to investigate the role of extracellular and stored Ca^{2+} on KCl, 5-HT, and PE-induced contraction. The following were the specific objectives for this chapter;

i) to study the role of external Ca^{2+} in KCl-induced contraction in calcium-free Krebs Ringer solution

ii) to investigate the role of external Ca^{2+} in 5-HT (5-HT_{2A} agonist) and PE (α_1-adrenoceptor agonist)-induced contraction in **a)** calcium-free Krebs Ringer solution, **b)** in the presence of nifedipine (a L-type Ca^{2+} channel inhibitor), **c)** in the presence of SKF 96365 (a non-selective TRPC channel inhibitor).

iii) to examine the role of stored Ca^{2+} in 5-HT (5-HT_{2A} agonist)- and PE-induced contraction in the presence of **a)** thapsigargin (a sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump inhibitor) **b)** 2-APB (an IP_3-induced calcium release inhibitor and store-operated calcium channel inhibitor).

4.3 Materials and methods

4.3.1 Drugs and Krebs-Ringer bicarbonate solution

All dissolved drugs were made into stock concentration of 0.1 M. A serial of ten-fold dilution was made for all dissolved drug solutions. Details on the drugs, solvents used and stock concentration for each drug are summarised in **Table 4.1.**

Table 4.1: Summary of drugs used, country manufactured, solvent and stock concentration used in the experiments.

Drugs	Brand / Country manufactured	Solvent	Stock concentration (M)
Phenylephrine hydrochloride	Sigma, USA	Distilled water	0.1
5-hydroxytryptamine	Nacalei Tesque, Japan	Distilled water	0.1
Nifedipine	Nacalei Tesque, Japan	100 % DMSO	0.1
1-[2-(4-methoxyphenyl)-2-[3-(4-methoxyphenyl)propoxy]ethyl]imidazole (SKF 96365)	Sigma, USA	100 % DMSO	0.1
Thapsigargin	Sigma, USA	100 % DMSO	0.1
2-aminoethoxydiphenyl borate (2-APB)	Sigma,UK	100 % DMSO	0.1

Calcium-containing Krebs solution (or normal Krebs solution) was prepared as described in **Section 2.3.1.** Calcium-free Krebs-Ringer solution was made in the similar composition as normal Krebs-Ringer solution but without the inclusion of $CaCl_2$ in the solution. The chelator ethylene glycol tetraacetic acid (EGTA), 0.1 mM was added to calcium free Krebs-Ringer solution, as a perfusion buffer in experiments to evaluate the role of external calcium.

4.3.2 Tissue preparation and organ bath assembly

Refer to **section 2.3.2**

4.3.3 Organ bath experimental protocols

4.3.3.1 Effects of contractile agents on the extracellular calcium in rat aorta

Following second KCl addition, concentration-response curves (CRC) to agonists, KCl, PE and 5-HT were constructed in both normal and calcium free Krebs-Ringer solution. The degree of response (tissue contraction) was measured as tension weight of the contraction achieved by increasing agonist concentrations. The tissue was exposed to increasing concentrations of the drugs, KCl (10 µM to 0.3 M), PE (0.1 nM-30 µM) and 5-HT (0.1 nM to 300 µM) after the response to previous concentration had reached its maximum. Only one CRC was performed per tissue.

In a separate protocol, to further deduce the effect of extracellular calcium on the agonist induced contraction (PE and 5-HT), the aortic rings were pre-incubated with nifedipine (10 μM) or SKF 96365 (100 μM), for at least 40 minutes in normal Krebs-Ringer solution, prior to the construction of PE or 5-HT CRC. The concentration of each inhibiting agent (nifedipine and SKF 96365) was chosen on the basis of its ability to cause 90% - 100% reduction in PE - induced tone. Tissue contraction to agonists were expressed as a percentage of 60 mM KCl-induced tone.

4.3.3.2 Effects of contractile agents on the stored calcium in rat aorta

Following second KCl stimulation, tissues were washed with calcium-free Krebs-Ringer solution containing 0.1 mM of EGTA and left to return to basal tone for 15 minutes. The tissues were then incubated with thapsigargin (1 μM) for at least 40 minutes. To empty residual cytosolic calcium, PE (10 μM) was added thereafter. The tissues were then washed with normal Krebs-Ringer solution followed by incubation with thapsigargin (1 μM) for the second time. This was to ensure the intracellular stores were emptied completely. After incubating for at least 40 minutes, CRCs to either PE or 5-HT were performed on rat isolated aortic rings. Tissue contraction to agonists were expressed as a percentage of 60 mM KCl-induced tone.

In a different set of experiment, following the second KCl stimulation, tissues were washed with normal Krebs-Ringer solution. The tissues were left to return to basal tone prior to pre-incubation with 2-APB (100 μM). After 40 minutes of incubation, the agonists (PE or 5-HT) CRC was performed. The concentration of 2-APB was chosen on the basis of its ability to cause > 90% reduction in PE -induced tone. Tissue contraction to agonists was expressed as a percentage of 60 mM KCl-induced tone.

4.3.4 Data analysis

Data were analysed and graphs were drawn using PRISM version 7.0 (GraphPad software). All data were expressed as mean ± standard error of mean (SEM) of *n* number of animals. The SEM was used to explicitly state the precision of true mean of the population as it takes into account both the value of the SD and the sample size. Maximum tissue contraction or relaxation response (E_{max}) and concentration to produce basal response by 50 % (pEC_{50}) was derived from nonlinear regression analysis of the obtained CRC. Statistical analyses were performed using Student's unpaired *t*-test. Unpaired *t*-test was used to compare the statistical differences from two experimental groups: one from a control condition while another from a treatment condition [PRISM Version 7 (Graph Pad Software)]. Results were considered statistically significant if $p < 0.05$.

4.4 Results

4.4.1 Membrane depolarisation by (KCl) involve mobilisation of extracellular calcium

In order to examine the role of extracellular calcium upon membrane depolarisation, KCl CRC were performed in normal or calcium-free Krebs solution. The maximal contraction of KCl in normal Krebs solution was (120.1 ± 4.1%) and in calcium-free Krebs the contraction dropped significantly to (49.8 ± 6.4 %; unpaired *t*-test, *p=0.0001*) (see **Figure 4.2**).

The KCl-induced contraction showed a slower onset and required a higher concentration of KCl to reach maximal response in rat isolated aortic rings. As the contraction was only noticed at higher concentrations, the potency (pEC_{50}) of the KCl response could not be determined. Interestingly, at the highest concentration, KCl-induced contraction could not be sustained but started to drop in normal Krebs solution (see **Figure 4.3**).

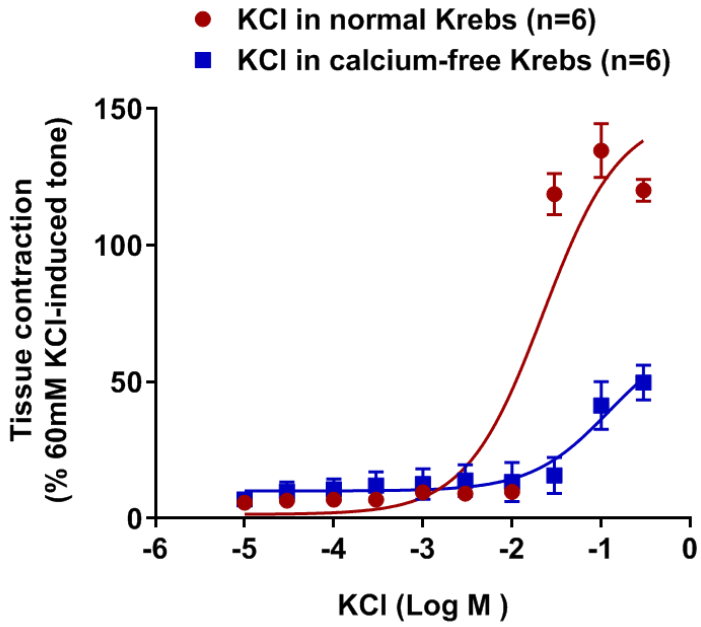


Figure 4.2: Effects of normal and calcium-free Krebs solution on KCl- concentration-response curve in rat aortae. Tissue responses have been expressed as a percentage of KCl-induced contraction and are shown as means ± SEM of 6 animals.

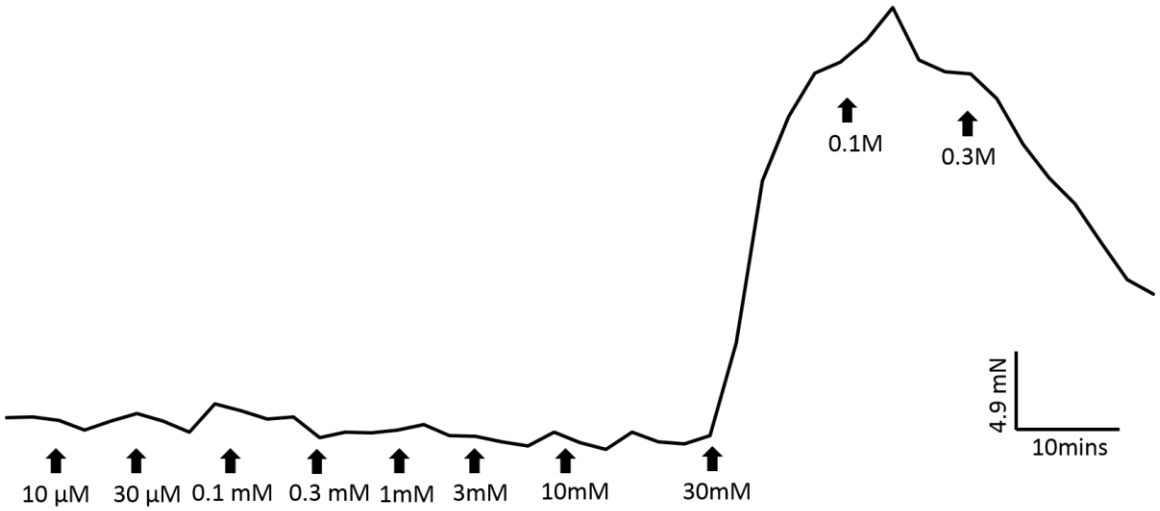


Figure 4.3: Representative trace recording of KCl concentration-response curve in rat isolated aortic rings. The contraction of KCl at the highest concentrations could not be sustained and started to drop. (Abbreviations: KCl = potassium chloride; mN = milliNewton; mins = minutes).

In a separate experiment, a single concentration of KCl (30 mM) was added in order to test the sustainability of KCl-induced contraction in normal Krebs-Ringer solution. The KCl-induced contraction dropped and could not be sustained after 15 minutes of addition (see **Figure 4.4**).

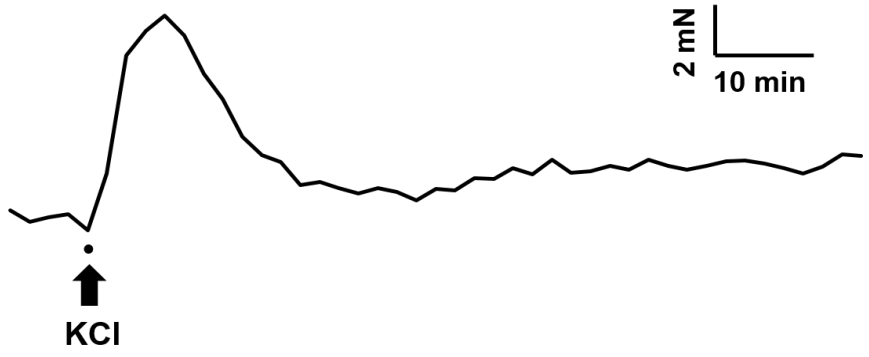


Figure 4.4: Representative trace recording of addition of single concentration of KCl (30 mM) in rat isolated aortic ring in normal Krebs solution. The contraction of KCl could not be sustained and started to drop. (Abbreviations: KCl = potassium chloride; mN = milliNewton; mins = minutes). The time of KCl added was regarded as 0 hour and the experiment was conducted for 1 hour and 30 minutes.

4.4.2 Activation of serotonin receptors linked to G_q-protein by 5-HT involves mobilisation of extracellular calcium

5-HT CRC was performed on the aortic rings in normal and calcium-free Krebs physiological solution. The contractions produced by 5-HT were markedly augmented in normal Krebs while the contraction was almost abolished in calcium-free medium (see **Figure 4.5**). The maximal contaction values are (5-HT in normal Krebs= 176.4 ± 8.8 %, calcium-free Krebs = 32 ± 4.1 %; $p < 0.0001$). The comparison in terms of potency (pEC_{50}) between and calcium-free group could not be determined as the contraction in calcium-free tissues were almost abolished.

To determine further the role of extracellular and intracellular calcium in sustaining the 5-HT contraction, a single concentration of 5-HT (10 µM) was added to aorta in normal Krebs Ringer solution. 5-HT-induced contraction could not be sustained and started dropping after 16 minutes of addition (see **Figure 4.6**).

To further confirm the role of external Ca^{2+}, in the 5-HT-induced contractile response, nifedipine was added prior to the construction of 5-HT CRC. The control group had shown to be devoid of any observable effects on the 5-HT-induced contraction (see **Figure 4.7A**) with maximal values of (control = 180 ± 9.8 %) when compared to 5-HT in normal Krebs (5-HT alone = 176.4 ± 8.8 %, n=6). This findings confirm that the depression observed in the contraction is due to the calcium channel antagonists.

Pre-incubation with nifedipine significantly depressed 5-HT contraction to (74.1 ± 13.9 %; $p = 0.0001$) compared to control group (see **Figure 4.7B**). Next, in order to explore other Ca^{2+} permeable channels involved in 5-HT-induced contraction the aortic rings were incubated with SKF 96365. SKF 96365 abolished the 5-HT induced contraction in the aortic rings (see **Figure 4.7B**).

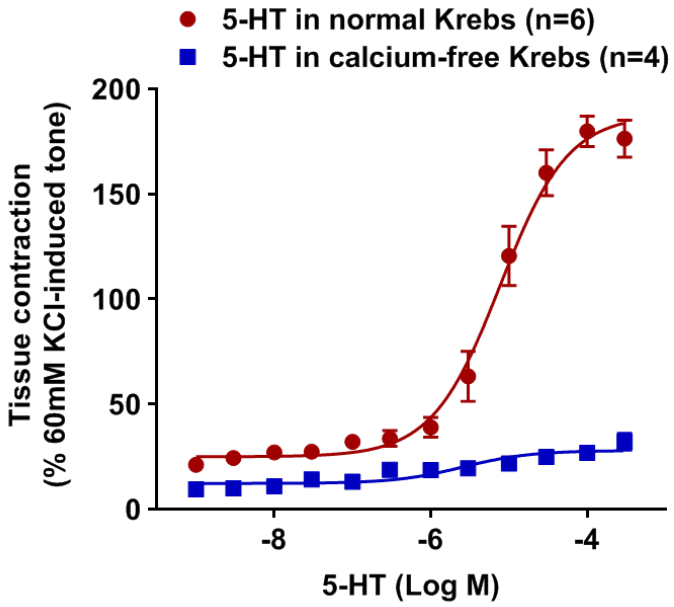


Figure 4.5: Effects of normal and calcium-free Krebs solution in 5-HT concentration-response curve on rat aortae. Tissue responses have been expressed as a percentage of KCl-induced contraction and are shown as means ± SEM of 4-6 animals.

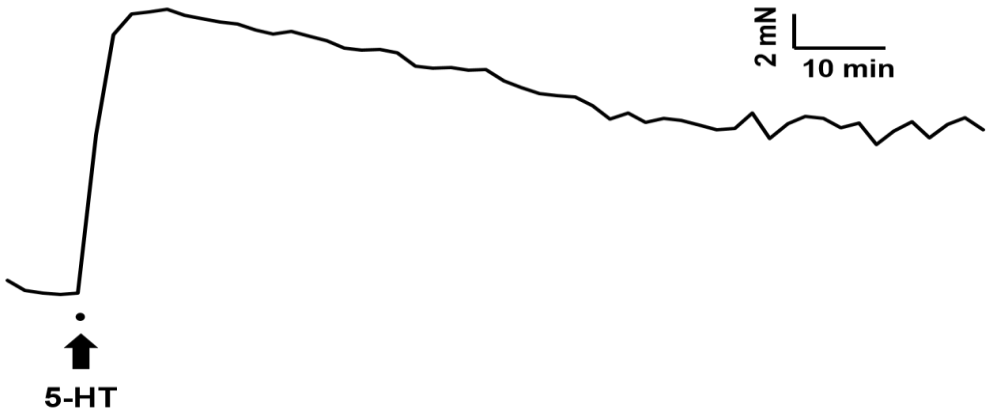


Figure 4.6: Representative trace recording of addition of single concentration of 5-HT (10 µM) in rat isolated aortic ring in normal Krebs solution. The contraction of 5-HT could not be sustained and started to drop after 16 minutes of addition. (Abbreviations: 5-HT = 5-hydroxytrptamine; mN = milliNewton; mins = minutes). The time of the 5-HT added was regarded as 0 hour and the experiment was conducted for 1 hour and 30 minutes.

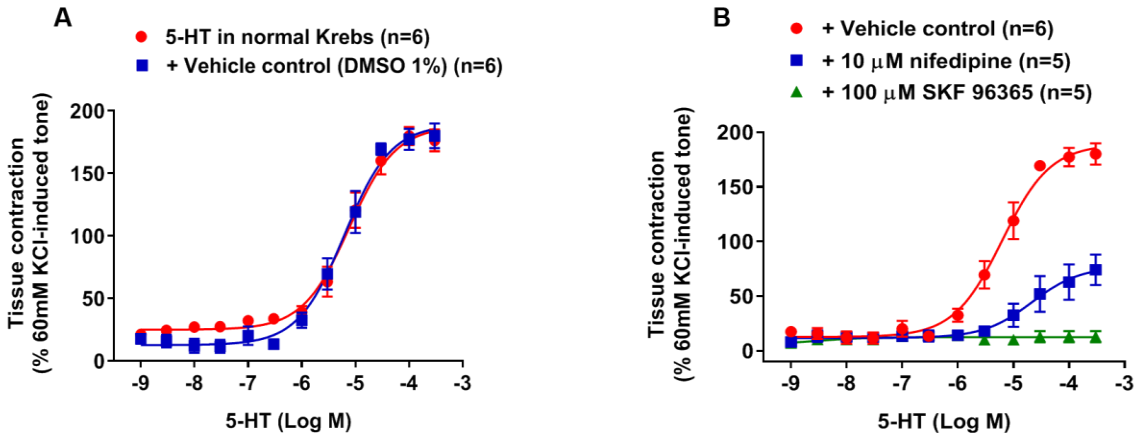


Figure 4.7: Effects of **A)** absence of vehicle control and presence of vehicle control (DMSO 1%) **B)** vehicle control, nifedipine (10 μM) and SKF 96365 (100 μM) pre-incubation on 5-HT concentration-response curves in rat aortae. Tissue responses have been expressed as a percentage of KCl-induced contraction and are shown as means ± SEM of 5-6 animals.

To investigate the role of stored Ca^{2+} in 5-HT contractile activity, the aortic rings were incubated with thapsigargin. Interestingly, incubation with thapsigargin did not affect the 5-HT contractility in aorta. Aortic rings in the absence of thapsigargin served as control. The contractions to 5-HT in treatment group were not significantly different from the control group (control = 158.7 ± 20.1 %, thapsigargin treated aortic rings= 140.1 ± 18.8 %; unpaired *t*-test, $p = 0.5$) (see **Figure 4.8**).

In the second series of experiment, in order to expound whether the contraction of 5-HT was related to the stored Ca^{2+}, 2-APB was used. Upon pre-incubation with 2-APB, the contraction to 5-HT was significantly reduced, (control = 180 ± 9.8 %, 2-APB treated aortic rings= 122.1 ± 23.2 %; unpaired *t*-test, $p = 0.04$) (see **Figure 4.8**).

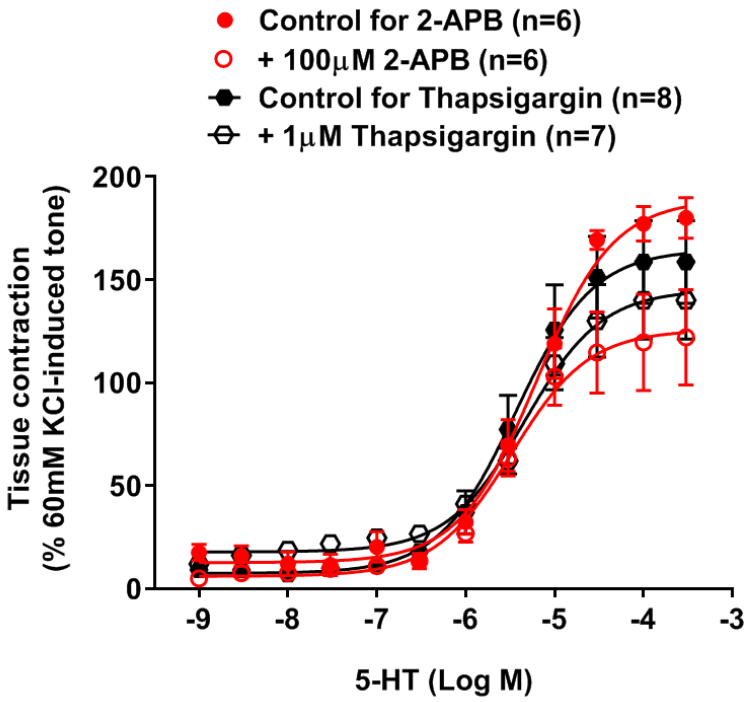


Figure 4.8: Effects of control for 2-APB (DMSO 1%), 2-APB, control for thapsigargin (DMSO 0.05%) and thapsigargin pre-incubation on 5-HT concentration-response curve in rat isolated aortae. Tissue responses have been expressed as a percentage of KCl-induced contraction and are shown as means ± SEM of 6-8 animals.

4.4.3 Activation of α-adrenoceptors linked to G_q-protein by PE involves mobilisation of both extracellular and stored calcium

In order to examine the role of external calcium on activation of α-adrenoceptors contraction, PE CRC was performed in normal or calcium-free Krebs solutions. The maximal contration of PE in normal Krebs-Ringer solution was (195.8 ± 13.9 %), whereas in calcium-free Krebs solution the contraction dropped significantly to (63.8 ± 12.4 %; unpaired *t*-test, *p<0.0001*) (see **Figure 4.9**).

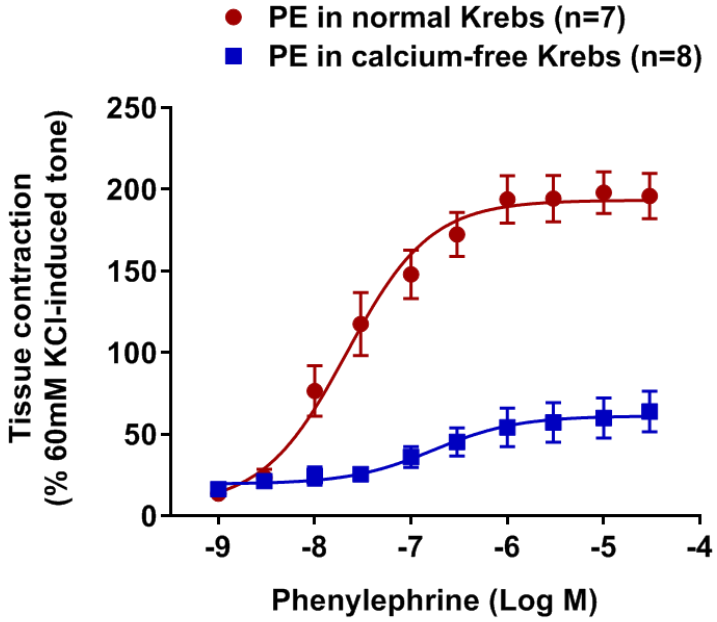


Figure 4.9: Effects of normal and calcium-free Krebs solution on PE concentration-response curve in rat aortae. Tissue responses have been expressed as a percentage of KCl-induced contraction and are shown as means ± SEM of 7 to 8 animals.

Since PE-induced contraction was observed and measurable in calcium-free Krebs Ringer solution, it prompted more experiments to be done to confirm the role of both intracellular and extracellular calcium in sustaining the contraction. A single concentration of PE was added to aorta in both normal and calcium-free Krebs solution. As expected, the contraction of PE in normal Krebs was higher with tension weight of 15.7 mN while the contraction in calcium-free Krebs was only 1.5 mN (see **Figure 4.10**). However, the contractile response in both medium was sustained throughout the experiment.

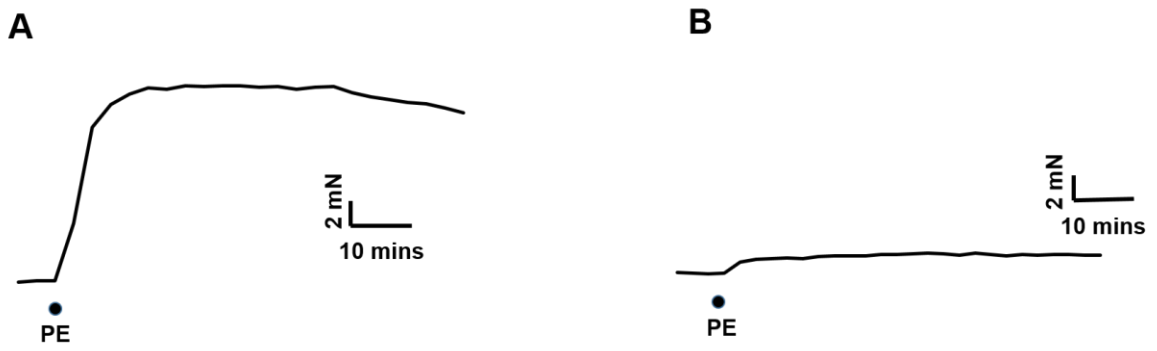


Figure 4.10: Representative trace recordings of addition of single concentration of PE (10 µM) in rat aortae in **A)** normal Krebs-Ringer solution and **B)** calcium-free Krebs-Ringer solution. The contraction of PE were present and sustained in both medium. The time of the PE added was regarded as 0 hour and the experiment was conducted for 1 hour and 30 minutes. (Abbreviations: PE = phenylephrine; mN = milliNewton; mins = minutes).

To further confirm the role of extracellular Ca^{2+} in the PE-induced contractile response, nifedipine was added into the bath prior to the construction of the PE CRC. Aortic rings treated with vehicle control (DMSO 1 %) served as controls. The control group had shown to be devoid of any observable effects on the PE-induced contraction (see **Figure 4.11A**), (E_{max}: control= 206 ± 19.5 %, PE alone= 195.8 ± 13.8 %).

The E_{max} of the PE CRC was reduced to (88.9 ± 17.3 %) in the presence of nifedipine. Whilst the presence SKF 96356, attenuated the PE contraction to (31.8 ± 14.4 %) (see **Figure 4.11B**).

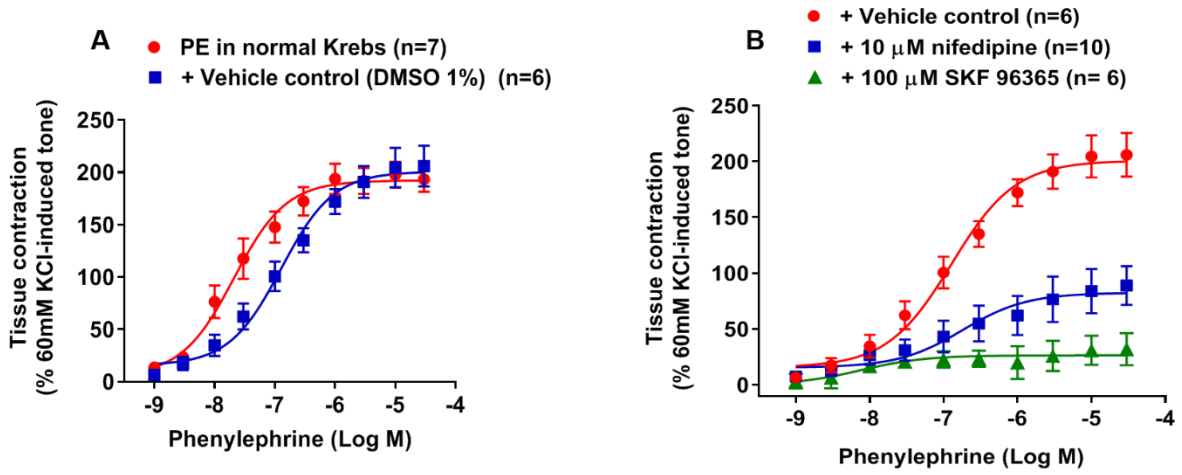


Figure 4.11: Effects of **A)** absence of vehicle control and presence of vehicle control (DMSO 1%) **B)** vehicle control, nifedipine and SKF 96365 pre-incubation on PE concentration-response curve in rat aortae. Tissue responses have been expressed as a percentage of KCl-induced contraction and are shown as means ± SEM of 6-10 animals.

Subsequent investigation on the role of stored calcium on PE-induced contraction was examined using thapsigargin, a SERCA pump inhibitor to deplete the sarcoplasmic calcium store. Aortic rings treated with (DMSO 0.05 %) were served as control. Thapsigargin treatment reduced PE contractile significantly, (control= 149.2 ± 10.2 %, thapsigargin treated aorta= 63.4 ± 7.7 %; *p=0.0001*) (see **Figure 4.12**). Interestingly, when a single concentration of PE was added into in calcium-free Krebs solution to ensure complete depletion of Ca^{2+} from the store, the contraction was relatively low, however, reintroduction of normal Krebs enhanced the PE-induced contraction before dropping back to basal tone. The contraction ceased and returned to basal tone after 15 minutes (see **Figure 4.13**).

To further confirm the role of stored calcium on PE contractile activity, 2-APB was employed. Aortic rings treated with (DMSO 1%) were served as control for this experiment. Upon pre-incubation with 2-APB, the contraction to PE was markedly reduced, (control= 206 ± 19.5 %, 2-APB treated aorta= 126.5 ± 8.7 %; *p=0.002*) (see **Figure 4.12**).

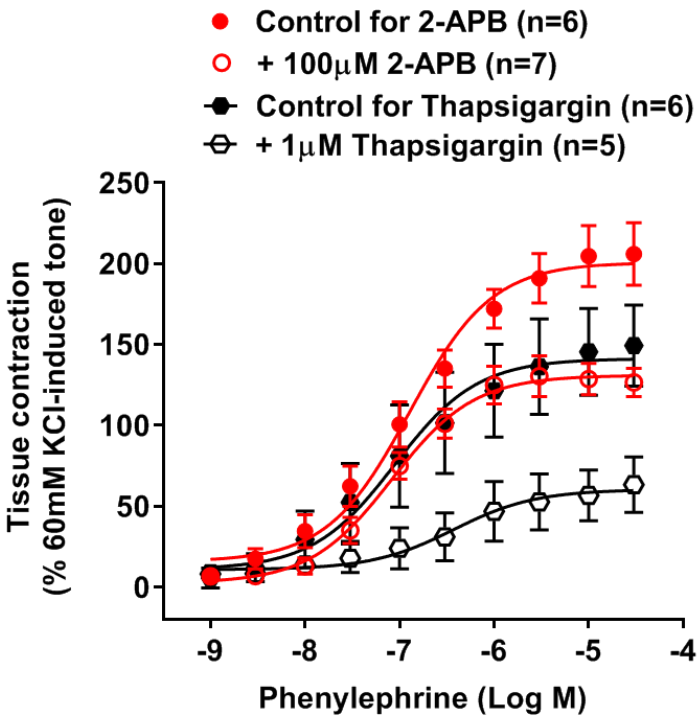


Figure 4.12: Effect of control for 2-APB (DMSO 1%), 2-APB, control for thapsigargin (DMSO 0.05%), and thapsigargin pre-incubation on PE concentration-response curves in rat aortae. Tissue responses have been expressed as a percentage of KCl-induced contraction and are shown as means ± SEM of 5-7 animals.

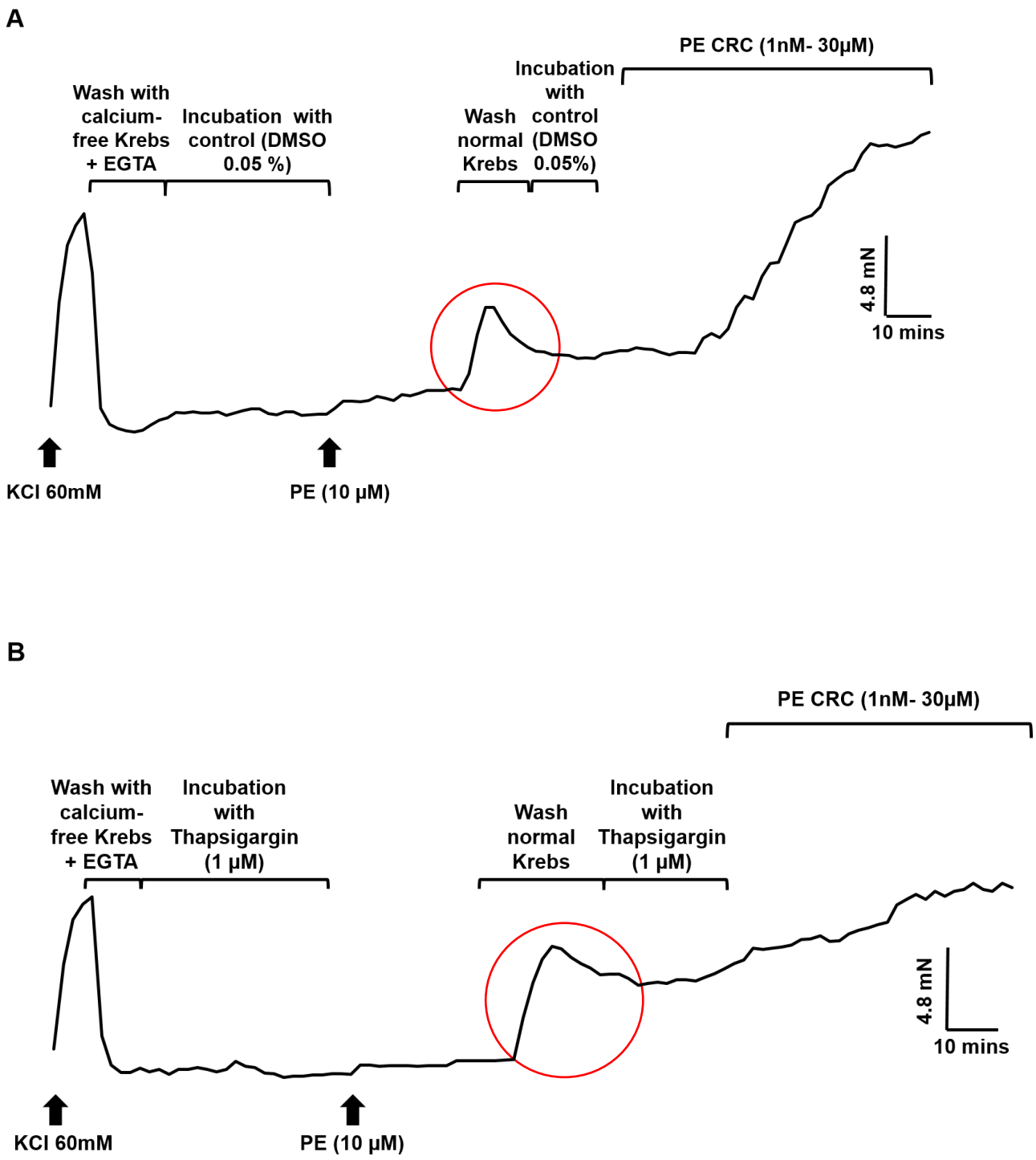


Figure 4.13: Representative trace recordings of PE contractile activity in the presence of **A)** vehicle control (DMSO 0.05%) **B)** thapsigargin (1 µM). Following second KCl, the tissues were washed with calcium-free Krebs solution and incubated with either vehicle control or thapsigargin (1 µM). Then, a single concentration of PE (10 µM) was added. The contraction was rather smaller in calcium-free Krebs, interestingly upon reintroduction of normal Krebs the PE contraction was enhanced (marked in red circle), before dropping back to basal tension. As the tissues returned to basal tension and stabilised, PE CRC were performed. (Abbreviations: KCl = potassium chloride; PE= phenylephrine; mN = milliNewton; mins = minutes)

4.5 Discussion

The present study examined the role of external and stored Ca^{2+} upon activation of different contractile agents, KCl, 5-HT, and PE on rat aortae.

In this study, KCl contraction showed a slower onset when compared to PE and 5-HT contractions, at which a higher concentration of KCl was needed to cause the maximal contraction. One possible explanation for this observation is increased extracellular K^+ levels result in depolarisation, while lower extracellular K^+ level causes hyperpolarisation, therefore, a higher amount of KCl was required for depolarisation of the membrane in order to initiate contraction (Levitan *et al*.,1995). This also shows that KCl does not act via GPCR, unlike the PE and 5-HT, but causes contraction via different calcium signaling pathways. The drop in KCl response at higher concentrations could be due to repolarisation or calcium desensitization (Ratz *et al.,* 2005; Tammaro *et al.,*2004). The excess extracellular K^+ inactivates sodium channels and opens K^+ channels, thus leading to refractory (Ratz *et al*. 2005). KCl, in general, cause contraction by depolarizing the aorta. As K^+ depolarises the aorta, this stimulates an enhanced uptake of calcium via voltage-dependent Ca^{2+} channels, which leads to contraction (Ratz *et al*. 2005). Despite the removal of extracellular Ca^{2+}, KCl contraction was still observable but smaller. This shows that KCl can also cause intracellular Ca^{2+} release. This finding is consistent with studies done in the past by (Kirschstein *et al*., 2009) on rat gut smooth muscle. However, the drop in KCl contractile activity observed at higher concentrations and after a single addition of KCl, suggest that the intracellular Ca^{2+} has little role in maintaining the contractile activity of KCl in rat aorta. This corroborates with studies done in the past on KCl in different smooth muscle tissues (Kannisto *et al*., 1987; Visser and Mastrigt 2000; Ratz *et al*.,2005). The present finding suggests that KCl depends mostly on extracellular Ca^{2+} to cause a contraction in the rat aorta.

Stimulation of G_q-coupled receptors leads to the release of stored and influx of extracellular Ca^{2+} via second messengers IP_3 and DAG, respectively. DAG via PKC activation, opens the TRPC channels to allow Ca^{2+} influx through TRPC3, TRPC6, or TRPC7 (Hoffman *et al*., 1999). The initial influx of Ca^{2+} via the TRPC channels depolarise the membrane and leads to the opening of L-type Ca^{2+} channels. In addition to this paradigm, the downward signaling also involves the activation of RhoA, a mediator of calcium sensitisation. Active RhoA interacts with Rho GEF to stimulate the downstream effector, ROCK, and PKC-related kinase, which contributes to smooth muscle contraction by involving the release of intracellular Ca^{2+} (Szasz and Webb, 2017). All G-proteins have a similar structure, and they operate in a similar way (Alberts *et al.*, 2002). However, it has been shown that many of the cellular responses

mediated by GPCRs do not only involve stimulation of conventional second-messenger-generating systems but instead result from the functional integration of an intricate network of intracellular signaling pathways (Marinissen and Gutkind, 2001).

5-HT induces vasoconstriction in the rat aorta mainly via 5-HT_{2A} subtype (Lu *et al.*, 2008). The 5-HT_2 receptors are linked to the G_q family of G-proteins and subsequently activating PLC. Since both PE and 5-HT bind to G_q-coupled protein receptors, it was envisaged that 5-HT would show similar contractile activity to PE in the aorta, but in the present study, they both reacted differently in terms of calcium dependency. The 5-HT elicited delayed onset of action when compared to PE contraction, which was rapid. These results confirm the existence of both α-adrenoceptors and serotonergic receptors in rat aortae and also suggest that α-adrenoceptors and serotonergic receptors do not act similarly. The finding is inconsistent with the study done previously on rabbit aorta, where both 5-HT and norepinephrine contractions appear to be mechanistically similar (van Breemen, 1969). Therefore it is interesting to speculate that the discrepancies observed could be due to the variation in the expression of the receptors and different signaling pathways of PE and 5-HT in rat aorta. The past study had reported that 5-HT_{2A} receptor stimulation leads to the activation of PLC, L-type Ca^{2+} channels as well as tyrosine kinases, including MEK. These pathways are activated somewhat independently of each other to stimulate aortic contraction (Masson *et al.*, 2012). The 5-HT cumulative concentration-response in normal Krebs was higher than in calcium-free Krebs, suggesting that 5-HT depends mostly on extracellular Ca^{2+} to cause contraction. This shows that the source of Ca^{2+} used to refill the intracellular store responsible for the early, phasic contraction in rat aorta was of extracellular origin, and this is consistent with the previous study done on 5-HT in the bovine ventricular coronary artery (Ratz *et al*.,1984). This notion is further supported by our present results obtained from a single addition of 5-HT, where the contraction could not be sustained, suggesting little or no contribution of intracellular Ca^{2+} in 5-HT-induced contraction. It is also important to note that the waning on contraction to 5-HT could be due to the desensitization of 5-HT_{2A} receptors in rat aorta (Leff and Martin 1988; BenHarari *et al*.,1991; Watts *et al*.,1994). Desensitization of 5-HT-induced contraction in rat aorta may result from (i) alteration in the coupling of receptors with GTP-binding proteins or effectors or (ii) inactivation of receptors due to phosphorylation (Watts *et al*.,1994). Besides that, desensitization could also be related to a lack of phosphoinositide (PI) hydrolysis (Watts *et al*.,1994). Therefore the decline in 5-HT over time could be due to lack of 5-HT-stimulated PI hydrolysis in rat aorta.

Pre-treatment with nifedipine and SKF96365 markedly reduced the effect of 5-HT. These results suggest that L-type and non-L-type Ca^{2+} influx, e,g TRPC channels are mostly involved in 5-HT_{2A} receptor-mediated constriction in rat aorta. Incubation with thapsigargin did not seem to affect 5-HT contraction. This result is inconsistent with the previous studies of 5-HT in the presence of thapsigargin on rat mesenteric resistant arteries (Sofola *et al*.,2003) and on rat aortic smooth muscle cells (Saini *et al.*, 2003). The discrepancy in the results of the present study could be due to different experimental conditions e.g., cells vs. tissues, artery type, and species variation. However, the exact signaling pathway remains elusive. Pre-treatment with 2-APB, reduced 5-HT contraction, confirming that serotonin receptor activation by 5-HT in rat aortic rings involve PI hydrolysis and produce a generation of IP_3, subsequently releasing Ca^{2+} from the intracellular store. This results, however, is contradictory to the results obtained using thapsigargin treatment. It is noteworthy to mention that 2-APB does not always act as an IP_3 receptor antagonist since it has multiple targets. The inhibition of IP_3-mediated Ca^{2+} release by 2-APB is exceptionally variable between studies. However, an almost universal observation is that 2-APB inhibits store-operated Ca^{2+} entry (Bootman *et al*.,2002). Besides that, 2-APB also had been shown to block some TRP isoforms while being a somewhat variable inhibitor of IP3-induced Ca^{2+} release (Iwaski *et al*.,2001). Thus explaining the contradictory observation in thapsigargin and 2-APB treated aorta. Collectively the present findings show that 5-HT contraction relies mainly on extracellular Ca^{2+} with little contribution from intracellular Ca^{2+}.

In the present study, there was a marked reduction of PE contractility in the calcium-free medium as compared to PE in normal Krebs- Ringer solution. Despite the significant reduction in the maximal responses in the calcium-free medium, the contraction to PE was still present and measurable. This suggests that extracellular Ca^{2+} is essential in PE-induced contraction, but it is not the only source of Ca^{2+} required to elicit contraction in the vascular tissue. Activation of G_q-protein in the smooth muscle involves a downstream Ca^{2+} influx through the TRPC and L-type Ca^{2+} channels (Iino *et al.,* 1994; Lee *et al.*, 2001; Hoffmann *et al.,* 1999). SKF 96365 markedly reduced the PE contraction. Nifedipine, which blocks the L-type Ca^{2+} channels also reduced contractions in the aorta but at a lower magnitude when compared to blockage of TRPC channels. This shows that the TRPC channels play a crucial role in the initial influx of Ca^{2+} before the second phase of Ca^{2+} entry via the L-type Ca^{2+} channels. The initial influx of Ca^{2+} promotes membrane depolarisation, which eventually leads to the opening of L-type Ca^{2+} channels. It is well established that L-type Ca^{2+} channels are essential for coupling membrane depolarization to the influx of Ca^{2+} in all excitable cells (Alexander *et al.*, 2017). The blockage of these channels by nifedipine stops external Ca^{2+} influx leading to the loss of sustained contractions, as shown in the result. The findings confirm that the role of TRPC channels and L-type Ca^{2+} channels are crucial in eliciting contraction mediated by PE

in rat aorta. Past findings have reported that TRPC3 channels are expressed in vascular smooth muscle and endothelial cells. They have been identified as a crucial player in Ca^{2+} signaling in human tissues (Senadheera *et al.,* 2012), and found to be highly up-regulated in cardiovascular diseases e.g., hypertension (Zou *et al*.,2015). However, no studies were done about TRPC3 expressions on rat aortic smooth muscle, particularly on Sprague Dawley rats.

The role of stored Ca^{2+} in regulating contractions was investigated by using thapsigargin to empty the internal Ca^{2+} store. Removal of stored Ca^{2+} affected the maximum response of PE. This finding supports our earlier observation that activation of G_q-protein in the vascular tissue requires the mobilization of both external and stored Ca^{2+}. Stored Ca^{2+} plays a vital role in the vasculature to maintain a specific tone for tight regulation of blood pressure (Leung *et al.*, 2008). As depicted in the result section (in **Figure 4.12**), the control group for the thapsigargin experiment displayed lower PE maximal response in comparison to the control group in other experiments. Although lower DMSO (0.05%) been used in this experiment, the reduction in PE maximal contraction could be due to two factors. First, the application of a single concentration of PE before performing PE CRC would have depleted the Ca^{2+} store, thereby decreasing subsequent PE-mediated contraction. Second, the first application of PE would have led to the desensitization of PE-mediated contraction, which is mediated by alteration of receptor coupling or blunting of phosphatidylinositol turnover. This observation was in accordance with research done in the past on rats and rabbits aortic rings (Lurie *et al*., 1985; Arce *et al*., 2017).

Despite the washing out the procedure (in **Figure 4.13**), the effect of PE from first addition (in calcium-free Krebs) remained and enhanced upon reintroduction of normal Krebs. The possible explanation for this could be due to a change in membrane potential upon the reintroduction of normal Krebs. Upon stimulation of PE in calcium-free Krebs, a decrease in K^+ conductance occurs to produce depolarization in the presence of an inward current (Lurie *et al*.,1985). Then the change in membrane potential induces sodium (Na^+) window current, which further propels the depolarization. The increase in Na^+ influx increase Ca^{2+} influx by activating the Na^+/ Ca^{2+} exchanger, thus explaining the transient contraction observed upon the reintroduction of extracellular Ca^{2+}.

Pre-incubation with 2-APB (SOCC inhibitor and IP_3R inhibitor) also reduced the PE-contraction, further confirming that the IP_3 pathway is involved in PE stimulation and highlighting its pivotal role in modulating Ca^{2+} handling and the contractile activity of PE. Previously, Lee *et al*., (2001) concluded that the refilling of the SR Ca^{2+} store through the SERCA pump, which is maintained by extracellular Ca^{2+} entry, is essential in sustaining PE-induced contraction. However, the present study shows that the intracellular Ca^{2+} alone in the absence of extracellular Ca^{2+} entry may be sufficient to cause contraction and sustain PE-induced contraction in the rat aorta, further reiterating the importance of stored Ca^{2+} in maintaining the vascular tone. This is in accordance with past studies that have shown that IP_3R-mediated Ca^{2+} release in VSMC is a critical player in cellular contraction (Lin *et al.*, 2016).

Conventionally, activation of a G_q-protein elicits entry of extracellular Ca^{2+} and release of stored Ca^{2+} to produce contraction in smooth muscle cells. From the present findings, it can be deduced that external and stored Ca^{2+} synergistically promote contraction in the aorta, but the removal of either source still enables a tone of the tissue to be maintained. This study successfully showed that different contractile agents activated by membrane depolarization and G_q-coupled protein utilize different signaling pathways to mobilize intracellular and extracellular Ca^{2+} to cause a contraction in the smooth muscle. However, the exact signaling pathways responsible for this remain elusive and warrants further investigations. In summary, (1) KCl-induced contraction depends on extracellular Ca^{2+} (2) 5-HT-induced contraction depends mostly on extracellular Ca^{2+} (3) PE-induced contraction depends on both extracellular and intracellular Ca^{2+} on rat aortic smooth muscle. This information will be useful to further the investigation of calcium-dependent mechanisms underlying FK70-induced relaxation. The details of the experiments will be discussed in-depth in the next chapter (chapter 5).

Chapter 5: The role of FK70 in calcium-mediated vascular responses in rat aortae

5.1 Introduction

In the previous chapter, the role of external and stored Ca^{2+} in the contractile activity of different contractile agents (KCl, 5-HT and PE), were discussed. The results of the investigation showed that different contractile agents mobilise external and stored Ca^{2+} differently to elicit smooth muscle contractions in the rat aortae. In Chapter 3, it was shown that FK70 relaxed the contractile agents-induced contraction and attenuated calcium-induced contraction. Therefore, this chapter was designed to further characterise the influence of FK70 in calcium-mediated responses in the aortae.

5.1.1 Calcium dynamics in vascular smooth muscle

Muscle cells, including vascular smooth muscle cells, generally contract when $[Ca^{2+}]_i$ rises. The changes in the contractile state of vascular smooth muscle either increases or decreases vascular diameter, which in turn alters the blood flow through the vessel and, subsequently, the vascularized tissue. Thus, events that influence $[Ca^{2+}]_i$ are critical regulators of vascular function with physiological and pathophysiological ramifications. The vascular smooth muscle cells integrate multiple signaling events that influence $[Ca^{2+}]_i$ either directly or indirectly (Amberg *et al.*,2013). The sources of Ca^{2+} for cytoplasmic signaling in vascular smooth muscle cells include Ca^{2+} influx through ion-permeable channels located in the plasma membrane and Ca^{2+} release from intracellular Ca^{2+} stores e.g., SR. Ca^{2+} influx in vascular smooth muscle is mediated primarily by voltage-dependent L-type Ca^{2+} channels with contributions from other channels, including T-type Ca^{2+} channels, transient receptor potential (TRP) superfamily of cation channels, and Ca^{2+}-permeable ligand-gated cation channels (Earley and Brayden, 2015). Ca^{2+} release from intracellular stores is mediated by two types of Ca^{2+}-permeable ion channels located in SR membranes, ryanodine receptors and inositol 1,4,5-trisphosphate (IP_3) receptors (Gordienko and Boulton, 2002; Foskett *et al.,*2007).

Increased intravascular pressure stimulates gradual membrane depolarization in vascular smooth muscle, thus leading to the opening of L-type Ca^{2+} channels (Sonkusare *et al.,*2006). Alternatively, the calcium-dependent contractile response can also be induced through the activation of receptors coupled to PLC. The hydrolysis of PLC leads to the activation of various forms of TRP ion channel family, particularly, TRPC channels (Earley and Brayden, 2015). These channels contribute to initial plasma membrane Ca^{2+} influx and subsequent membrane depolarization. Besides the external Ca^{2+} source, the store-operated Ca^{2+} channels (SOCC) also contribute to the source of Ca^{2+} to maintain a long term Ca^{2+} signal. They are activated upon a decrease in internal store Ca^{2+}. Reduction in the Ca^{2+} in SR results in dissociation of Ca^{2+} from STIM1 (stromal interaction molecule), a Ca^{2+} sensor, then the STIM1 molecules oligomerize and translocate to specialized reticulum compartments adjacent to the plasma membrane (Shen and Demaurex, 2012). Then STIM1 activates domains bound to and cluster the Orai1 proteins to open Ca^{2+} channels. The TRPC channels have also been demonstrated to participate in SOCC through interactions with STIM1 and Orai proteins (Yuan *et al*.,2012).

The calcium signal is terminated by membrane hyperpolarisation and cytosolic Ca^{2+} removal. First, the calcium sparks resulting from increased cytosolic Ca^{2+} activate large-conductance Ca^{2+}-sensitive K^+ (BK_{Ca}) channels. Then, the resulting spontaneous transient outward currents (STOC) hyperpolarize the membrane and decrease the opening of L-type Ca^{2+} channels (Nelson *et al*.,1995). The cytosolic Ca^{2+} is extruded at the level of plasma membrane by plasma membrane Ca^{2+} ATPase (PMCA) and Na^+/Ca^{2+} exchanger (NCX) (Strehler *et al*.,2007). More than 70% of cytosolic Ca^{2+} is re-uploaded to the internal store.

5.2 Study aim and objectives

FK70 exhibited prominent relaxation effect in the vascular smooth muscle tissues. Based on our findings in (Chapter 3), the evidence point towards its involvement of calcium dynamics in isolated tissue. Therefore, the main aim of the present study was to scrutinise the role of FK70 on calcium-induced responses. Our experiments only focused on the functional (relaxation and contractions) responses of the blood vessel *in-vitro*. The specific objectives were;

i) to study the effects of FK70 on calcium release by concentration-dependent contractile agents (PE, 5-hydroxytryptamine and KCl).

ii) to compare the concentration-dependent relaxation effect of FK70 with known calcium channel blockers, nifedipine (a L-type Ca^{2+} channel inhibitor) and SKF 96365 (a non-selective TRPC channel inhibitor).

iii) to investigate the effects of FK70 on external Ca^{2+} **a)** in the presence of nifedipine (L-type Ca^{2+} channel inhibitor), **b)** verapamil (L- and T-type Ca^{2+} channel inhibitor) **c)** in the presence of SKF 96365 (non-selective putative TRPC channel inhibitor)

iv) to investigate the effects of FK70 on stored Ca^{2+} in calcium-free Krebs

5.3 Materials and methods

5.3.1 Drugs and Krebs-Ringer bicarbonate solution

(R)-(−)-phenylephrine hydrochloride (Sigma), 1-[2-(4-methoxyphenyl)-2-[3-(4-methoxyphenyl)propoxy]ethyl]imidazole (SKF 96365) (Sigma, USA), 5-hydroxytryptamine (5-HT) (Nacalei Tesque, Japan), nifedipine (Nacalai Tesque, Japan), (±)-verapamil hydrochloride (Acros organics, UK) and potassium chloride (KCl) were used. All dissolved drugs were made into stock concentration of 0.1 M. A serial of ten-fold dilution was made for all dissolved drug solutions. Details on the drugs, solvents used and stock concentration for each drug are summarised in **Table 5.1**.

Table 5.1: Summary of drugs used, country manufactured, solvent and stock concentration used in the experiments.

Drugs	Brand / Country manufactured	Solvent	Stock concentration (M)
Phenylephrine hydrochloride	Sigma, USA	Distilled water	0.1
5-hydroxytryptamine	Nacalei Tesque, Japan	Distilled water	0.1
Nifedipine	Nacalei Tesque, Japan	100 % DMSO	0.1
1-[2-(4-methoxyphenyl)-2-[3-(4-methoxyphenyl)propoxy]ethyl]imidazole (SKF 96365)	Sigma, USA	100 % DMSO	0.1
(±)-Verapamil hydrochloride	Acros organics, UK	100 % DMSO	0.1

Calcium-containing Krebs solution (or normal Krebs solution) was prepared as described in **Section 2.3.1.**

5.3.2 Tissue preparation and organ bath assembly

Refer to **section 2.3.2**

5.3.3 Organ bath experimental protocols

5.3.3.1 Effect of FK70 on concentration-dependent contractile agents

In order to investigate the effect of FK70 on calcium release by contractile agents, FK70 (30µM) was incubated prior to performing cumulative concentration-response curve to PE (1 nM to 30 µM), KCl (10 µM to 0.1 M) and 5-HT (1 nM to 300 µM). The concentration of FK70 for incubation was chosen on the basis of its ability to cause 100% reduction in PE -induced tone.

5.3.3.2 Effects of FK70 on PE-induced contraction

In order to assess the characteristics of FK70, the aortae were pre-incubated with different concentrations of FK70 (10 µM, 30 µM and 100 µM), prior to performing PE concentration-response curves.

In a separate experiment to assess whether FK70 block PE-induced contraction reversibly or irreversibly, FK70 CRCs were performed on aortic rings pre-constricted with PE (0.1 μM). Thereafter, the tissues were washed with normal Krebs for 30 minutes prior to adding single concentration of PE (10 μM).

5.3.3.3 Effects of FK70 and other known calcium channel blockers

FK70 concentration dependent relaxation effect was compared with relaxation effect of known calcium channel blockers nifedipine and SKF 96365. The aortic rings were pre-constricted with PE (0.1 μM) prior to performing concentration-response curve of FK70, nifedipine and SKF 96365. The tissues were exposed to increasing concentrations of the drugs, after the response to previous concentration had stabilised. The concentration range used was 1nM-100μM. Only one CRC was performed per tissue.

5.3.3.4 Effect of FK70 on L-type and T-type Ca^{2+} channels

Firstly, the involvement of L-type Ca^{2+} channels in FK70 –induced relaxation was tested by pre-incubating the aortic rings with nifedipine (30 μM) for 40 minutes. Then, the rings were pre-constricted with PE (0.1 μM) prior to performing FK70 CRC.

To further investigate the involvement of L-type Ca^{2+} channels, series of experiments were carried out as following:

i) Following the second KCl stimulation, tissues were washed with normal Krebs-Ringer solution. Then they were treated with a single concentration of PE (10 μM). Once the response has stabilised, the tissues were washed for 15 mins. The tissues were then incubated with nifedipine (30 μM) for 40 minutes, followed by addition of a single concentration of PE (10 μM). Thereafter, a single concentration of FK70 (30 μM) was added to the bath and left for 1 hour. The concentration of nifedipine and FK70 used in this experiment were chosen based on their (IC_{90}) ability to cause 90%-100% relaxation in rat aorta.

ii) In a different set of experiment, the drugs mentioned above were added in a reverse order. The experiments were repeated as above but incubated with FK70 (30μM) first for 40 minutes, followed by addition of PE (10 μM). Thereafter, a single concentration of nifedipine (30 μM) was added to the bath and left for 1 hour.

To examine the involvement of T-type Ca^{2+} channels in FK70-induced relaxation, series of experiments were carried out as following;

i) the involvement of T-type Ca^{2+} channels in FK70-induced relaxation was tested using verapamil (Singh *et al*.,2010). Firstly, the aortic rings were pre-incubated with verapamil (30 μM) for 40 minutes. Then, the rings were pre-constricted with PE (0.1 μM) prior to performing FK70 CRC.

ii) Following the second KCl stimulation, tissues were washed with normal Krebs-Ringer solution. Then they were treated with a single concentration of PE (10 μM). Once the response has stabilised, the tissues were washed for 15 mins. The tissues were then incubated with verapamil (30 μM) for 40 minutes, followed by addition of a single concentration of PE (10 μM). Thereafter, a single concentration of FK70 (30 μM) was added to the bath and left for 1 hour. The concentration of verapamil and FK70 used in this experiment were chosen based on their ability to cause 100 % relaxation in rat aorta.

5.3.3.5 Effect of FK70 on TRPC channels

To examine the involvement of TPRC in FK70-induced relaxation, series of experiments were carried out as following:

i) Firstly, the involvement of TRPC in FK70 –induced relaxation was tested by pre-incubating the aortic rings with SKF 96365 (30 μM) for 40 minutes. Then, the rings were pre-constricted with PE (0.1 μM) prior to performing FK70 CRC

ii) In a separate experiment, the aortic rings were treated with a single concentration of PE (10 μM). Once the response has stabilised, the tissues were washed for 15 mins. The tissues were then incubated with SKF 96365 (30 μM) for 40 minutes, followed by addition of a single concentration of PE (10 μM). Thereafter, a single concentration of FK70 (30 μM) was added to the bath and left for 1 hour.

5.3.3.6 Effect of FK70 on stored calcium

Following the second KCl stimulation, the tissues were washed with calcium-free Krebs-Ringer solution. Then they were incubated with FK70 (30 μM) for 40 minutes, followed by addition of EGTA 0.1 mM (10 μM) into the bath for 10 minutes before stimulating the tissues with a single concentration of PE (10 μM) and left for 1 hour (Guan *et al*., 1988; Low *et al*.,1992).

5.3.4 Data analysis

Data were analysed and graphs were drawn using PRISM version 7.0 (GraphPad software). The aortic contraction was expressed as a percentage of PE / KCl (60mM)-induced contraction except for aortic responses in experimental result section **5.4.4** and **5.4.5**. Their contraction to PE were expressed in mN (=tension weight x 9.8) due to lower contractile magnitude to 60 mM KCl than PE-induced contractile response. All data were expressed as mean ± standard error of mean (SEM) of *n* number of animals. Maximum tissue contraction or relaxation response (E_{max}) and concentration to produce basal response by 50 % (pEC_{50}) were derived from nonlinear regression analysis of the obtained CRC. Statistical analyses were performed using Student's unpaired *t*-test, paired *t*-test and one-way ANOVA. Unpaired *t*-test was used to compare the statistical differences from two experimental groups: one from a control condition while another from a treatment condition [PRISM Version 7 (Graph Pad Software)]. Paired *t*-test was used to compare the statistical differences between different treatments from same experimental group. For comparing more than two treatment groups, their mean differences were tested using one-way ANOVA followed by Dunnett's multiple comparisons test (PRISM Version 7; Graph Pad Software). Results were considered statistically significant if $p < 0.05$.

5.4 Results

5.4.1 FK70 attenuates the contractile activity of PE, 5-HT and KCl

Aortic rings in the absence of FK70 were treated as control. Pre-incubation with FK70 reduced the maximal contractile response curves of PE, 5-HT and KCl significantly and shifted the curves to the right (see **Figure 5.1** and refer **Table 5.2**)

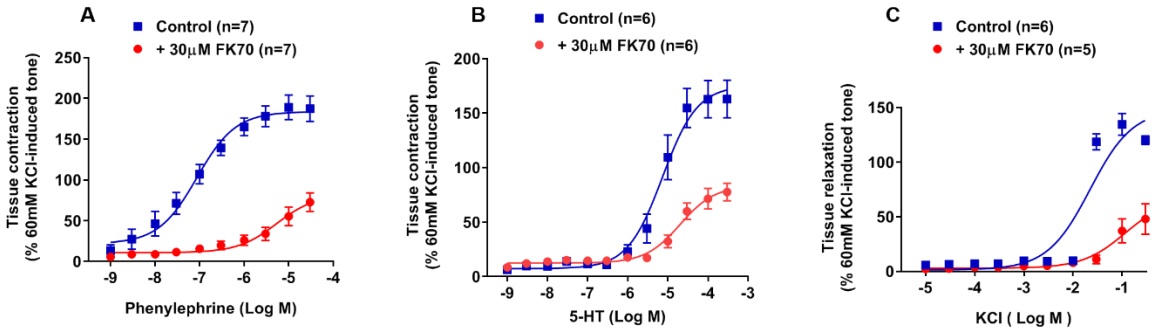


Figure 5.1: Effects of FK70 against **A)** PE concentration-response curve **B)** 5-HT concentration-response curve **C)** KCl concentration-response curve in rat isolated aortae. Tissue responses have been expressed as a percentage of KCl-induced tone and are shown as means ± SEM of 5 to 7 animals.

Table 5.2: Summary of PE, 5-HT and KCl contractile response in the presence of control and FK70 (30 μM) in rat aortae. Tissue responses have been expressed as a percentage of KCl-induced contraction and are shown as means ± SEM of 5 to 7 animals.

Agonist	n (number of animals)	Maximal contraction of control at (3×10^{-5}M) (%)	n (number of animals)	Maximal contraction at (3×10^{-5}M) in the presence of FK70 (%)
PE	7	187.3 ± 15.7	7	72.7 ± 11.4
5-HT	6	163 ± 17.2	6	77.6 ± 7.9
KCl	6	120.1 ± 4.0	5	47.9 ± 13.9

5.4.2 FK70 inhibits PE contraction irreversibly

Based on the experiment (in **section 5.4.1**), it was evident that FK70 inhibit PE contraction. Therefore, the next step of the experiment was to evaluate the characteristics of FK70. The suppressive effect of FK70 on PE CRC is concentration- dependent. FK70 at the highest concentration tested abolished the PE contractile responses (see **Figure 5.2** and refer **Table 5.3**).

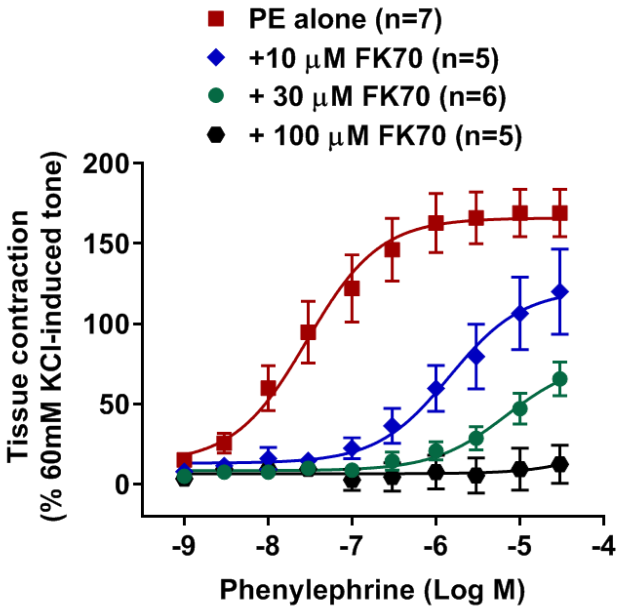


Figure 5.2: Effects of different concentrations of FK70 against PE-induced contractions. Tissue responses have been expressed as a percentage of KCl-induced tone and are shown as means ± SEM of 5-7 animals.

Table 5.3: Summary of PE alone and pre-incubation with different concentrations of FK70 on PE-induced contraction in rat aortae. FK70 reduced PE-induced contraction in a concentration-dependent manner. Tissue responses have been expressed as a percentage of KCl-induced contraction and are shown as means ± SEM of 5 to 7 animals. The one-way ANOVA followed by Dunnett's multiple comparison test compared the contraction between PE alone and FK70 (10 µM, 30 µM, and 100 µM). (***p*<0.01, *** *p*<0.001, *****p*<0.0001, a *p* value of >0.05 is considered no significant difference).

	n (number of animals)	**Maximal contraction at (3×10^{-5} M) (%)**	***p* value**
PE alone	7	169.1 ± 14.8	
FK70 (10 µM)	5	120.1 ± 26.6	0.11
FK70 (30 µM)	6	65.6 ± 10.5	0.0004***
FK70 (100 µM)	5	12.5 ± 11.0	0.0001****

To further investigate the characteristics of FK70 against PE induced-contraction a different experimental protocol was carried out, where the tissues were washed after pre-treatment with FK70 prior to adding PE. Aortic rings treated with DMSO (0 – 1 %) were served as control in this experiment. In FK70 treated aorta, the PE-induced contraction was markedly reduced when compared to the control group, despite washing out with normal Krebs (see **Figure 5.3**).

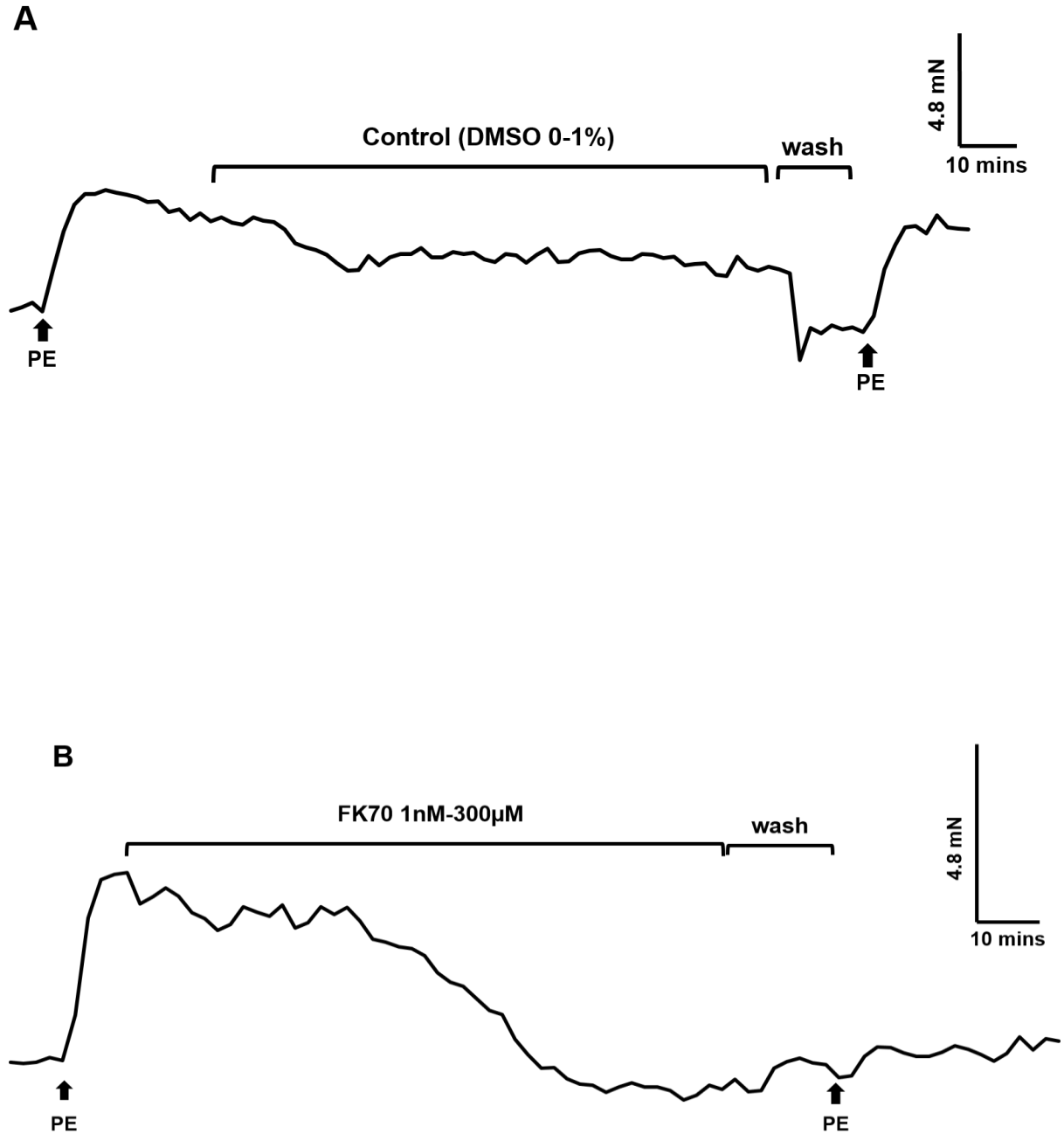


Figure 5.3: Representative trace recordings showing effects of **A)** vehicle control and **B)** FK70 (1 nM – 300 µM) on PE contractile response. After the washing out step, FK70 treated group abolished the PE-induced contraction (Abbreviations: PE = phenylephrine; mN = milliNewton; mins = minutes).

5.4.3 FK70 shows similar relaxation responses as a putative TRPC channel blocker (SKF 96365)

Aortic rings treated with vehicle control (DMSO 0-1%) served as control for this experiment. FK70 exhibited similar maximal relaxation response as SKF 96365 (see **Figure 5.4** and refer **Table 5.4**).

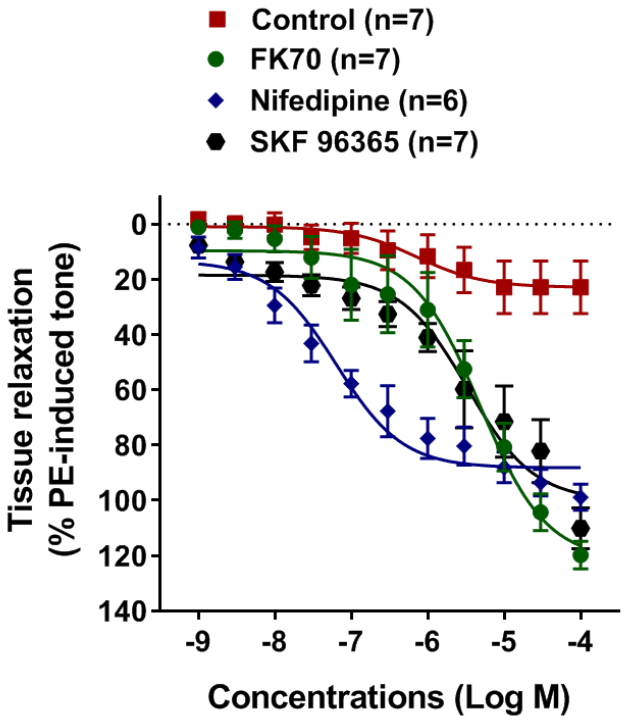


Figure 5.4: Effects of vehicle control, FK70, nifedipine and SKF 96365 in rat isolated aortic rings pre-contracted with PE. Tissue responses have been expressed as a percentage of PE-induced tone and are shown as means ± SEM of 6 to 7 animals.

Table 5.4: Summary of vehicle control, FK70, nifedipine and SKF 96365-induced relaxation in rat aortae. Tissue responses have been expressed as a percentage of PE-induced contraction and are shown as means ± SEM of 6 to 7 animals. The one-way ANOVA followed by Dunnett's multiple comparison test compared the relaxation effect between FK70, and all other treatment groups: control, nifedipine and SKF 96365 (*p<0.05, ****p<0.0001, a p value of >0.05 is considered no significant difference).

	n (number of animals)	**Maximal relaxation at (10^{-4} M) (%)**	***p* value**
Vehicle control	7	22.8 ± 9.4	0.0001****
FK70	7	119.8 ± 4.9	
Nifedipine	6	98.8 ± 4.7	0.45
SKF 96365	7	110 ± 7.2	0.74

5.4.4 FK70-induced relaxation mechanism may not involve blockage of L / T-type Ca^{2+} channels.

In order to examine the role of L / T- type Ca^{2+}channels on FK70 induced relaxation, series of experiments using known calcium channel blockers, nifedipine and verapamil were performed.

In the first set of experiment, the aortic rings were pre-incubated with nifedipine prior to performing FK70 concentration-response curve. Aortic rings without the presence of nifedipine served as control for this experiment. Pre-treatment with nifedipine did not seem to affect FK70 induced relaxation on aortic rings pre-contracted with PE with relaxation values of (control = 119.8 ± 4.9 %, nifedipine treated = 128.8 ± 6.7 %; unpaired *t*-test, *p*= 0.3) (see **Figure 5.5**).

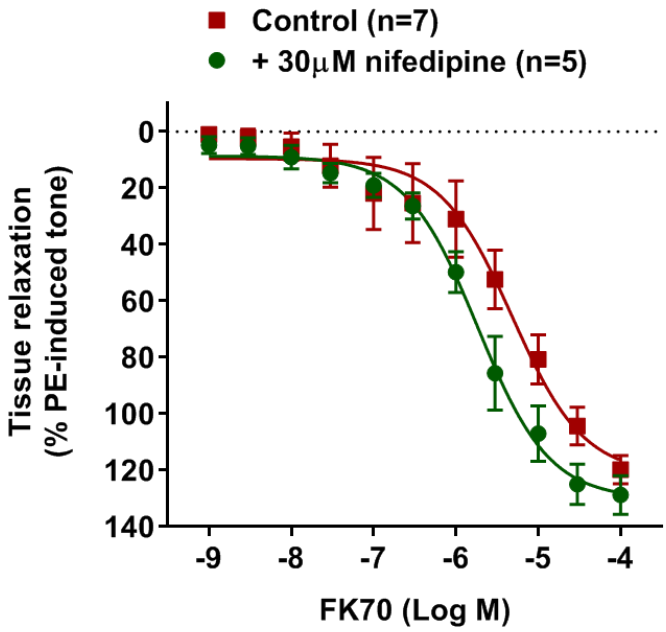


Figure 5.5: Effects of control and nifedipine on FK70 concentration-response curve in rat aortae. Tissue responses have been expressed as a percentage of PE-induced contraction and are shown as means ± SEM of 5 to 7 animals.

To further study the involvement of L-type Ca^{2+}channels in FK70-induced relaxation, a different experimental protocol involving pre-incubation with nifedipine and single addition of FK70 on PE-induced contraction were performed. Aortic rings treated with DMSO (0.22%) served as control.

In a separate series of experiment, the same protocol as above was repeated but in a reverse order where the aortic rings were incubated with FK70 first, then a single concentration of nifedipine was added. This was done to further confirm the involvement of L-type Ca^{2+} channels in FK70-induced relaxation on PE contractile activity. Pre-incubation with vehicle control did not seem to affect PE-induced contraction, while pre-incubation with nifedipine and FK70 reduced PE-induced contraction significantly (see **Figure 5.6** and refer **Table 5.5**)

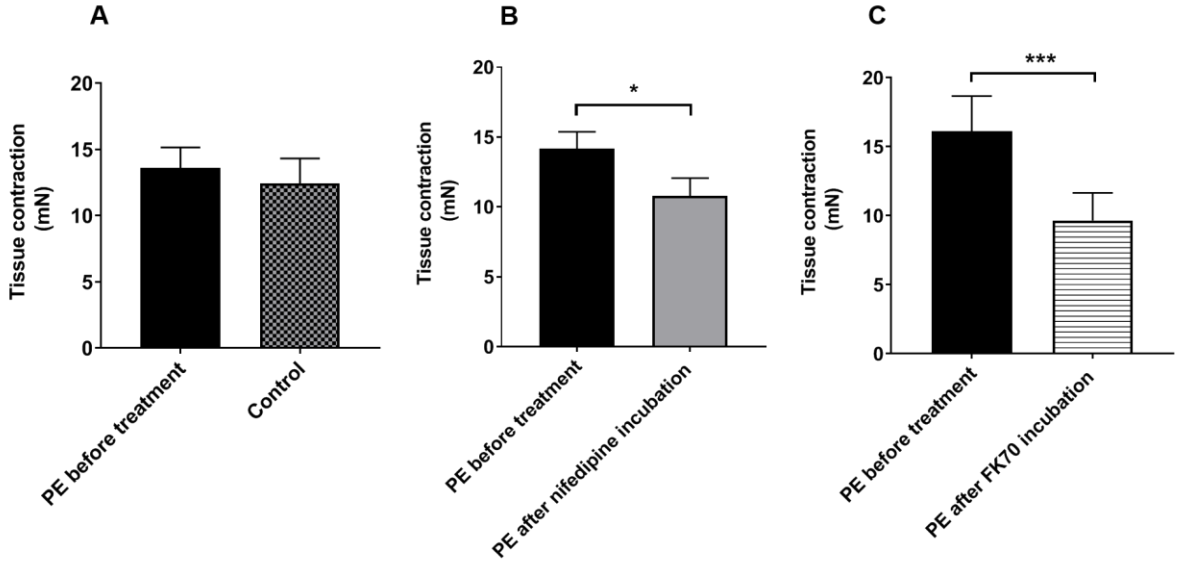


Figure 5.6: Effect of pre-incubation with **A)** vehicle control **B)** nifedipine (30 µM) **C)** FK70 (30 µM) on PE-induced contractions. Tissue responses have been expressed as a force of contraction in force mN and are shown as means ± SEM of 5 to 6 animals. (paired Student's *t*-test : **p*<0.05, ****p*<0.001).

Table 5.5: Summary of (E_{max}) prior to and after incubation with vehicle control, nifedipine and FK70 on PE-induced contractions. Tissue responses have been expressed as force of contraction in force mN and are shown as means ± SEM of 5 to 6 animals. The paired *t*-test compared the contraction of PE prior to incubation and after incubation (**p*<0.05).

Incubation treatment	**PE contraction prior to incubation (mN)**	**PE contraction after incubation (mN)**	***p* value**
+ Vehicle Control (DMSO 0.22 %)	13.6 ± 1.6	12.5 ± 1.9	0.53
+ 30 µM nifedipine	14.2 ± 1.3	10.8 ± 1.3*	0.02
+ 30 µM FK70	16.1 ± 2.6	9.7 ± 2.0***	0.0002

When FK70 was added thereafter to the tissues pre-incubated with nifedipine (in tissue from **Figure 5.6B**), the PE-tone was rapidly reduced after 10 minutes of addition to 3.5 ± 0.8 mN (p= 0.0017) while addition of vehicle did not significantly affect the PE-induced contraction (8.1 ± 1.4 mN; p= 0.22) (see **Figure 5.7**).

When nifedipine was added to tissues pre-incubated with FK70 (in tissue from **Figure 5.6C**), the PE-tone dropped sharply after 10 minutes of addition and sustained. The PE contraction was reduced to 4.1 ± 0.7 mN (p= 0.007), while addition of vehicle reduced PE-tone to 7.7 ± 1.7 mN (p= 0.002) (see **Figure 5.8**).

Addition of FK70 produced a rapid relaxation to the baseline while addition of nifedipine produced a slow and sustained relaxation. The distinct relaxation responses of FK70 and nifedipine suggest that the mechanism of nifedipine and FK70 to cause relaxation in the aorta are not the same.

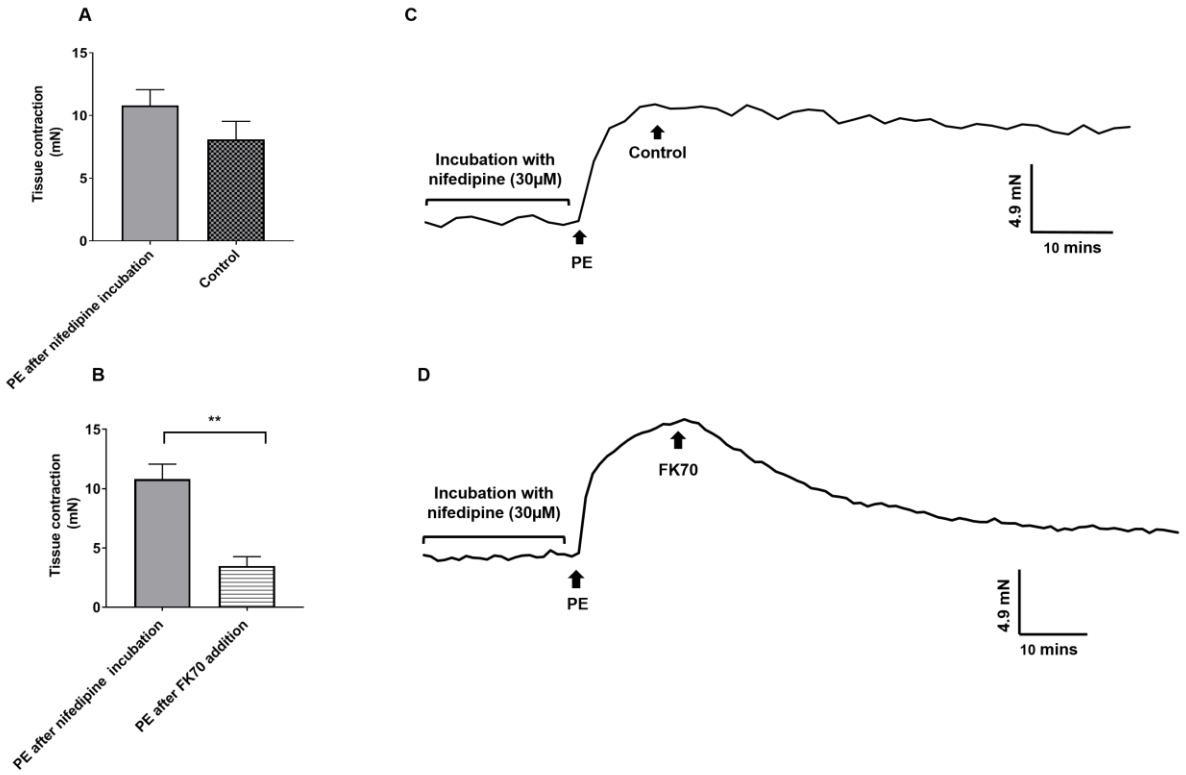


Figure 5.7: Effect of pre-incubation with nifedipine (30 μM) followed by addition of **A)** vehicle control **B)** FK70 (30 μM) on PE-induced contractions. Representative trace recordings of effect of pre-incubation with nifedipine (30 μM) followed by addition of **C)** vehicle control **D)** FK70 (30 μM) on PE-induced contractions. Tissue responses have been expressed as a force of contraction in force mN and are shown as means ± SEM of 5-6 animals. (paired *t*-test : ** $p<0.01$). (Abbreviations: PE = phenylephrine; mN = milliNewton; mins = minutes).

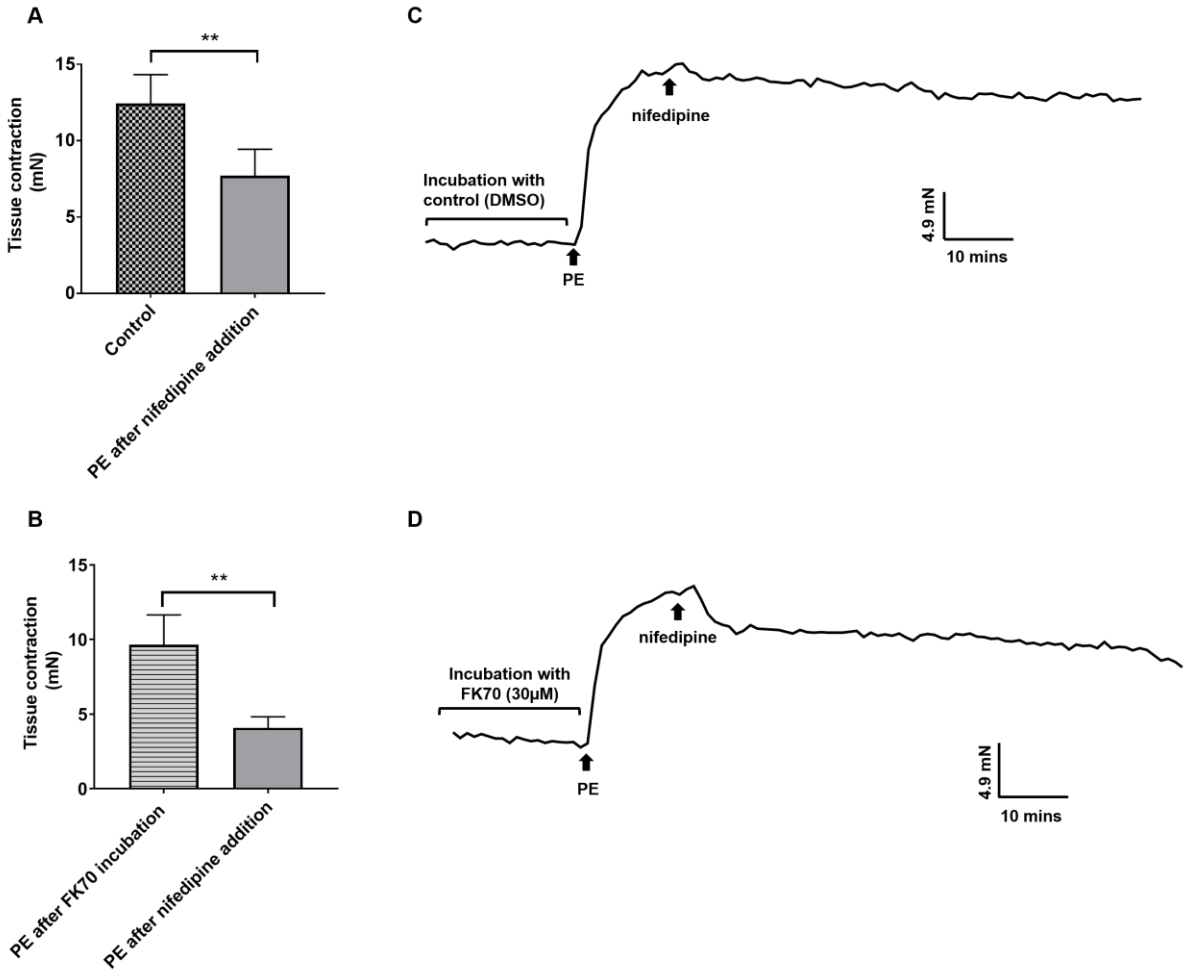


Figure 5.8: Effects of pre-incubation with **A)** vehicle control and **B)** FK70 (30 µM) followed by addition of nifedipine (30 µM) on PE-induced contractions. Representative trace recordings of effect of pre-incubation with **C)** vehicle control and **D)** FK70 (30 µM) followed by addition of nifedipine (30 µM) on PE-induced contractions. Tissue responses have been expressed as a force of contraction in force mN and are shown as means ± SEM of 5-6 animals. (paired *t*-test : ** $p<0.01$). (Abbreviations: PE = phenylephrine; mN = milliNewton; mins = minutes).

The next step of the experiment was to examine the involvement of T-type Ca^{2+} channels on FK70 mechanism using verapamil and FK70. In the first set of experiment, the aortic rings were pre-incubated with verapamil prior to performing FK70 concentration-response curve. Aortic rings without the presence of verapamil served as control for this experiment. Pre-treatment with verapamil did not seem to affect FK70 induced relaxation on aortic rings pre-contracted with PE with maximal response, (control = 119.3 ± 4.4 %, verapamil treated = 120.5 ± 7.8 %; unpaired *t*-test, *p*= 0.9) (see **Figure 5.9**). In terms of potency no significant difference was observed between control and verapamil treated group, with pEC_{50} values of: (control = 5.2 ± 0.2, verapamil treated = 5.0 ± 0.01; unpaired *t*-test, *p*=0.2).

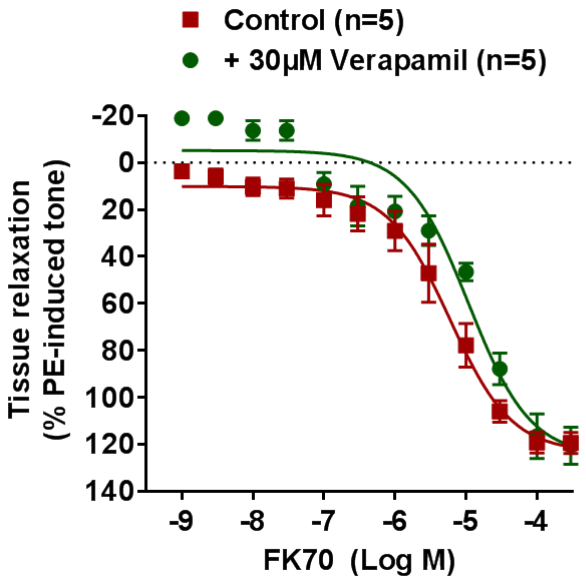


Figure 5.9: Effects of control and verapamil on FK70 concentration-response curve in rat aortae. Tissue responses have been expressed as a percentage of PE-induced contraction and are shown as means ± SEM of 5 animals.

To further study the involvement of T-type Ca^{2+} channels in FK70-induced relaxation, a different experimental protocol involving pre-incubation with verapamil and single addition of FK70 on PE-induced contraction was performed. Aortic rings treated with DMSO (0.22%) served as the vehicle control. PE-induced contraction prior to verapamil incubation was (11.8 ± 0.7 mN). However, upon incubation with verapamil, the PE contraction was reduced to 6.7 ± 0.3 mN (*p*= 0.0008) (see **Figure 5.10**).

Thereafter, FK70 or vehicle control were added to the same tissue. Addition of control reduced PE contraction to (4.8 ± 1.0 mN; *p*= 0.03), whilst addition of FK70 reduced the PE contraction rapidly after 10 minutes of addition to (1.8 ± 0.5 mN; *p*< 0.0001) (see **Figure 5.11**).

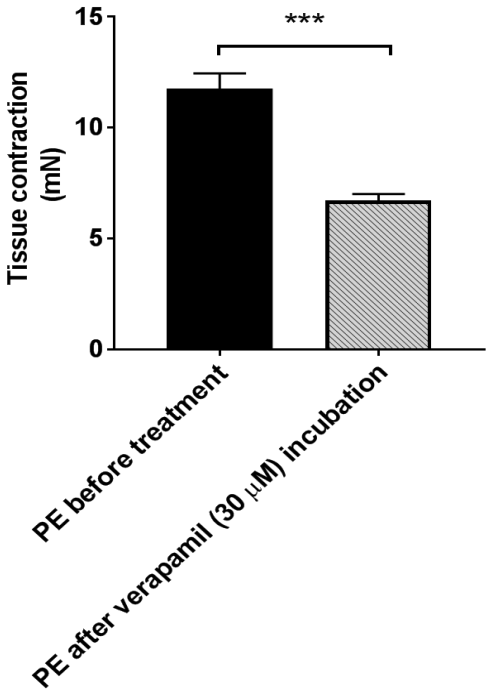


Figure 5.10: Effect of verapamil (30 μM) on PE-induced contractions Tissue responses have been expressed as a force of contraction in force mN and are shown as means ± SEM of 5- 6 animals. (paired Student's *t*-test : *** *p*<0.001).

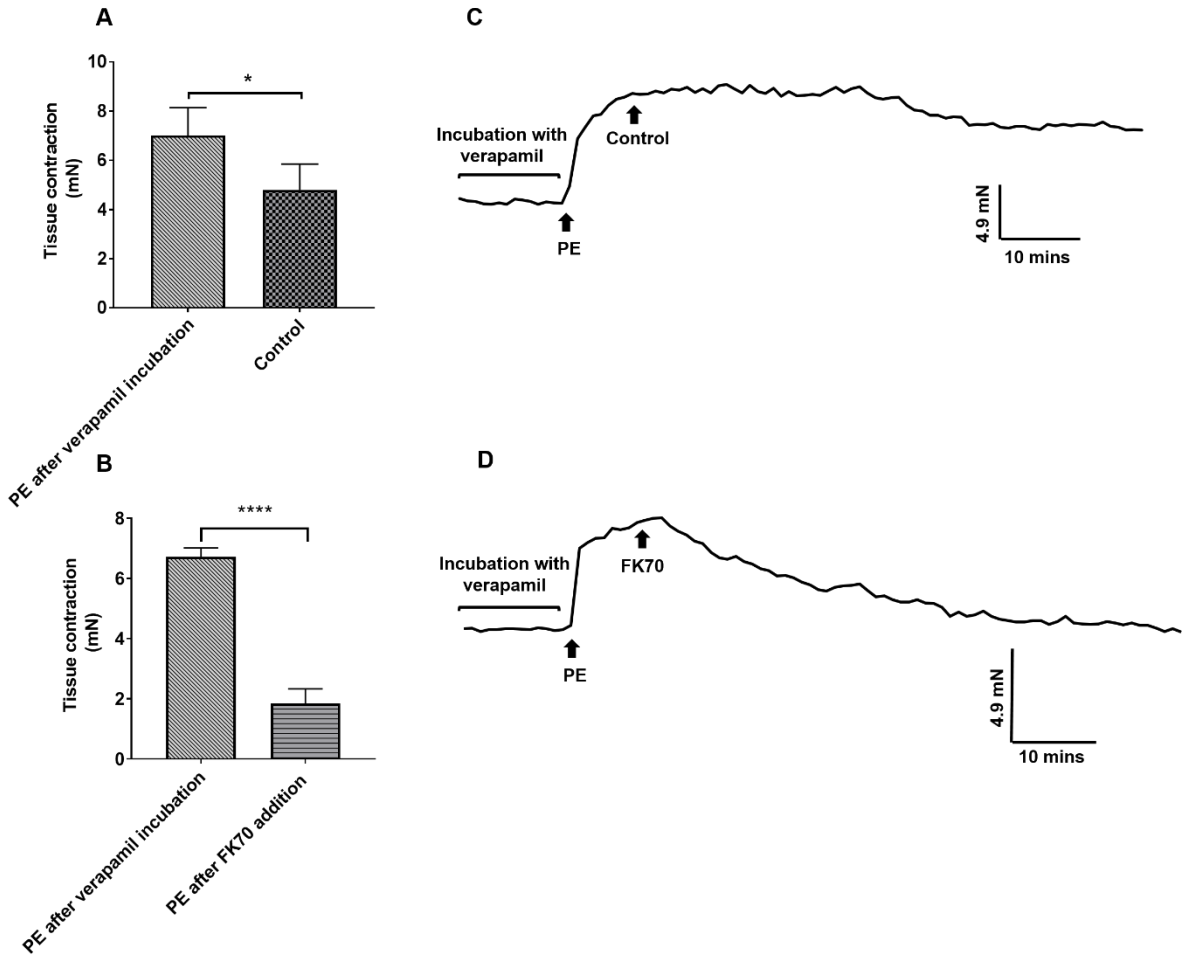


Figure 5.11: Effect of pre-incubation with verapamil (30 µM) followed by addition of **A)** vehicle control **B)** FK70 (30 µM) on PE-induced contractions. Representative trace recordings of PE contractile activity after addition of **C)** vehicle control **D)** FK70 (30 µM). Tissue responses have been expressed as a force of contraction in force mN and are shown as means ± SEM of 5-6 animals. (paired *t*-test : * $p<0.05$, *** $p<0.001$). (Abbreviations: PE = phenylephrine; mN = milliNewton; mins = minutes)

5.4.5 FK70-induced relaxation mechanism may not involve modulation of TRPC channels

The next step was to examine the involvement of TRPC channels on FK70-induced relaxation using SKF 96365 and FK70 on PE-induced contraction.

In the first set of experiment, the aortic rings were pre-incubated with SKF 96365 prior to performing FK70 concentration-response curve. Aortic rings without the presence of SKF 96365 served as control for this experiment. Pre-incubation with SKF 96365 did not seem to affect the maximal response of FK70-induced relaxation, (control: = 119.3 ± 4.4 % vs SKF 96365 treated = 111.6 ± 3.9 %; unpaired *t*-test, *p*= 0.2). The results also showed no significant difference in potency between the control and SKF 96365 treated group (see **Figure 5.12**).

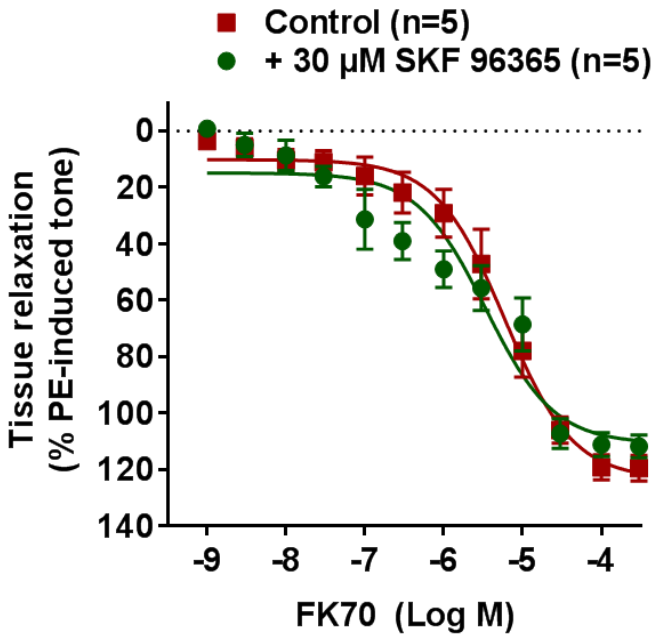


Figure 5.12: Effects of control and SKF 96365 on FK70 concentration-response curve in rat aortae. Tissue responses have been expressed as a percentage of PE-induced contraction and are shown as means ± SEM of 5 animals.

In a separate experiment, the involvement of TRPC channels on FK70-induced relaxation was further examined using SKF 96365 and FK70 on PE-induced contraction. The PE-induced contraction prior to incubation with SKF 96365 was (16.5 ± 2.2 mN), however upon incubation with SKF 96365, the contraction was reduced to (8.0 ± 0.9 mN; *p*= 0.0059) (see **Figure 5.13A**). When FK70 was added thereafter to same tissues (in **Figure 5.13A**), the PE contraction dropped rapidly after 10 minutes of addition to (-0.07 ± 0.6 mN; *p*= 0.0052) whilst addition of control instead of FK70 reduced PE contraction to (4.0 ± 0.2 mN; *p*= 0.01) (see **Figure 5.14**)

When the experiment was reversed similar response was observed, pre-incubation with FK70 reduced PE-contraction from (15.9 ± 1.6 mN) to (7.0 ± 1.1 mN, *p*= 0.0001) (see **Figure 5.13B**). Thereafter, addition of SKF 96365 to the same tissue, reduced the PE contraction rapidly to (0.7 ± 0.3 mN; *p*= 0.002) (see **Figure 5.14C** and **F**).

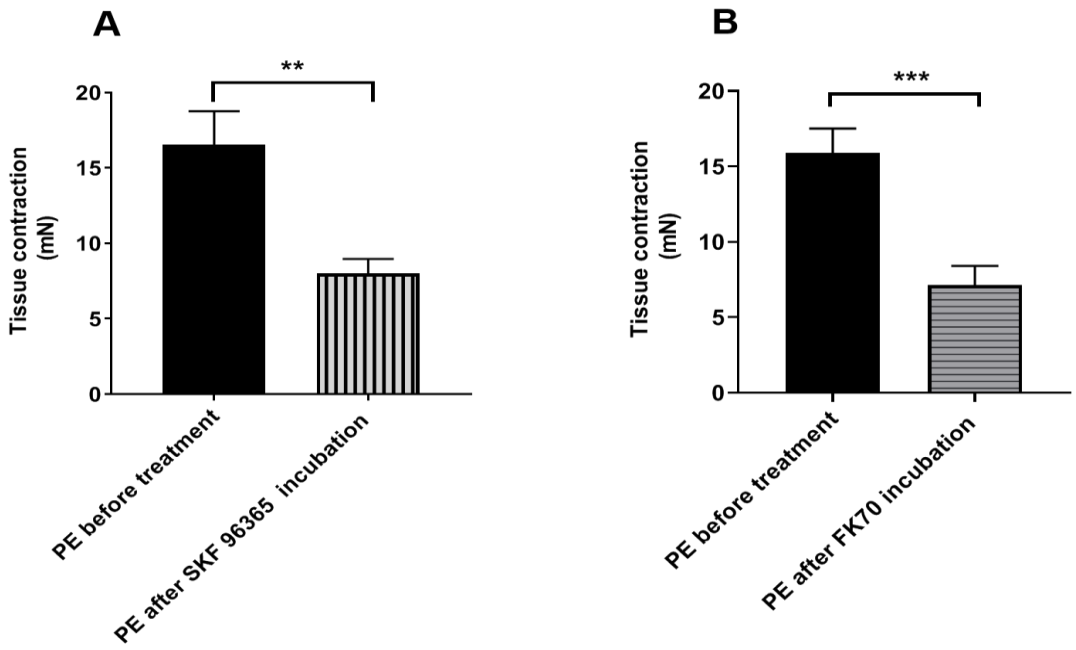


Figure 5.13: Effects of **A)** SKF 96365 (30 µM) and **B)** FK70 (30 µM) on PE-induced contractions. Tissue responses have been expressed as a force of contraction in force mN and are shown as means ± SEM of 5- 6 animals. (paired Student's *t*-test: ** *p*<0.01, *** *p*<0.001).

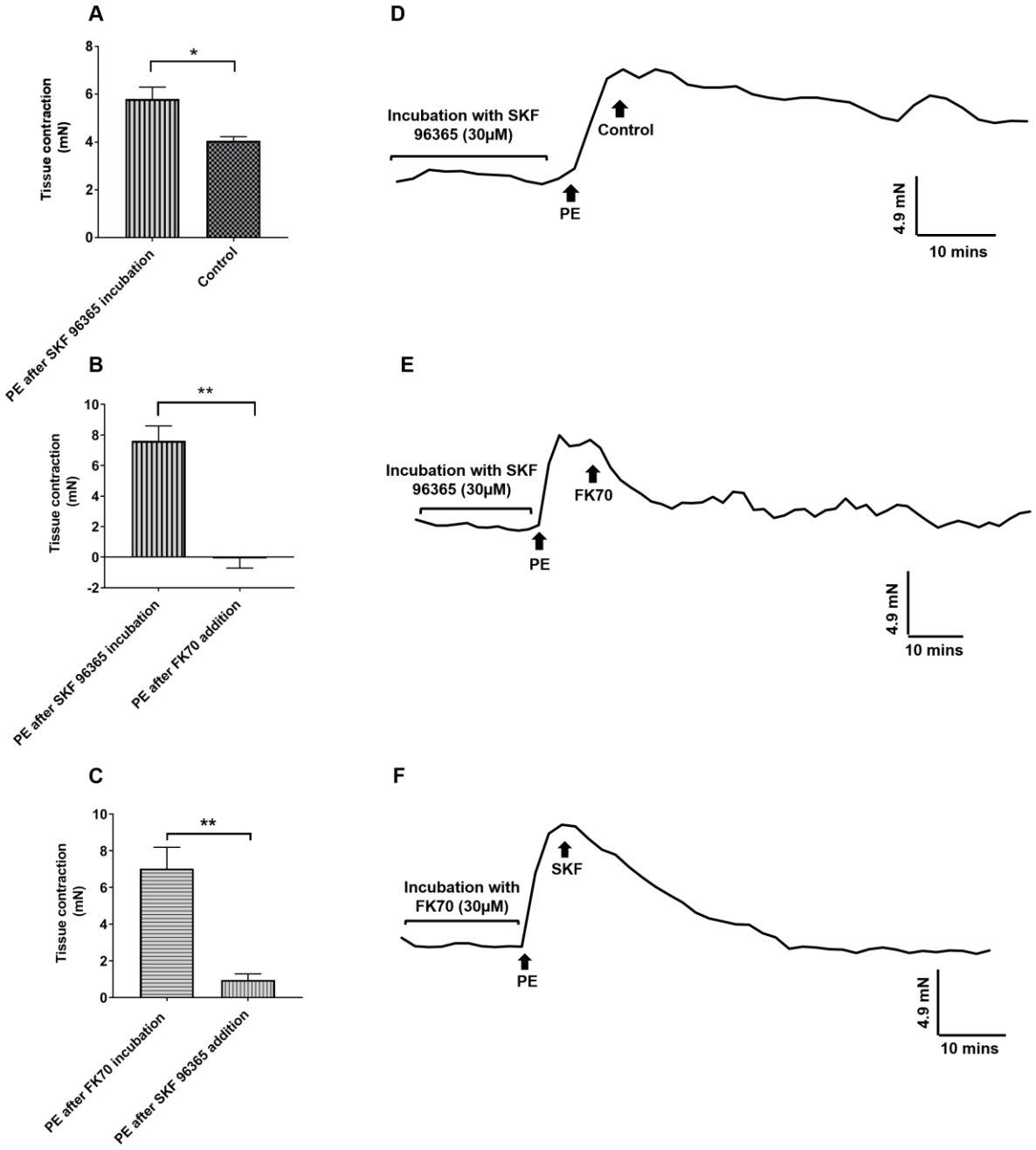


Figure 5.14: Effect of pre-incubation with SKF 96365 (30 µM) followed by addition of **A)** vehicle control **B)** FK70 (30 µM) on PE-induced contractions **C)** Effect of pre-incubation with FK70 (30 µM) followed by addition of SKF 96365 (30 µM) on PE-induced contractions. Representative trace recordings of PE contractile activity after addition of **D)** vehicle control **E)** FK70 (30 µM) **F)** SKF 96365. Tissue responses have been expressed as a force of contraction in force mN and are shown as means ± SEM of 5-6 animals. (paired *t*-test : * $p<0.05$, ** $p<0.01$). (Abbreviations: PE = phenylephrine; mN = milliNewton; mins = minutes)

5.4.6 FK70 has no effect on intracellular calcium

Aortic rings without prior treatment to FK70 served as control. FK70 did not seem to affect PE induced contraction in calcium-free Krebs solution, E_{max}: (control= 25.6 ± 4.0 % vs FK70= 21.3 ± 3.8 %; *p*=0.45) (see **Figure 5.15**). Despite no difference in the magnitude of initial PE contraction (phasic contraction) in both the groups, the PE contraction could not be sustained in aortic rings treated with FK70 (see **Figure 5.16A** and **B**).

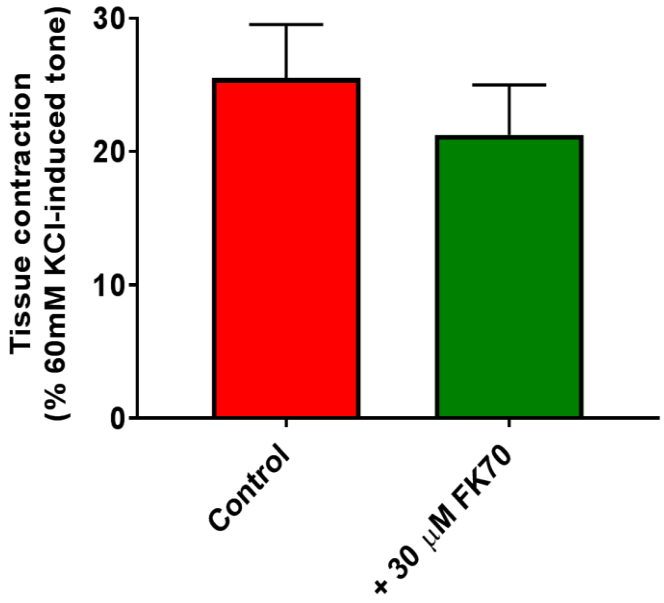


Figure 5.15: Effects of vehicle control and FK70 (30 μM) on maximal contractile response of PE in calcium-free Krebs-Ringer solution. Tissue responses have been expressed as a percentage of KCl-induced tone and are shown as means ± SEM of 5-6 animals.

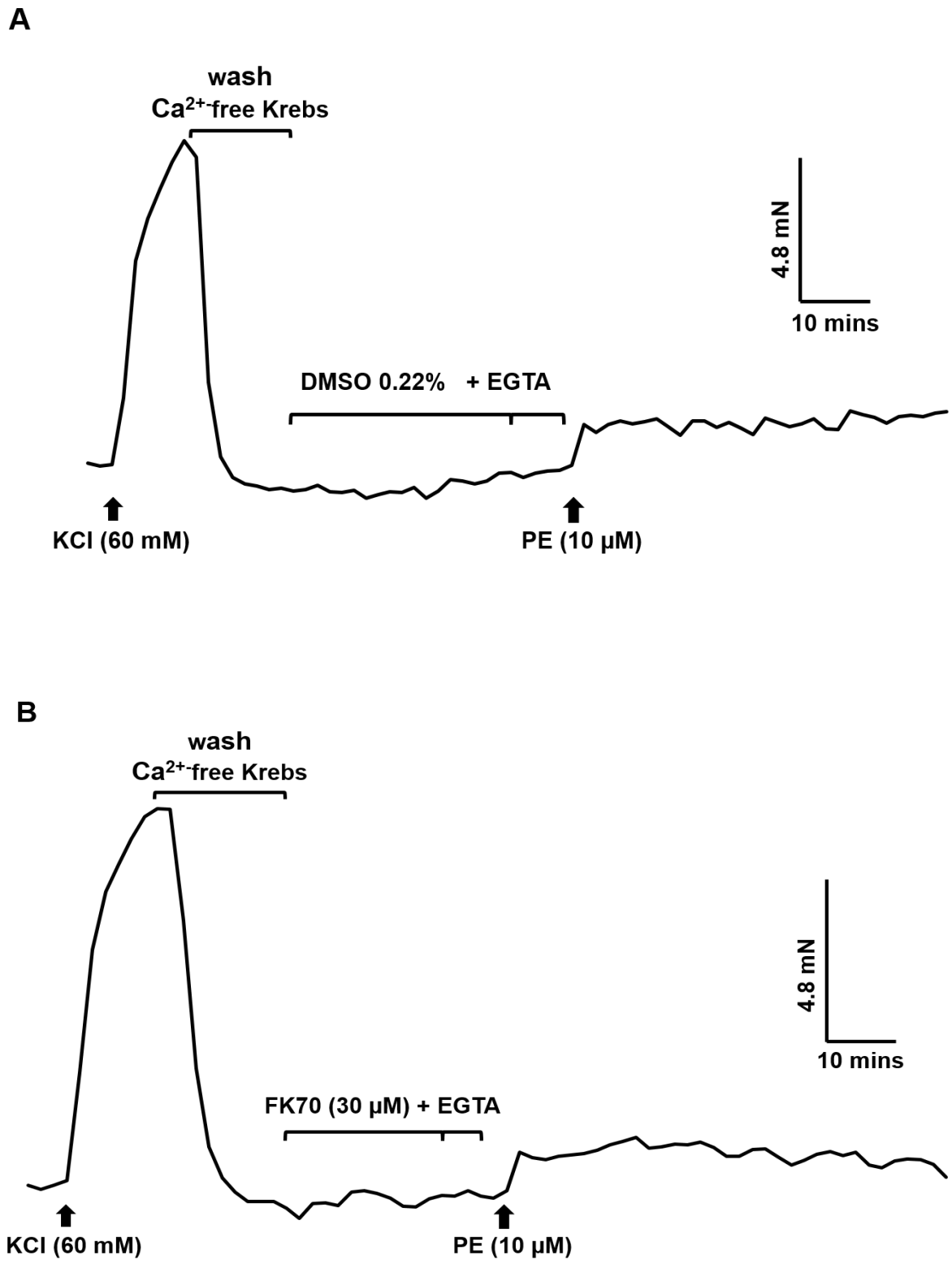


Figure 5.16: Representative trace recordings of PE contractile activity **A)** in the presence of vehicle control (DMSO 0.22%) and EGTA (0.1mM) in calcium-free Krebs solution. The contraction of PE is sustained throughout the experiment **B)** in the presence of FK70 (30 µM) and EGTA (0.1mM) in calcium-free Krebs solution. The PE contraction could not be sustained and started dropping towards the end of 1 hour. (Abbreviations: KCl= potassium chloride, PE = phenylephrine; mN = milliNewton; mins = minutes).

5.5 Discussion

This chapter aimed to characterize the calcium-mediated mechanism of FK70-induced relaxation on rat aorta. Previous chapter (chapter 3) have demonstrated that FK70-induced relaxation may involve a calcium-mediated mechanism, thus prompting subsequent experiments undertaken. The most apparent finding that emerged from this study was that FK70 blocks extracellular calcium entry.

The initial phase of contraction induced by contractile agents is known as the phasic component. It is characterised by a fast initial peak due to increases in the cytosolic Ca^{2+} concentration. This will then be followed by a decline to lower maintained tension level, which is known as the tonic component. The phasic component has been attributed to an IP_3-evoked release of Ca^{2+} from the sarcoplasmic reticulum (SR) store while the tonic component from Ca^{2+} entry via the sarcolemma following depolarisation, independently of IP_3 (Baron *et al*., 1984; Somlyo *et al.*, 1985; Kobayashi *et al.*, 1989).

Pre-incubation with FK70 followed by the addition of concentration-dependent contractile agents, PE, KCl, and 5-HT on rat isolated aortae resulted in the reduction of the maximal response and shifted the curves of the contractile agents to the right. PE, an α_1-adrenergic receptor agonist, stimulates contractions in the vascular smooth muscle by stimulating the activity of PLC, which cleaves the membrane phospholipid. Phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolyse into inositol 1,4,5 triphosphate (IP_3) and diacylglycerol (DAG) (Exton, 1985; Utz *et al.,*1999). The IP_3 binds to IP_3Rs on the ER / SR to cause the release of Ca^{2+} into the cytosol. DAG activates Ca^{2+} influx through TRPC 3, TRPC 6, or TRPC 7 (Venkatachalam and Montell, 2007; Kiselyov and Patterson 2009; Svobodova *et al.,*2016). This suggests that FK70 might affect the tonic and phasic components of PE-induced contraction. Besides that, 5-HT induces vasoconstrictions in the rat aorta mainly via 5-HT_{2A} subtype (Lu *et al.*, 2008) and the 5-HT_{2A} receptors are also linked to the G_q family of G-proteins and subsequently activating PLC. Albeit growing evidence suggest that 5-HT_{2A} utilize the same calcium signaling pathway as PE, the findings from previous (chapter 4), confirmed that 5-HT depends mostly on extracellular Ca^{2+} to cause contraction. KCl, on the other hand, causes contraction by depolarizing the aorta. As K^+ depolarises the aorta, this stimulates an enhanced uptake of calcium by activating voltage-dependent Ca^{2+} channels (Ratz *et al.* 2005) to cause contraction. Based on the findings in Chapter 4, the contraction of PE, 5-HT, and KCl depend mostly on extracellular Ca^{2+} entry than intracellular Ca^{2+}. Collectively, these findings suggest that FK70 may impose an effect on the tonic component of the contractile agents,

particularly on extracellular Ca^{2+} entry, as they depend mostly on extracellular Ca^{2+} to cause a contraction in rat aorta.

The next step of the experiment was to identify the possible characteristics of FK70 as an inhibitor of PE. Increasing concentrations of FK70 reduced the maximal response and the slope of PE contractile responses. In a separate experiment, despite the washing out procedure, PE-induced contraction in FK70 treated group was suppressed. Based on these observations, two hypotheses can be drawn. First, FK70 could be a partial agonist at α_1-adrenoceptors as one of the critical properties of partial agonists is that they display both agonistic and antagonistic effects. However, in the presence of full agonist (PE in this case), a partial agonist will act as an antagonist, competing with the full agonist for the same receptor and thereby reducing the ability of the full agonist to produce its maximum effect (Kenakin, 2012). FK70, however, could not be a partial agonist as it has been shown in (Chapter 3) that it did not act directly via the α-adrenoceptors to cause relaxation in the aortic rings. Second, FK70 could be an irreversible antagonist as it reduced the E_{max} and pEC_{50} of PE. An irreversible antagonist forms covalent bonds with receptor protein and thus prevent binding of agonists. They will not produce a parallel shift in the dose-response curve of an agonist, but instead reduces the maximum effect (efficacy) and the slope of the agonist dose-response curve, as well as producing the rightward shift (Paul, 2005; Dougall and Unitt, 2015). FK70 could have covalently bonded to the receptor involved in PE- induced contraction, therefore, could not be displaced by either higher amount of PE or washing.

In the present study, the vasodilator activity of FK70 on rat aorta was compared with known calcium channel blockers, nifedipine (an L-type Ca^{2+} channel blocker), verapamil (an L-and T-type Ca^{2+} channel blocker), and SKF 96365 (a non-selective TRPC channel blockers). FK70 behaved and exhibited relaxation effect comparable to calcium channel blockers in aortic rings pre-constricted with PE, confirming the link between Ca^{2+} and FK70-induced relaxation.

Pre-incubation with nifedipine did not seem to affect FK70 concentration-dependent relaxation. In a separate experiment, combined treatment with FK70 and nifedipine considerably reduced the PE contraction. These observations suggest that FK70 induced relaxation unlikely to involve L-type Ca^{2+} channels. Interestingly, when the same experiment was done in the reverse manner, where the tissues incubated with FK70 first followed by the addition of nifedipine, the PE contraction did not drop to the basal line. There are a few possible explanations for this observation. First, FK70 might partially block L-type Ca^{2+} channels. If the binding of FK70 to L-type Ca^{2+} channels is part of the mechanism of FK70-induced relaxation, it is possible to expect an enhanced relaxation effect in the combined

treatment of FK70 and nifedipine. It is, however, noteworthy to mention that the concentrations of FK70 and nifedipine used in this experiment ought to block 90 -100 % of PE contraction based on IC_{90} values determined in the previous experiment in this study. Assuming that the concentrations of antagonists used in this experiment occupy 90-100 % of the desired receptors, it seems unlikely that FK70 would have blocked L-type Ca^{2+} channels. Second, FK70 displayed rapid onset of relaxation response when compared to nifedipine. Previous studies had shown that nifedipine is known to block mainly the VOCCs in blood vessels, but the physiological response of blood vessels depends to a large extent on receptor-operated channels (Kashiwabara *et al*., 1991; Wang *et al*., 2018). This suggests that the possible mechanisms underlying the inhibitory effect of FK70 and nifedipine on PE contraction are not the same. However, the exact mechanism is yet to be found.

In the control group, albeit no significant difference and more sustained PE contraction were observed, there was a slight reduction in PE contraction before and after incubation of vehicle control. There are two possible explanations for this observation. First, the first application of PE would have increased the eNOS to produce nitric oxide, thereby decreasing subsequent PE-mediated contraction (Han *et al*., 2014). Second, the first application of PE would have led to the desensitization of PE-mediated contraction, which is mediated by alteration of receptor coupling or blunting of phosphatidylinositol turnover. This observation was in accordance with research done in the past on rats and rabbits aortic rings (Lurie *et al*.,1985; Arce *et al*., 2017).

Pre-incubation with verapamil reduced PE contraction verapamil produced more significant inhibition of PE contraction when compared to nifedipine. Verapamil blocks both L-type and T-type Ca^{2+} channels with a higher affinity for depolarized channels than for resting channels. The affinity of verapamil for L-type depolarized channels is on the order of 10-fold higher than for closed channels (Johnson *et al*., 1996) whilst for T-type channels, the state-dependence is less dramatic, with less than 2-fold more block of depolarized channels than closed channels in the presence of 10 μM verapamil (Freeze *et al*., 2006). Moreover, verapamil at the concentration of 30 μM had been shown to block T-type Ca^{2+} channels in rat aortic smooth muscle cells (Kuga *et al*., 1990). This is also in accordance with another study which had shown that verapamil blocks T-type Ca^{2+} channels with micromolar affinity and has modestly increased affinity at depolarized potentials (Bergson *et al*., 2011). The dual and selective effect of verapamil possibly explains the higher inhibition effect of verapamil when compared to nifedipine in PE contraction, suggesting that T-type Ca^{2+} channels are also involved in PE contraction. Past *in-vitro* and *in-vivo* physiological studies had supported that T-type Ca^{2+} channels do not contribute significantly to arterial vasoconstriction except renal microcirculation due to the argument that the currents through these channels are tiny and

transient (Matsuda *et al.,*1990; Quingnard *et al.,*2001; Chevalier *et al*.,2006). However, growing evidence accumulating that points to the involvement of T-type channel Ca^{2+} in vascular function (Cribbs, 2006; Ball *et al*., 2009; Kuo *et al*., 2011). As shown in the result section (in **Figure 5.11**), combined treatment of FK70 and verapamil produced more significant inhibition in PE contractile response, suggesting that FK70 induced relaxation may not involve the T-type Ca^{2+} channels in rat aorta. Albeit the present study rules out the possible involvement of L/T-type Ca^{2+} on FK70 induced relaxation, more research is needed to verify the present findings (e.g., using a patch-clamp technique) conclusively. Taken together, these results suggest that, FK70 in the presence of verapamil/nifedipine causes additional relaxation effect, which presumably resulted from other mechanisms independent of L/T- type Ca^{2+} channels.

Based on the previous findings in (Chapter 4), the initial phase of PE contractile activity is majorly dependent on Ca^{2+} influx through TRPC channels. It was therefore tempting to speculate that FK70 blocks extracellular Ca^{2+} entry via TRPC channels. To refute the hypothesis, further experiments involving FK70 and SKF 96365 were carried out. The findings imply that FK70 induced relaxation might not inhibit Ca^{2+} influx via the TPRC channels. According to previous studies, SKF 96365 blocks not only TRPC channels but also high voltage-activated Ca^{2+} channels at typically utilized test concentrations, and even more potently inhibit T-type Ca^{2+} channels (Singh *et al*.,2010). However, the involvement of T-type Ca^{2+} channels could be ruled out as FK70 did cause further relaxation in the presence of verapamil. Early studies on SKF 96365 conducted on human embryonic kidney cells (HEK293) have shown that SKF 96365 in the micromolar range (2–100 µM) inhibits Ca^{2+} influx through TRPC channels (Okada *et al*.,1998; Zhu *et al*.,1998). That said, the concentration of SKF 96365 (30 µM) used to carry out this set of experiment fell well within the range. However, the acceptable concentration range for SKF 96365 blocking TRPC channels only, in rat tissues is not known and yet to be found. Considering the amount of SKF 96365 used for this set of experiments, SKF 96365 (30 µM) may have unlikely acted on other channels except TRPC channels. Taken together, the results from the findings suggest that the FK70-induced relaxation mechanism might not involve the TRPC channels.

Besides, TRPC and L-type Ca^{2+} channels, it is also essential to consider the intracellular Ca^{2+} pathway. PE upon binding stimulates the formation of IP_3, which binds to IP_3R in SR membrane induces intracellular Ca^{2+} release to cause phasic contraction. In the present study, FK70 did not seem to affect the phasic contraction in calcium-free Krebs; however, the contraction could not be sustained when compared to control. In calcium-free Krebs solution, the small contraction observed may not altogether attributable to intracellular Ca^{2+} release but could also be due to activation of C-kinase (Khalil and Van Breeman, 1988). Therefore the inability of FK70 treated aortic rings to sustain PE phasic contraction could due to inhibition of the residual contraction, which underlay the phasic contraction. However, the exact mechanism warrants further study.

This study offers some insight into understanding the calcium-dependent mechanism underlying FK70-induced relaxation and elucidate the pathway responsible for the attenuation of contractile agents-induced contraction. From this experiment, it can be deduced that FK70 had caused attenuation of PE-induced contractions as a result of inhibition of extracellular calcium influx involving mechanism independent of L/T-type Ca^{2+} channels and TRPC channels. As aforementioned, calcium plays a critical role in the biochemical control of vascular smooth muscle tone, and thus of blood pressure homeostasis. Therefore, the role of FK70 in inhibiting calcium entry better than the known calcium channel blockers should prove to be particularly valuable in hypertensive studies. The effects of FK70 on spontaneously hypertensive rats (SHR) are elucidated in Chapter 6.

Chapter 6: Role of FK70 in calcium-mediated responses in Spontaneously hypertensive rats (SHR) aortae

6.1 Introduction

Hypertension is a major risk factor for many common cardiovascular diseases such as heart failure, heart attack, stroke, vascular dementia, and chronic kidney disease. The pathophysiology of hypertension includes increased vascular resistance due to increased vascular contraction and arterial remodeling. The vascular smooth muscle contraction is highly dependent on intracellular free calcium concentration, and essential hypertension is associated with profound alterations in Ca^{2+} homeostasis.

Findings from **Chapter 5**, suggest that FK70 inhibits extracellular calcium entry in the aorta of normotensive rats, and it may not be via L/T-type Ca^{2+} channels or the putative TRPC channels. This activity will also be explored in the aortic rings of SHR. Therefore, this study was designed to examine the vasoactivity and the underlying mechanisms of FK70 in spontaneously hypertensive rats (SHR). Genetic hypertension in SHR is generally characterized by increased activity of the sympathetic nervous system (SNS) and by alterations in Ca^{2+} homeostasis in vascular smooth muscle cells, thus making it an ideal model to study the calcium-dependent mechanism underlying FK70-induced relaxation in hypertension. A brief introduction of SHR and abnormal calcium regulation in SHR are discussed in the context.

6.2 Spontaneously hypertensive rats

Spontaneously hypertensive rats (SHR) are one of the most common animal models that have been used extensively for the past 40 years to study the mechanism of essential hypertension in humans. The spontaneously hypertensive outbred model was developed by the national institute of health (NIH) in 1966 from Wistar Kyoto outbred stock from Okamoto, Kyoto School of Medicine (Okamoto and Aoki,1963). The male SHR exhibit average systolic blood pressures higher than 200 mmHg by 3-4 months of age. A male rat with spontaneously high systolic blood pressures of 150 to 175 mmHg persisting for more than one month and a female rat with blood pressures slightly above the average, 130 to 140 mmHg, were selected from 68 normal Wistar strain rats and mated to produce F1 rats. Those F1 rats, males, and females with hypertension (blood pressure exceeding 150 mmHg) persisting for more than a month (mostly over two months) were mated to produce F2 rats. The procedure was repeated to obtain F3, F4, F5, and F6 rats totaling 380 animals. The incidence of severe hypertension

increased with each generation, that in the male population it increased from 9% in F2 to 35%, in F3, 42%, in F4, and 56% in F5, and in female animals from 3% in F2, 16% in F3, 33% in F4, and 37% in F5 (Okamoto and Aoki, 1963). The spontaneous occurrence of hypertension leads to the name "spontaneously hypertensive rats''. Spontaneous hypertension in Wistar Kyoto rats occurred without any prior treatment or diet to induce hypertension. However, under specific environmental conditions, such as when fed a high-fructose diet, the SHR also develops additional features of the metabolic syndrome, including insulin resistance and dyslipidemia (Pravenac *et al*.,1999). Therefore, they have similar characteristics to human essential hypertension. The kinetics of blood pressure changes in SHR can be divided into three phases, namely, pre-hypertensive phase, labile-hypertension phase, and established-hypertension phase. In the pre-hypertensive phase (up to the 4th postnatal week), their blood pressure is similar to that of control normotensive rats. In the labile-hypertension phase (5th to 14th week of age), there is a progressive increase in blood pressure. Finally, in the established-hypertension phase (starting from the 15th week), steady and high blood pressure levels are observed until the end of life (Okamoto and Aoki,1963).

The male SHR is commonly used as a model of established human hypertension and to test new antihypertensive medication (Lund-Johansen, 1990; Takata *et al.,*1996; Zhang *et al.,*2016). The main reason for the usage of SHR models is because human hypertension is challenging to study, as there are many substantial individual variations (Doggrell, 1998). For instance, human hypertension is commonly triggered by two elements known as the polygenetic disposition and excitatory environmental factors. These factors lead to many variations in the effects on the cardiovascular system, which are difficult to differentiate. Therefore to address this problem, researchers have commonly resorted to the use of SHRs for hypertension studies. SHRs have, within each colony, a uniform polygenetic disposition and excitatory factors that produce uniform changes in the indirect and direct effects on the cardiovascular system (Doggrell, 1998). Besides lacking inter-individual variation, another advantage of the SHR is that it follows the same progression of hypertension as human hypertension with pre-hypertensive, developing, and sustained hypertensive phases with each phase lasting at least several weeks (Folkow, 1993).

Notwithstanding those mentioned above, no animal model mimics exactly all the symptoms of the human disease, partly because many of the changes in the human disease are not thoroughly understood. Albeit being a useful model of genetic high blood pressure, the progress in human genetic hypertension is still lacking. That said, the genetic basis of hypertension in SHR remains an important objective. The SHRs offer an opportunity to understand what type of genetic variation is at work, how the causative genetic variants interact to create disease, and how they overcome normal physiological counter-regulation in understanding the pathogenesis of high blood pressure (Doris, 2017). The significant deficiency in many rat models is reproducing the slow onset of human disease. Most animal models do not reproduce the concurrent activation of homeostatic mechanisms in humans, especially neurohumoral changes, and many rat models produce pathophysiological changes that are rarely seen in the human disease state. Therefore, a thorough understanding of both the advantages and disadvantages of each model is necessary in order to provide relevant and translatable scientific data to humans.

6.2.1 Structure of aorta in SHR

Essentially, both SHR and normotensive rats' aorta have a similar structural arrangement with an outer layer to the lumen, namely tunica adventitia, tunica media, and tunica intima. They possess similar mechanical properties; however, in SHR, the wall is submitted to a higher level of stress (Bezie *et al*., 1998), suggesting that sustained hypertension is associated with the restructuring of the arterial wall that produces the mechanical adaptation of the arterial wall. The vascular remodeling in SHR causes structural changes to the arterial wall in terms of arterial diameter, arterial mechanics (e.g., stiffness), medial thickness e.g., changes in the smooth muscle cell-elastic lamella and deteriorated endothelial function. The size of the internal radius of aortic rings of SHR is smaller than that of normotensive rats, 520 ± 50 μm and 595 ± 18 μm, respectively (Bezie *et al*.,1998). Besides that, the medial thickness to body weight ratio was higher in SHR than in normal rats (116 ± 11 versus 80 ± 3 μm/kg) (Bezie *et al*.,1998). Generally, the muscle cells of media have prominent dense plaques encrusting the cell membrane and projecting deep into the cell. Smooth muscle cells to elastic lamina connections are defined as elastin expansions that spanned obliquely from the elastic laminae to the surface of the smooth muscle cell, where they are attached in a region occupied by membrane-associated dense plaque. Although there is no difference in the number of total dense plaques between Wistar rats and SHR, the percentage of cell surface occupied by dense plaques was increased in SHR (Bezie *et al*.,1998). The increase in the arterial thickness of SHR can be associated with cellular hypertrophy and extracellular matrix accumulation.

Moreover, the aortic stiffness in SHR is higher than in normotensive rats. It is well known that hypertension is a highly age-related human disease. Although the association between aortic stiffness and hypertension is well established, whether aortic stiffening is a cause or a consequence of hypertension remains controversial. The American Heart Association (AHA) asserts that aortic stiffening is a cause rather than a consequence of hypertension in middle-aged and older individuals (Townsend *et al.,* 2015). The aortic stiffness in the aorta is attributable to vascular smooth muscle cells and has two components: intrinsic and extrinsic (Hays *et al.,*2018). The intrinsic component results from the alteration of the smooth muscle itself, including increased stiffness of the individual cell and decreased adaptability to the extracellular matrix. The extrinsic component is the modulation of extracellular matrix by smooth muscle cells, via both synthesis and degradation of collagen, and by facilitating the reorganization of the extracellular matrix. The extracellular effects of aortic smooth muscles in SHR are regulated by signaling pathways, which involve the upregulation of SRF / myocardin, cytoskeleton/integrin β1, and bone morphogenetic protein-1 and lysyl oxidase (BMP1/LOX) (Hays *et al*., 2018).

The vascular endothelium plays a vital role in the regulation of arterial tone by producing dilator mediators, which include endothelium-derived relaxing factor e.g., nitric oxide (NO), prostacyclin (PGI2) and endothelium-derived hyperpolarizing factors (EDHF). Several reports have shown that the endothelial function is impaired in hypertension, thus affecting the endothelium-dependent relaxations in SHR (Widlansky *et al.*,2003; Dharmashankar, 2010). Endothelial dysfunction occurs at an initial step in the pathogenesis of hypertension and is also considered as a consequence of hypertension. The mechanisms underlying endothelial dysfunction can be caused by a loss of NO bioactivity and abundant formation of reactive oxygen species (ROS) in the vessel wall (Cai *et al*., 2000). NO plays a pivotal role in regulating basal vascular tone by rendering the actions of potent endothelium-derived vasoconstrictors such as ANG II and endothelin-1 (Verma *et al*.,2003). ROS contributes to the pathogenesis of numerous cardiovascular diseases and conditions, including hypertension. ROS reduces NO bioactivity through the rapid chemical reaction with NO, thereby resulting in the formation of peroxynitrite (Rubio *et al*., 2004), a species that participates in endothelial dysfunction. The NADPH oxidase (NOX), is an enzyme complex that has been regarded as a significant ROS source in vascular cells. NOX reduces oxygen (O_2) by the addition of one electron to generate the superoxide anion (O_2^-). The O_2^- interferes with the signal transduction mechanisms of vascular smooth muscle relaxation mediated by NO. In SHR, increased NOX activity and O_2^- production were reported when compared to the aorta of normotensive Wistar-Kyoto rats (Zalba *et al*., 2000; Wind *et al*.,2010). Excessive ROS formation alters metabolic activity, proteins, and DNA, which in turn cause cellular dysfunction, cell death, and ultimately lead to

numerous cardiovascular diseases (Elahi *et al*.,2009). The NO scavenges superoxide anions and contributes to improved vascular endothelial function (Rochette *et al.,*2013).

6.3 Abnormal calcium regulation in vascular smooth muscle of SHR

Calcium (Ca^{2+}) is essential to many component systems required for normal cardiovascular homeostasis including the central and peripheral nervous systems, cardiac, renal, and vascular smooth muscle cell function. Alterations in calcium sensitivity have been implicated in the pathogenesis of primary hypertension (Touyz *et al.,*2018). The alteration in calcium sensitivity in vessels from SHR could be located at several possible sites: 1) a change in membrane permeability to calcium 2) changes in intracellular calcium handling mechanisms and 3) a change in the calcium sensitivity of the contractile mechanisms.

Ca^{2+} influx across the external cellular membrane in smooth muscle cells and cardiomyocytes plays a crucial role in the control of cellular excitation-contraction and impulse propagation. Maintenance of high intracellular $[Ca^{2+}]_i$ levels in the vascular smooth muscle of SHR is genetically regulated and is one of the mechanisms in this strain. The abnormal calcium regulation in the vascular smooth muscle is exhibited even before overt hypertension (Sugiyama *et al*., 1990).

The intracellular Ca^{2+} is a significant regulator of contraction and relaxation of the vascular smooth muscle. The level of intracellular Ca^{2+} is determined by Ca^{2+} influx through the VOCC, pumping out of the cell by Na^+ / Ca^{2+} exchange, release of Ca^{2+} from the sarcoplasmic reticulum (SR) by Ca^{2+}-induced Ca^{2+} release, and the release of Ca^{2+} from SR by inositol 1,4,5-trisphosphate (IP_3)-induced Ca^{2+} release. It has been reported that the increased function of VOCC in the artery is involved in the increase of peripheral resistance in hypertension (Holloway and Bohr, 1973; Asano *et al*.,1995; Arii *et al*.,1999). The long-lasting Ca^{2+} (Ca_L) channels of the $Ca_{v1.2}$ gene family are heteromultimeric structures that are minimally composed of a pore-forming α_{1C} subunit and regulatory β and $\alpha_{2\delta}$ subunits in vascular smooth muscle cells. The Ca_L channels are the primary pathways for the VOCC Ca^{2+} influx that trigger excitation-contraction coupling in small resistance vessels. The vascular smooth muscle cells of hypertensive rats showed an increased expression of Ca_L channel α_{1C} subunits, which is associated with elevated Ca^{2+} influx and the development of abnormal arterial tone (Sonkusare *et al*., 2006). Studies using the whole-cell voltage-clamp technique have also demonstrated that L-type Ca^{2+} channel activity is increased in vascular smooth muscle cells of SHR (Kubo *et al*.,1998).

Besides that, past studies have also reported that the calcium-activated potassium current density is increased in the aortas of hypertensive rats compared to normotensive rats (Rusch *et al*.,1992) and single-channel Ca^{2+} activated K^+ current is increased in arterial smooth muscle cells from rats with genetic or renal hypertension (England *et al*.,1993). Evidence from past studies suggest that the increased BK_{Ca} current in aortic SMCs of SHR and salt-sensitive hypertensive rats was as a result of increased Ca^{2+} influx. The increased Ca^{2+} influx have a more substantial inhibitory effect on K_V currents and a larger augmenting effect on BK_{Ca} channels, thus explaining the higher expression of BK_{Ca} channels (Cox *et al*., 2003).

The plasma membrane of vascular smooth muscle cells also expresses another Ca^{2+} transport system, known as the Na^+/Ca^{2+} exchanger (NCX). The Na^+/Ca^{2+} exchanger (NCX), operates in parallel with the Ca^{2+}-selective channels and the ATP-driven Ca^{2+} pumps. The NCX can move Ca^{2+} either into or out of cells, depending on the net electrochemical driving force across the exchanger. The past study had shown that the acceleration of Ca^{2+} efflux from vascular smooth muscle of SHR was at least partly due to the enhancement of the functional activity of NCX1 (Taniguchi *et al*.,2004). The NCX may play a vital role in the regulation of $[Ca^{2+}]_i$ stimulated by ANG II in a hypertensive state. However, the role of NCX in Ca^{2+} maintenance in SHR remains to be elucidated.

The Ca^{2+} influx is also mediated by calcium-permeable ion channels, known as the transient receptor potential canonical channels (TRPC). The TRPC channel-mediated trans–plasma membrane currents have been identified in several cell types, including those in the resistance arteries, aortic tissue, endothelium, and vascular smooth muscle, as well as in peripheral blood cells (Earley and Brayden, 2015). TRP channels belong to the superfamily of cation channels formed by tetramers of six transmembrane domain subunits. Unlike voltage-gated ion (Ca^{2+} and K^+) channels, TRP subunits do not possess a voltage-sensing moiety, making their activity insensitive to changes in membrane potential. TRP channels, therefore, function as voltage-independent, non-selective cation channels that are permeable to Na^+, K^+, Cs^+, Li^+, Ca^{2+}, and Mg^{2+}. Abnormal expression and dysfunction of TRPC channels have been reported in both hypertensive patients and animal models (Liu *et al*.,2014). Several studies had shown that the expression of TRPC3 channels was increased in the aortic smooth muscle tissues of SHR (Liu *et al*.,2009; Chen *et al*.,2010).

6.4 Study aim and objectives

Based on the findings from the previous chapter (chapter 5), FK70 caused a relaxation effect in the aorta of normotensive rats by blocking extracellular calcium (Ca^{2+}) entry. Therefore, the present study was designed to investigate the effect of FK70 and the calcium-mediated mechanisms underlying FK70-induced relaxation in the isolated aorta of SHR. The specific objectives of this chapter were as following;

i) To investigate the endothelium integrity of SHR and role of endothelium on FK70-induced relaxation

ii) To examine the contractile response to phenylephrine (PE) and involvement of calcium channels in PE-induced contraction by using nifedipine (an L-type Ca^{2+} channel inhibitor) and SKF 96365 (a non-selective TRPC channel inhibitor).

iii) To investigate the role of FK70 in calcium-mediated responses by conducting the following experiments ;

   a) to compare the concentration-dependent relaxation effect of FK70 with known calcium channel blockers, nifedipine, verapamil (L- and T-type Ca^{2+} channel inhibitor) and SKF 96365 on aortic rings pre-contracted with PE

   b) to investigate the effects of FK70 on external Ca^{2+} in calcium-free Krebs Ringer solution

   c) to investigate the effects of FK70 on calcium release by concentration-dependent PE

   d) to examine the role of L-type Ca^{2+} channel and TPRC channels on FK70-induced relaxation.

6.5 Method and materials

6.5.1 Drugs and Krebs-Ringer bicarbonate solution

(R)-(−)-phenylephrine hydrochloride (Sigma), 1-[2-(4-methoxyphenyl)-2-[3-(4-methoxyphenyl)propoxy]ethyl]imidazole (SKF 96365) (Sigma, USA), nifedipine (Nacalai Tesque, Japan), (±)-verapamil hydrochloride (Acros organics,UK), carbachol (Nacalei Tesque, Japan) and potassium chloride (KCl) were used.

Normal Krebs-Ringer solution was prepared as described in **Section 2.3.1**.

6.5.2 Tissue preparation and organ bath assembly

Ethical approval was obtained from the University of Nottingham's Animal Welfare and Ethics Review Body (UNMC_00006_KNT and UNMC18). All applicable international, national and/or institutional guidelines for the care and use of animals were followed. Male Wistar-Kyoto rats (336-497 g; 3-4 months old) purchased from Monash University, Malaysia campus were euthanized using carbon dioxide and sacrificed on the day of experiment. Rat aorta were removed and excised into 4 mm rings, respectively.

For organ bath assembly refer to **section 2.3.2.**

6.5.3 Blood pressure measurement

The systolic and diastolic blood pressure were measured with Mouse and Rat Tail Cuff Blood Pressure System (IITC Life Sciences, USA). The rats were placed into a plastic re-strainer one at a time to restrict their movement during measurement. Then a tail-cuff with a pulse transducer was applied around the tail of the restrained rats. The rats were then placed into a well-ventilated chamber equilibrated at 32°C for 15-20 minutes in order to facilitate the dilatation of caudal arteries. Finally, the systolic and diastolic blood pressure measurements were made in triplicate and averaged. The blood pressure of the rats was measured one week before the experiment. The blood pressure (systolic / diastolic) of SHRs used for this study ranged between systolic (284-338 mmHg) / diastolic (169-308 mmHg).

6.5.4 Organ bath experimental protocols

6.5.4.1 Examination of endothelium integrity of SHR and role of endothelium on FK70-induced relaxation

The endothelial integrity of SHR was verified by the presence of the relaxant response to carbachol (1 µM) on aortic rings pre-contracted with PE (0.1 µM). The concentrations of carbachol and PE were chosen on the basis of their ability to cause 80% relaxation (carbachol) and contraction (PE) which were determined from their respective concentration- response curves. The aortic rings which induced relaxation greater than 80% were considered normal while the aortic rings which produced less than 10% were considered damaged (Arsyad 2016). All aorta used in this experiment were intact unless specifically labelled as denuded aorta.

In a separate experiment, in order to further confirm the involvement of endothelium-derived factor on FK70-induced relaxation, sodium nitroprusside (SNP), a nitric oxide donor was used. The endothelium intact aortic rings were incubated with FK70 (30 µM) followed by cumulative-addition of SNP in aortic rings pre-constricted with PE (0.1 µM). The concentration range of the SNP used was between (0.01 nM – 1 µM).

6.5.4.2 Role of calcium channels in PE-induced contraction

Following second KCl addition, concentration-response curves (CRC) to PE was constructed in normal Krebs-Ringer solution. The tissue was exposed to increasing concentrations of PE (0.1 nM-30 µM) after the response to previous concentration had reached its maximum. The degree of response (tissue contraction) was measured as a tension weight of the contraction achieved by increasing PE concentrations. Only one CRC was performed per tissue.

In a separate protocol, to further deduce the effect of extracellular calcium on the PE-induced contraction, the aortic rings were pre-incubated with nifedipine (30 µM) or SKF 96365 (100 µM), for at least 40 minutes in normal Krebs-Ringer solution, prior to the construction of PE CRC. The concentration of each inhibiting agent (nifedipine and SKF 96365) was chosen on the basis of its ability to cause 90% - 100% reduction in PE -induced tone (determined from CRCs to respective drugs). Tissue contraction to PE was expressed as a percentage of 60mM KCl-induced tone.

6.5.4.3 Role of FK70 in calcium-mediated responses in aortae

FK70 concentration-dependent relaxation effect was compared with relaxation effect of known calcium channel blockers nifedipine, verapamil and SKF 96365. The aortic rings were pre-constricted with PE (0.1 μM) prior to performing concentration response curve of FK70, nifedipine, verapamil and SKF 96365. The tissues were exposed to increasing concentrations of the drugs, after the response to previous concentration had stabilised. Only one CRC was performed per tissue. The concentration range of the drugs used was between (1 nM-300 μM).

(a) In order to investigate the concentration dependent effect of FK70 on $CaCl_2$-induced contraction, the tissues were equilibrated for 30 minutes in calcium-free Krebs, and then the tissues were pre-incubated with either FK70 (10 μM or 30 μM) for 40 minutes. This was then followed by addition of 60 mM KCl to confirm the viability of tissues in calcium-free Krebs solution. Once the KCl-induced contractile response plateaued, a concentration-response curve to $CaCl_2$ was constructed. The concentrations of FK70 for pre-incubation were chosen based on their cumulative concentration-dependent relaxant effect in pre-constricted aorta. The concentration range of $CaCl_2$ used was between (0.1 μM to 3 mM).

(b) In order to investigate the effect of FK70 on calcium release by PE, the tissues were pre-incubated with either FK70 (10 μM or 30 μM) prior to performing concentration-response curve to PE. The concentration range of PE used was between (1 nM to 30 μM).

(c) i) In order to confirm the involvement of L-type Ca^{2+} channels, following the second KCl stimulation, the tissues were washed with normal Krebs-Ringer solution. Then they were treated with a single concentration of PE (10 μM). Once the response has stabilised, the tissues were washed for 15 min. The tissues were then incubated with nifedipine (30 μM) for 40 minutes, followed by addition of a single concentration of PE (10 μM). Thereafter, a single concentration of FK70 (30 μM) was added to the bath and left for 1 hour.

The same protocol was repeated in a reverse manner where the aortae were pre-incubated with FK70 followed by a single addition of nifedipine. The concentration of nifedipine and FK70 used in this experiment were chosen based on their ability to cause 90 %-100 % relaxation in rat aorta.

ii) In order to examine the effects of FK70 on TRPC channels, following the second KCl stimulation, tissues were washed with normal Krebs-Ringer solution. Then they were treated with a single concentration of PE (10 μM). Once the response has stabilised, the tissues were washed for 15 mins. The tissues were then incubated with SKF 96365 (30 μM) for 40 minutes, followed by addition of a single concentration of PE (10 μM). Thereafter, a single concentration of FK70 (30 μM) was added to the bath and left for 1 hour.

6.5.5 Data analysis

Data were analysed and graphs were drawn using PRISM version 7.0 (GraphPad software). The aortic contraction was expressed as a percentage of PE / KCl (60mM)-induced contraction except for aortic responses in experimental result **section 6.6.8** and **section 6.6.9**. Their contraction to PE was expressed in mN (=tension weight x 9.8) due to lower contractile magnitude to 60mM KCl as compared to PE-induced contractile response. All data were expressed as mean ± standard error of mean (SEM) of *n* number of animals. Maximum tissue contraction or relaxation response (E_{max}) and concentration to produce a basal response by 50 % (pEC_{50}) were derived from nonlinear regression analysis of the obtained CRC. Statistical analyses were performed using Student's unpaired *t*-test and one-way ANOVA. Unpaired *t*-test was used to compare the statistical differences from two experimental groups: one from a control condition while another from a treatment condition [PRISM Version 7 (Graph Pad Software)]. Paired *t*-test was used to compare the statistical differences from the same experimental group. For comparing more than two treatment groups, their mean differences were tested using one-way ANOVA followed by Dunnett's multiple comparisons test (PRISM Version 7; Graph Pad Software). Results were considered statistically significant if $p < 0.05$.

6.6 Results

6.6.1 The endothelial function of SHR aortae was impaired

The endothelium function of SHR and SD aortic rings was investigated. The SHR aortic rings relaxed a marginal 8.3 ± 3.4 % whilst the SD rat's segments produced a 72.7 ± 6.4 % carbachol-induced relaxation. However, when the endothelium of the SD rat's aorta was removed the relaxation magnitude produced was 6.6 ± 1.4 % (see **Figure 6.1**). This implies the impairment of the function of endothelium in SHR rats' aortae.

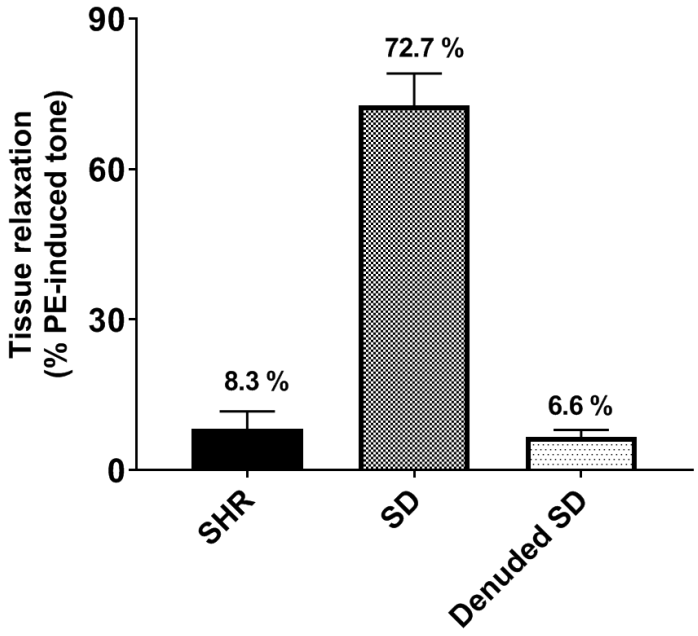


Figure 6.1: Effect of carbachol-induced relaxation assessing the endothelium integrity of aortae pre-contracted with PE from SHR, normotensive SD, and endothelium-denuded SD rats. Tissue responses have been expressed as a percentage of PE-induced tone and are shown as means ± SEM of 5 to 8 animals. (Abbreviations: SHR = spontaneously hypertensive rats; SD = Sprague Dawley; PE =phenylephrine).

6.6.2 Endothelium impairment did not affect PE-induced contraction in SHR

The PE sensitivity of aortic rings from SHR was not significantly different from that of endothelium-intact aortic rings from SD and Wistar rats (see **Figure 6.2** and **Table 6.1**). This suggests that the contraction induced by PE is endothelium-independent.

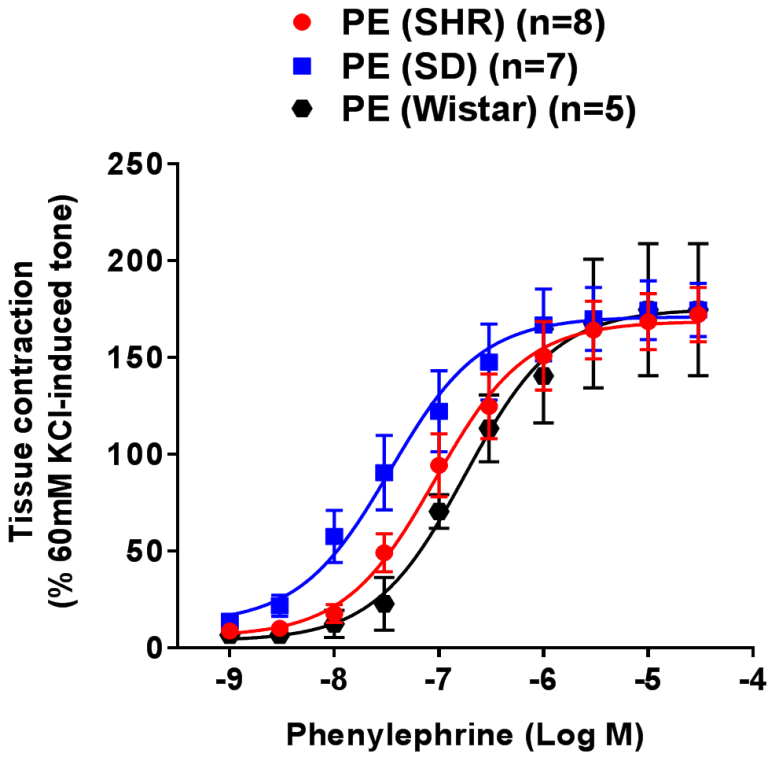


Figure 6.2: Effects of PE concentration-response curve in intact SHR, normotensive SD and normotensive Wistar rats aortae. Tissue responses have been expressed as a percentage of KCl-induced tone and are shown as means ± SEM of 5 to 8 animals.

Table 6.1: Summary of PE concentration-response curves (E_{max} and pEC_{50}) in SHR, SD and Wistar rats isolated aortic rings. Tissue responses have been expressed as a percentage of KCl-induced contraction and are shown as means ± SEM of 5 to 8 animals. The one-way ANOVA followed by Dunnett's multiple comparison test compared the PE contractile response between SHR, SD and Wistar (a *p* value of >0.05 is considered no significant difference).

Rat type	n (number of animal)	E_{max} (%)	pEC_{50}	*p* value	
				E_{max}	pEC_{50}
SHR	8	172.2 ± 14.1	7.03 ± 0.1		
SD	7	174.6 ± 13.8	7.46 ± 0.2	0.98	0.24
Wistar	5	174.7 ± 19.7	6.75 ± 0.1	0.99	0.74

6.6.3 PE-induced contraction depends on extracellular calcium

The involvement of calcium channels in PE-induced contraction was examined using nifedipine and SKF 96365. In the presence of nifedipine, the PE contraction was reduced and the curve was shifted to the right when compared to control. While SKF 96365 treatment abolished the PE contraction (see **Figure 6.3** and refer **Table 6.2**).

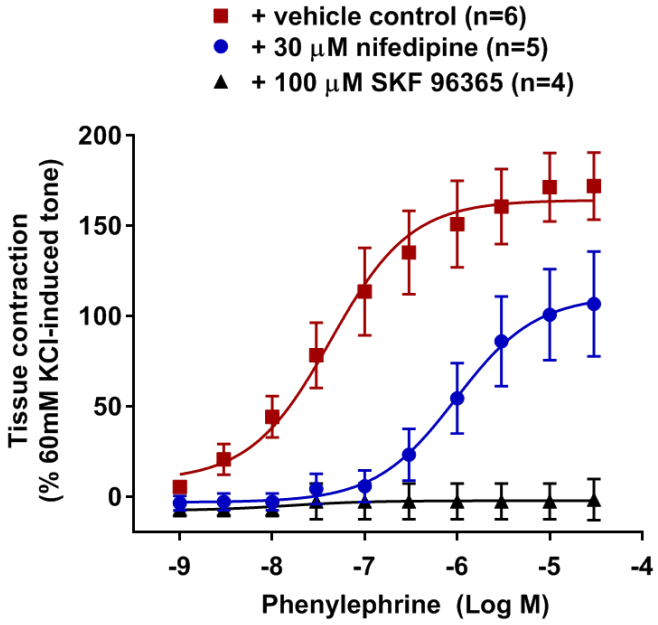


Figure 6.3: Effects of vehicle control, nifedipine (30 μM) and SKF 96365 (100 μM) pre-incubation on PE concentration-response curve in SHR aortae. Tissue responses have been expressed as a percentage of KCl-induced contraction and are shown as means ± SEM of 4-6 animals.

Table 6.2: Summary of effects of vehicle control, nifedipine (30 μM) and SKF 96365 (100 μM) on PE concentration-response curves on SHR isolated aortic rings. Tissue responses have been expressed as a percentage of PE-induced contraction and are shown as means ± SEM of 4 to 6 animals. The one-way ANOVA followed by Dunnett's multiple comparison test compared the relaxation effect with control (****p<0.0001, a p value of >0.05 is considered no significant difference).

	n (number of animals)	**Maximal contraction at (3×10^{-5} M) (%)**	***p* value**
+ Vehicle control	6	171.8 ± 18.6	
+ 30 μM nifedipine	5	106.6 ± 29.1	0.07
+ 100 μM SKF 96365	4	Abolished	0.0005****

6.6.4 FK70-induced relaxation is endothelium and nitric oxide independent in SHR

The aortic rings treated with vehicle control (DMSO 0.22%) served as control for this experiment. The aortic rings were pre-contracted with PE (0.1 μM) prior to performing SNP cumulative concentration-response curves. The maximal relaxation response of SNP in the presence of FK70 was similar to that of aortic rings from control group (see **Figure 6.4** and refer **Table 6.3**).

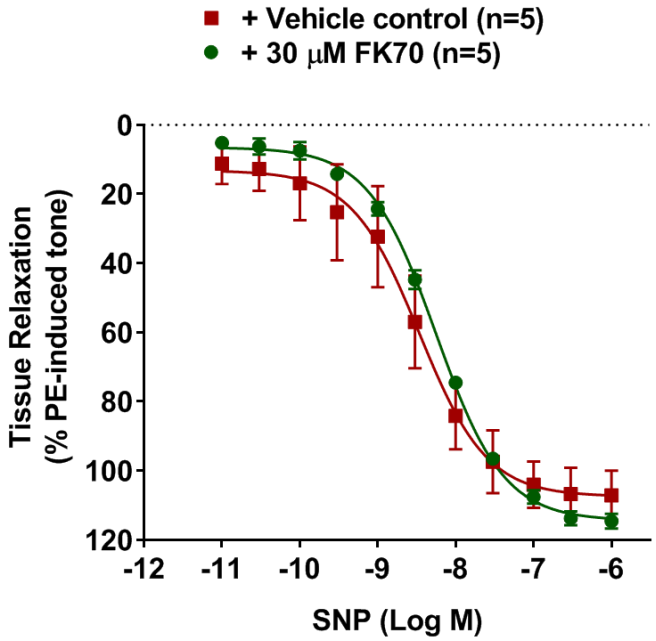


Figure 6.4: Effects of vehicle control and FK70 on SNP concentration-response curves. Tissue responses have been expressed as a percentage of PE-induced tone and are shown as means ± SEM of 5 animals.

Table 6.3: Summary of effects of vehicle control and FK70 on SNP on concentration-response curves in SHR isolated aortic rings. Tissue responses have been expressed as a percentage of PE-induced contraction and shown as means ± SEM of 5 animals.

	n (number of animals)	Maximal relaxation at (10^{-6} M) (%)	*p* value
Vehicle control	5	107.1 ± 7.1	
+ 30 μM FK70	5	114.6 ± 2.1	0.34

6.6.5 FK70 shows similar relaxation responses as a putative TRPC channel blocker (SKF 96365)

FK70 displayed relaxation effect in SHR aortic rings and the relaxation magnitude was comparable to that of known calcium channel blockers nifedipine, verapamil and SKF 96365 (see **Figure 6.5**). Aortic rings treated with vehicle control were served as control. The summary of maximal responses in SHR aortae were provided in **Table 6.4.**

The maximal response of FK70 and other calcium channel blockers were slightly elevated in SHR aortae when compared to the maximal responses of the calcium channel blockers in SD rats (see **Figure 6.6**).

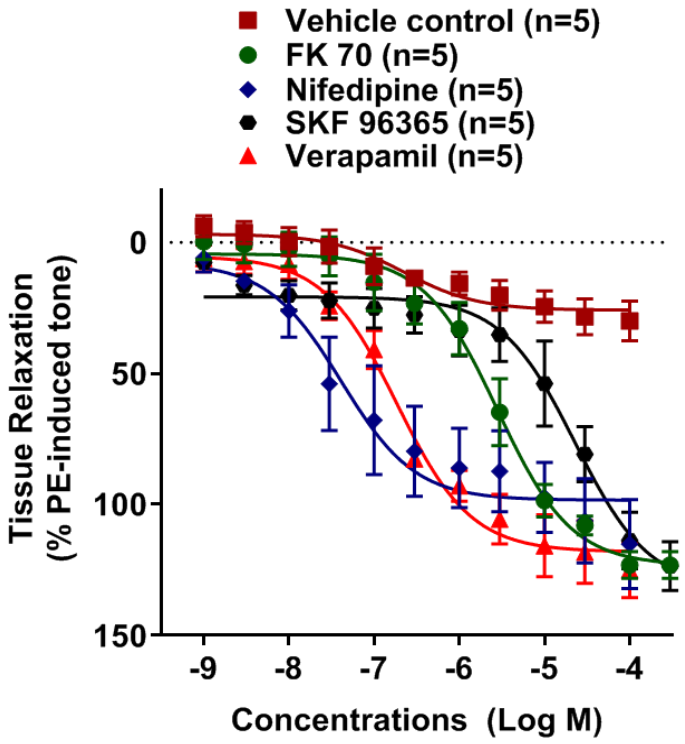


Figure 6.5: Effects of concentration-response curves of vehicle control, FK70, nifedipine, SKF 96365, and verapamil in SHR aortae pre-contracted with PE. Tissue responses have been expressed as a percentage of PE-induced tone and are shown as means ± SEM of 5 animals.

Table 6.4: Summary of effects of vehicle control, FK70, nifedipine, verapamil, SKF 96365-induced relaxation in SHR isolated aortic rings. Tissue responses have been expressed as a percentage of PE-induced contraction and are shown as means ± SEM of 5 animals. The one-way ANOVA followed by Dunnett's multiple comparison test, compared the relaxation effect between FK70, control, nifedipine, verapamil, and SKF 96365 (****p<0.0001, a p value of >0.05 is considered no significant difference.

	n (number of animals)	**Maximal relaxation at (3×10^{-4} M) (%)**	***p* value**
Vehicle control	5	29.9 ± 7.4	<0.0001****
FK70	5	123.1 ± 5.1	
Nifedipine	5	115.1 ± 16.8	0.95
Verapamil	5	125.0 ± 10.5	0.99
SKF 96365	5	123.5 ± 9.2	0.91

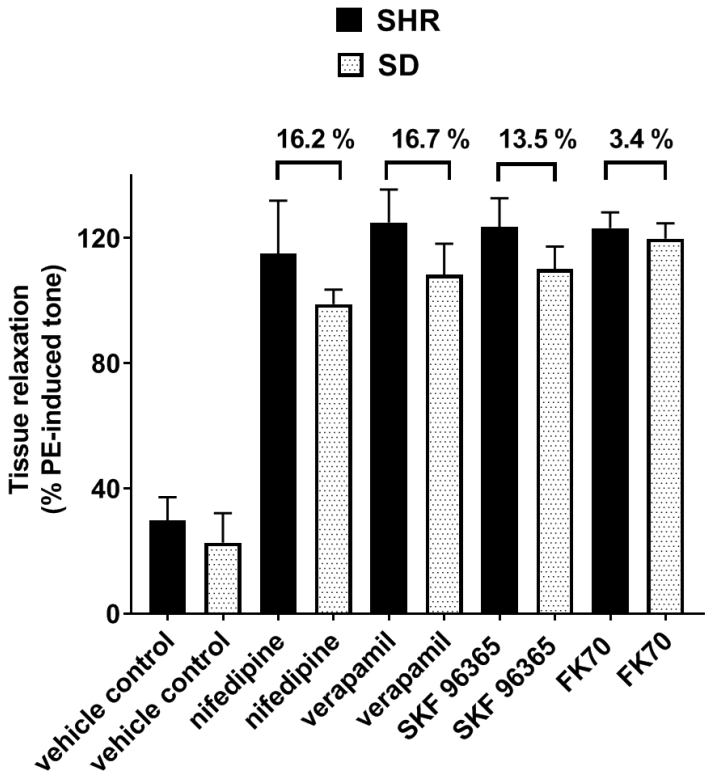


Figure 6.6: Effects of vehicle control, nifedipine, verapamil, SKF 96365 and FK70-induced relaxation in SHR and SD isolated aortic rings pre-contracted with PE. Tissue responses have been expressed as a percentage of PE-induced tone and are show as means ± SEM of 5-8 animals.

6.6.6 FK70 inhibited calcium-induced contraction in SHR

Aortic tissues pre-treated with vehicle control (DMSO 0.22%) served as control. The contractile response to $CaCl_2$ following pre-treatment with FK70 (10 and 30 µM) was concentration-dependent, with complete abolishment observed in the presence of FK70 (30 µM) (see **Figure 6.7A**) when compared to control (see **Table 6.5**). Similar observation was reported in the presence of nifedipine and SKF 96365, where pre-incubation with SKF 96365 and nifedipine reduced the calcium-induced contraction significantly (see **Figure 6.7B**). The pEC_{50} for each treatment could not be determined due to the abolishment of contraction.

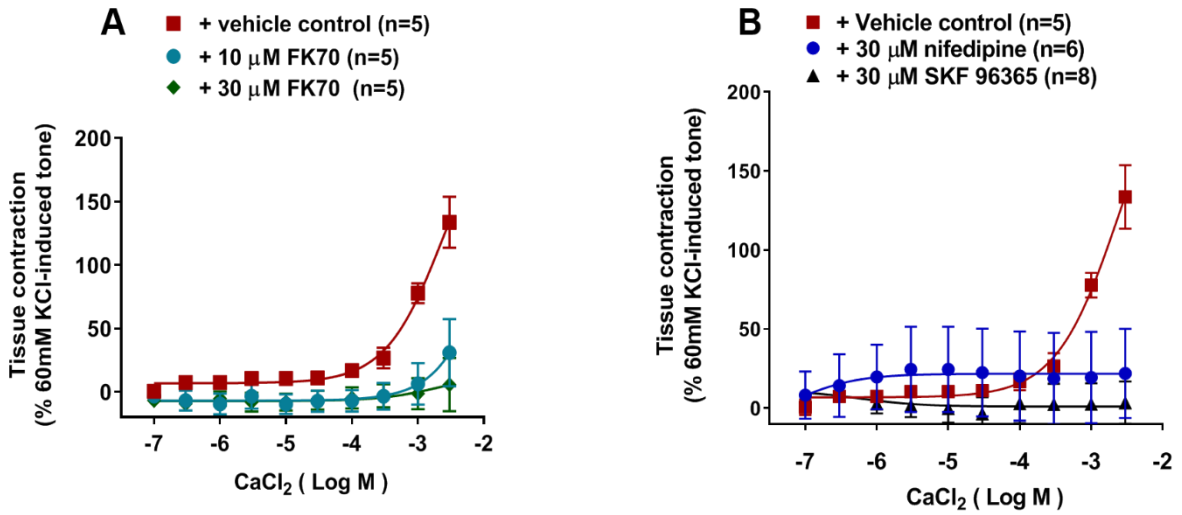


Figure 6.7: Effects of **A)** vehicle control, FK70 (10 µM) and FK70 (30 µM) **B)** vehicle control, nifedipine (30 µM), and SKF 96365 (30 µM) on $CaCl_2$ cumulative concentration response in SHR isolated aortae. Tissue responses have been expressed as a percentage of KCl-induced tone and are shown as means ± SEM of 5-8 animals.

Table 6.5: Summary of effects of vehicle control, FK70 (10 µM and 30 µM), nifedipine and SKF 96365 on calcium-induced contraction in calcium-free Krebs solution. Tissue responses have been expressed as a percentage of KCl-induced contraction and are shown as means ± SEM of 5-8 animals. The one-way ANOVA followed by Dunnett's multiple comparison test compared the relaxation effect between control, FK70, nifedipine, and SKF 96365 (*p<0.05, **p=0.01).

Treatment	n (number of animals)	Maximal relaxation at (3×10^{-3} M) (%)	p value
+ Vehicle control	5	133.6 ± 20.2	
+ 10 µM FK70	5	30.8 ± 26.5	0.02*
+ 30 µM FK70	5	5.7 ± 1.1	0.0045**
+ 30 µM nifedipine	6	21.9 ± 15.1	0.0072**
+ 30 µM SKF 96365	8	3.4 ± 1.1	0.0015**

6.6.7 FK70 reduced PE-induced contraction

Aortic rings treated with vehicle control served as control for this experiment. Incubation with vehicle control did not have significant effect on PE concentration-response curve when compared to PE concentration response curve without any prior treatment in SHR (control: 171.8 ± 18.6 %, PE alone: 172.2 ± 14.1 %; unpaired *t*-test, *p*=0.98) (see **Figure 6.8A**).

The contractile response to PE following pre-treatment with FK70 was concentration dependent, with significant reduction observed in the presence of highest concentration of FK70 (see **Figure 6.8B** and refer **Table 6.6**).

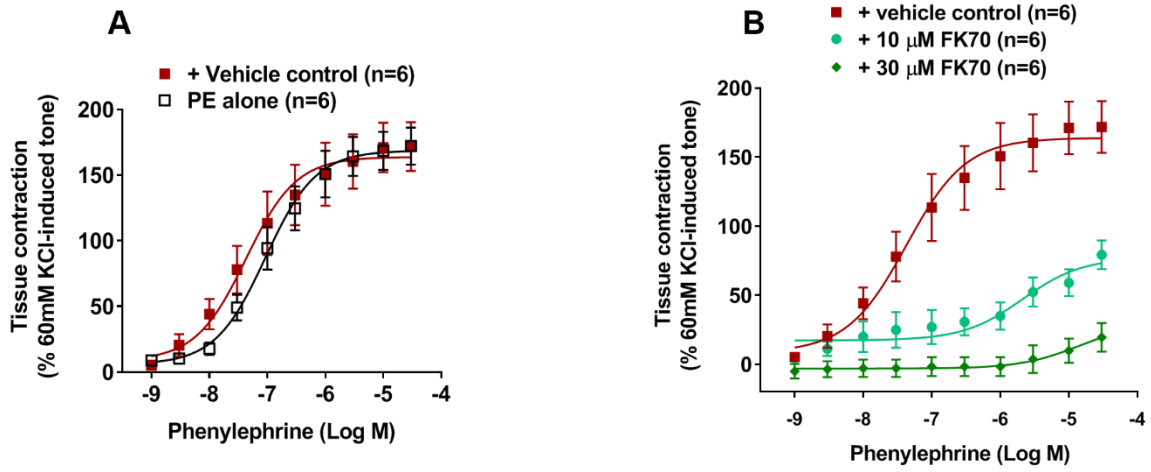


Figure 6.8: Effects of **A)** vehicle control (DMSO 0.22 %) and PE alone **B)** vehicle control (DMSO 0.22 %), FK70 (10 µM) and FK70 (30 µM) pre-incubation on PE concentration-response curve in SHR aortae. Tissue responses have been expressed as a percentage of KCl-induced contraction and are shown as means ± SEM of 6 animals.

Table 6.6: Summary of effects of vehicle control, FK70 (10 µM and 30 µM) on PE concentration-response curves in SHR isolated aortic rings. Tissue responses have been expressed as a percentage of PE-induced contraction and are shown as means ± SEM of 6 animals. The one-way ANOVA followed by Dunnett's multiple comparison test compared the relaxation effect with control (***p<0.001, ****p<0.0001, a p value of >0.05 is considered no significant difference).

	n (number of animals)	**Maximal contraction at (3×10^{-5} M) (%)**	***p* value**
+ Vehicle control	6	171.8 ± 18.6	
+ 10 µM FK70	6	79.2 ± 10.5	0.0005***
+ 30 µM FK70	6	19.5 ± 10.3	0.0001 ****

6.6.8 FK70-induced relaxation mechanism unlikely to involve blockage of L type Ca^{2+} channels.

In order to examine the role of L-type Ca^{2+} channel on FK70-induced relaxation in aorta, combined treatment with nifedipine and FK70 was performed. The PE contraction prior to incubation of nifedipine was (10.6 ± 2.3 mN). Upon incubation with nifedipine, the PE-induced contraction dropped to (6.6 ± 0.7 mN; *p*= 0.09)(see **Figure 6.9A**). While pre-incubation with FK70 instead of nifedipine reduced the PE contraction to (6.0 ± 1.2 mN; paired *t*-test, *p*=0.05) (see **Figure 6.9B**).

When FK70 was added thereafter to the same tissue (tissue in **Figure 6.9A**), the PE contractile response was significantly reduced to (0.09 ± 0.7 mN; paired *t*-test, *p*< 0.0001) when compared to addition of control (4.1 ± 0.4 mN) (see **Figure 6.10**).

When the experimental protocol was reversed, nifedipine addition thereafter (tissue in **Figure 6.9B**), the PE tone dropped and sustained after 10 minutes of addition. The PE contractile response was reduced to (2.8 ± 1.1 mN; paired *t*-test, *p*=0.006) (see **Figure 6.11**).

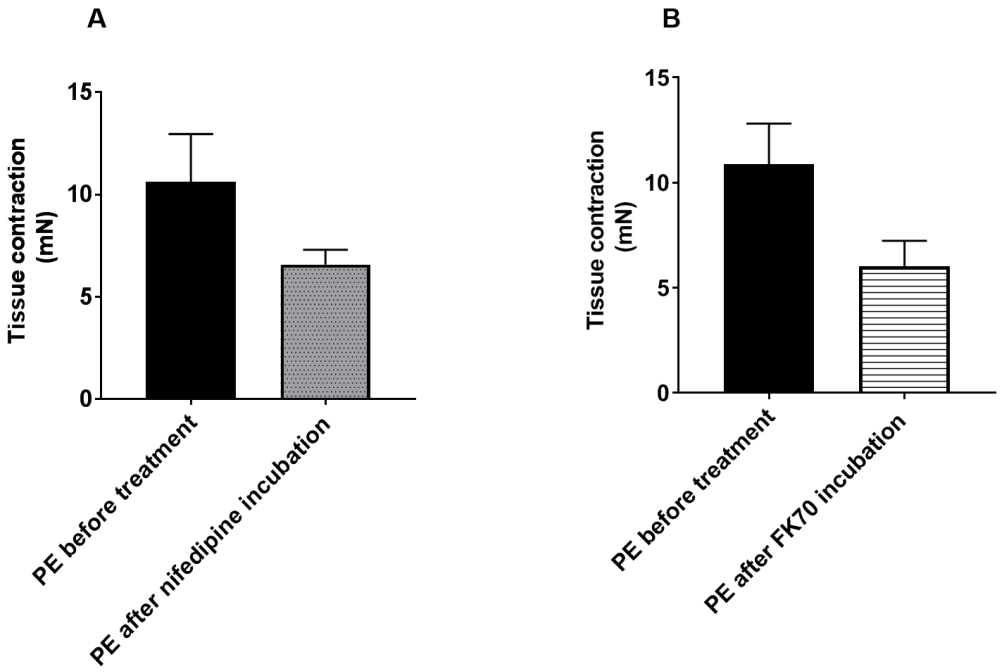


Figure 6.9: Effects of pre-incubation with **A)** nifedipine and **B)** FK70 (30 µM) on contractile response of PE. Tissue responses have been expressed as a force of contraction in force mN and are shown as means ± SEM of 5- 6 animals

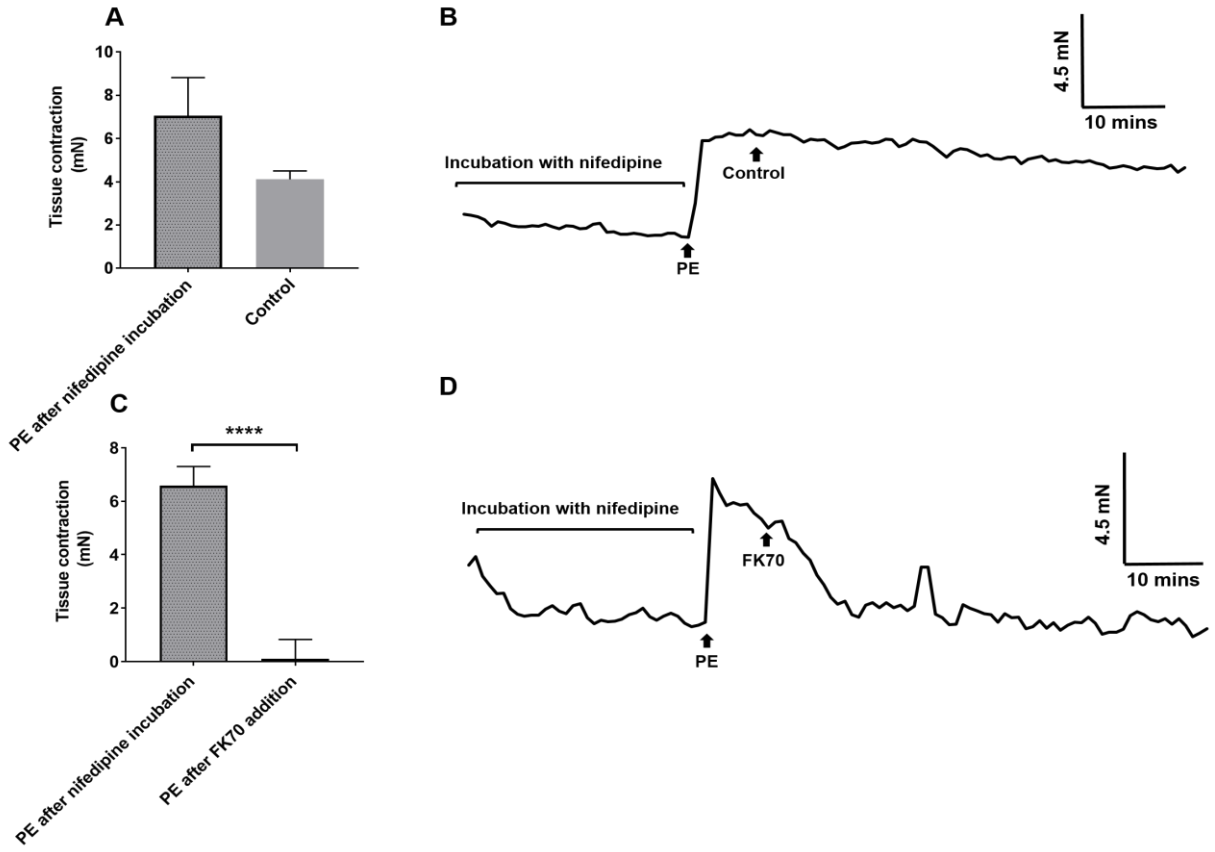


Figure 6.10: Effect of pre-incubation with nifedipine (30 µM) followed by addition of **A)** vehicle control **B)** FK70 (30 µM) on PE-induced contractions. Representative trace recordings of effect of pre-incubation with nifedipine (30 µM) followed by addition of **C)** vehicle control **D)** FK70 (30 µM) on PE-induced contractions. Tissue responses have been expressed as a force of contraction in force mN and are shown as means ± SEM of 5-6 animals. (paired *t*-test: **** $p<0.0001$). (Abbreviations: PE = phenylephrine; mN = milliNewton; mins = minutes).

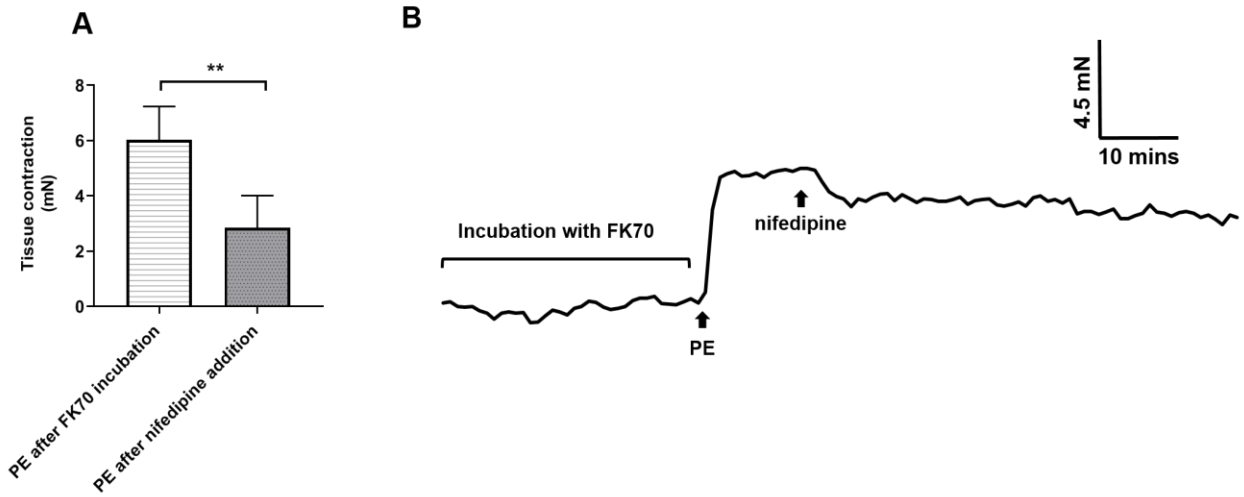


Figure 6.11: A) Effect of pre-incubation with FK70 (30 µM) followed by addition of nifedipine on PE-induced contractions **B)** Representative trace recordings of effect of pre-incubation with FK70 (30 µM) followed by addition of nifedipine on PE-induced contractions. Tissue responses have been expressed as a force of contraction in force mN and are shown as means ± SEM of 5-6 animals. (paired *t*-test : ** $p<0.01$). (Abbreviations: PE = phenylephrine; mN = milliNewton; mins = minutes).

6.6.9 FK70-induced relaxation may not involve modulation of TRPC channels

The next step was to examine the involvement of TRPC channels on FK70-induced relaxation. The experiments were conducted using SKF 96365, a non-selective putative TRPC channel blocker and FK70. The PE contraction prior to any treatment was (11.2 ± 0.84 mN). Incubation with SKF 96365 reduced PE-induced contraction to (6.84 ± 0.46 mN; paired *t*-test, *p*=0.01) (see **Figure 6.12**).

Thereafter, addition of FK70 to the same tissue (tissue in **Figure 6.12**), the PE tone dropped sharply after 10 minutes of FK70 addition and reduced the PE contraction to (-0.39 ± 0.6 mN; *p*= 0.0002). The control group did not display significant effect on PE-induced contraction, paired Student's *t*-test, two tailed *p*= 0.05 (see **Figure 6.13**).

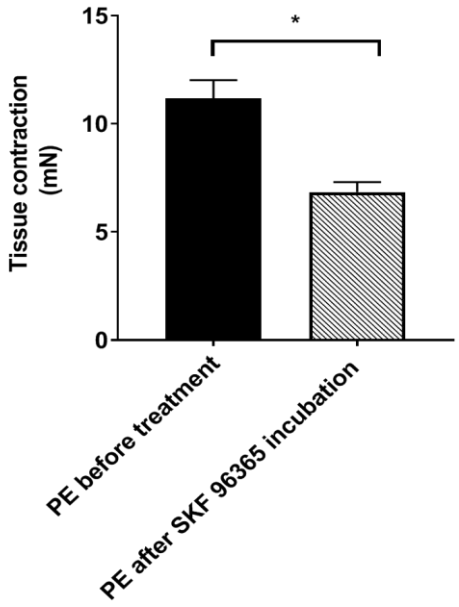


Figure 6.12: Effect of pre-incubation with SKF 96365 (30 µM) on contractile response of PE. Tissue responses have been expressed as a force of contraction in force mN and are shown as means ± SEM of 5 animals. (paired Student's *t*-test : **p*<0.05).

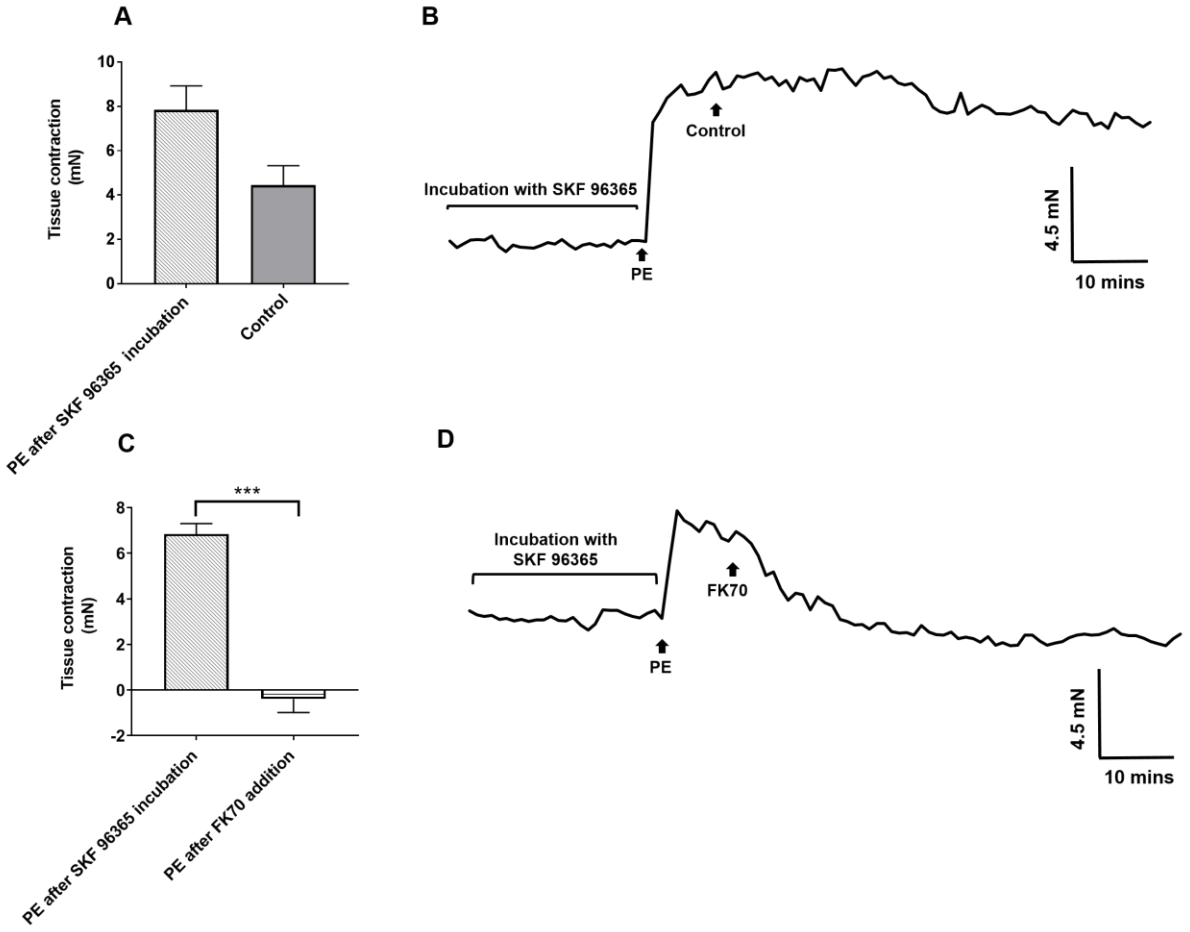


Figure 6.13: Effect of pre-incubation with SKF 96365 (30 µM) followed by addition of **A)** vehicle control **B)** FK70 (30 µM) on PE-induced contractions. Representative trace recordings of effect of pre-incubation with SKF 96365 (30 µM) followed by addition of **C)** vehicle control **D)** FK70 (30 µM) on PE-induced contractions. Tissue responses have been expressed as a force of contraction in force mN and are shown as means ± SEM of 5-6 animals. (paired *t*-test: ****p*<0.001). (Abbreviations: PE= phenylephrine; mN= milliNewton; mins= minutes)

6.7 Discussion

In the previous chapter (Chapter 5), it was evident that FK70-induced relaxation in aortae involves calcium-mediated responses. The results had demonstrated that FK70 could inhibit PE, KCl and 5-HT contraction and indicated that FK70-induced relaxation was mediated mainly through the inhibition of extracellular Ca^{2+} and had little to no effect on intracellular calcium release. The relaxant mechanism of FK70 in SD rats appears to be independent of L-type Ca^{2+} channels and TRPC channels based on the vascular function responses. One of the key mechanisms of antihypertensive drugs is to lower the vascular resistance by directly dilating the blood vessels (Sun *et al*., 2011). Vasodilation occurs as a result of the relaxation of smooth muscle cells within the blood vessel walls. The experimental results in this study showed that FK70 could relax the SHR aorta pre-contracted by PE, suggesting that FK70 possesses potential antihypertensive properties. Therefore, the current chapter was designed to further explore the vasorelaxant effect and the underlying mechanisms of FK70 in SHR aortic rings. Besides, the role of vascular endothelium in the regulation of contractile sensitivity and how this may be altered in hypertension was also examined.

There were five significant findings from this study. First, the endothelium integrity of intact SHR aortic rings was affected and the percentage of carbachol-induced relaxation was relatable to endothelium-denuded SD aortic rings. The carbachol-induced relaxation in rat aorta is mainly attributed to NO (Shimokawa *et al*., 1996). The reduced carbachol-induced relaxation in SHR rats suggests that the endothelial layer of SHR aortic tissue was severely affected and has lost its ability to release any relaxation factors. The blunted endothelium-dependent relaxation of aortic rings to acetylcholine in SHR observed in this study has been documented repeatedly in the aortae of different experimental models of hypertension by various investigators (Lockette *et al.,*1986; Vanhoutte *et al.,*1995; Rodrigo *et al.,*1997). Endothelial dysfunction is the hallmark of hypertension (Silva *et al.,*2012). Data from past literature indicate that, in general, endothelial dysfunction is not the cause of hypertension but rather a secondary that results from high blood pressure (Bernatova, 2014). The relaxation induced by NO, prostacyclin, and the endothelium-derived hyperpolarization factor (EDHF) is generally impaired in hypertension. The impaired ability of endothelial cells to release NO has been described to contribute to the endothelium dysfunction, which appears to lead to several cardiovascular diseases (Lockette *et al.,*1986; Silva *et al.,*2012).

Following the endothelium assessment test, further experiments were conducted in order to assess the PE sensitivity in SHR. It is generally presumed that α_1-agonists (in this case PE) activate adrenoceptors in the endothelial cells and thereby promote the release of a relaxing factor (NO) to inhibit vascular smooth muscle contraction (Dora *et al.,*2000; Fransen *et al*.,2016). Removal or impairment of endothelium would abolish the release of this putative inhibitory substance, and adrenergic agonists would activate only adrenoceptors in the muscle to cause vasoconstriction (Carrier and White, 1985). Therefore, it was envisaged that severe impairment in endothelium would render those aortae to be more sensitive to PE. Interestingly, in contrast to results obtained by other investigators (Zhao *et al*.,2012; Potje *et al*., 2019), no evidence for alterations in smooth muscle sensitivity to PE was found in this model of hypertension. Reasons for this discrepancy are not clear, although one possibility is the use of different strains to compare (Wistar Kyoto versus Sprague Dawley and Wistar rats). It is, however, noteworthy to mention that the inbred Wistar-Kyoto (WKY) rats were derived from the same ancestral outbred Wistar rat and have been used as the closest genetic control for the SHRs (James *et al,* 2013). There are also other possible explanations for this. First, although the endothelium was severely affected, the endothelium function is still present and not absent. Second, previous studies have suggested that the PE response in SHR aortae could also be attributable to eNOS-independent NO. Although NO accounts for the majority of NO produced in the vasculature, other sources such as nitrate and nitrite have been identified. The nitrate and nitrite on the extravascular tissues can be reduced to form NO under anoxic or acidotic conditions by different enzymes located in various tissues (Li *et al*., 2006). That said, the nitrate and nitrite were found to be higher in SHR aortae (Zhao *et al*., 2012). Third, PE contraction per se may not be a mechanism for NO release in the aorta or the functional α_1-adrenoceptors linked to an increase in $[Ca^{2+}]_i$ may not be located on the endothelial cells.

SNP is NO donor, which induce vascular smooth muscle relaxation mainly through the activation of the soluble guanylate cyclase (sGC) and the subsequent increase in cGMP levels (Cogolludo *et al.,*1999). The absence of variation between the vasorelaxation induced by SNP in FK70 and control-treated intact SHR aortic rings confirms the independence of the FK70 effect on the endothelium and suggests that the relaxant effect of FK70 was mostly exerted on the vascular smooth muscle cells. Moreover, the unaltered PE contraction in SHR aortae, when compared with normotensive rats aortae, adds more justification to this recent claim.

The primary aim of this study was to establish the calcium-blocking effect of FK70 in SHR aortae. In this study, it was evident that FK70 caused a relaxation effect in PE pre-contracted SHR aortae. Similar observations were also noted in other known calcium channel blockers, nifedipine, verapamil, and SKF 96365. The findings of this investigation complement the study done in previous (chapter 5), where the maximal relaxation response of FK70 was comparable to that of known calcium channel blockers. A plethora of past studies have conclusively demonstrated that an upregulation of L-type Ca^{2+} ($Ca_{V1.2}$) channel function in vascular smooth muscle cell is a hallmark feature of hypertension (Sonkusare *et al*.,2006; Joseph *et al.,* 2013) and the elevated Ca^{2+} currents observed in vascular smooth muscle during hypertension is related to the increased number of $Ca_{V1.2}$ channel openings (Ohya *et al*., 1998). Thus explains the significant difference observed in terms of the maximal relaxation response in SHR than in SD rats for nifedipine and verapamil. The higher relaxation response of SKF 96365 observed in SHR as compared to SD could be due to the heightened expression of TRPC channels in SHR. Consistent with this proposal, the up-regulation of TRPC6 and TRPC3 channels have been detected in several hypertensive animal models (Bae *et al*., 2007; Mita *et al*., 2010; Zulian *et al*., 2010; Wang *et al.*, 2017). From this experiment, it was shown that FK70 relaxes both SD and SHR at an almost similar degree when compared to other calcium antagonists tested. Therefore this suggests that FK70 could be inhibiting Ca^{2+} entry via a mechanism independent of L-type Ca^{2+} channels and TPRC channels. However, the exact mechanism by which FK70 demonstrated higher response in SHR is yet to be found.

The effect of FK70 on extracellular Ca^{2+} influx was investigated by carrying out the $CaCl_2$ re-addition experiment in calcium-free Krebs solution. FK70 reduced the potency and maximal response of $CaCl_2$ re-addition, confirming that FK70 affects calcium sensitivity and reduces calcium influx in SHR aortae. The present data also displayed a concentration-dependent blockade of Ca^{2+}-induced contractions after KCl-induced contraction, which argues for the blockade VOCC as part of the vasodilating effects of FK70. These results were then verified in the presence of nifedipine, a known L-type Ca^{2+} blocker and SKF 96365, a non-selective TRPC channel blocker.

It is well documented that the underlying mechanism of PE-induced contraction involves the opening of voltage-gated Ca^{2+}channels, especially L-type Ca^{2+} channels and TPRC channels in SHR (Paoletti and Govoni 1987; Auguet *et al*.,1989; Chitra Devi *et al.,*2012). In this study, FK70 treatment resulted in a rightward shift of concentration-response curves of PE in a concentration-dependent manner. It is, therefore, suggesting that FK70 affects both intracellular Ca^{2+} and extracellular Ca^{2+} entry to cause relaxation. It was, however, highlighted in the previous (Chapter 5) that FK70 unlikely to affect intracellular Ca^{2+} mechanism in normotensive rats. If this observation could be generalized to SHR aortae, FK70 might not affect the intracellular Ca^{2+} mechanism in SHR, and maybe the blocking of extracellular Ca^{2+} is a critical mechanism of FK70 in relaxing the aortae. Interestingly, the inhibitory effect of PE-induced contraction by nifedipine (30 µM) was lower than that of FK70 (30 µM). The reason for this is not apparent, but it might be due to L-type Ca^{2+} channel upregulation in SHR vascular tissues. Therefore, more research is warranted to verify the proposed hypothesis.

Numerous studies have focused on the relationship between primary hypertension and TRPC channels. TRPC1, TRPC3, and TRPC6 channels have been shown to play a pathophysiological role in hypertension (Dietrich *et al*.,2010; Fuchs *et al*.,2010; Gopal *et al*.,2015). Previous studies have suggested that the TRPC3 levels were elevated in patients with hypertension as well as in the pressure-overload rat and the SHR models (Liu *et al*.,2009; Thilo *et al*.,2009). Overexpression of TRPC6, on the other hand, strengthened agonist-mediated vascular smooth muscle contractility and companied with increased mean blood pressure (Bae *et al*., 2007). SKF 96365, as a non-selective TPRC channel blocker, abolished the PE-induced contraction supporting an essential role of calcium influx through TRPC channels in SHR. Collectively, this data suggests that activation of the α_1-adrenergic receptor by PE involves calcium influx through TRPC and L-type Ca^{2+} channels in this SHR model. Pre-treatment with FK70 at a lower concentration managed to reduce PE contraction comparable to SKF 96365 at a higher concentration. This further suggests that the FK70-induced relaxation mechanism in SHR may not involve the L-type Ca^{2+} channels or TRPC channels.

The experiments discussed above prompted more experiments to be carried out to clarify the calcium channels involved in FK70-induced relaxation. It was evident that the underlying mechanism of PE-induced contraction involves the opening of L-type Ca^{2+} channels and TPRC channels. Therefore, in the present study, nifedipine and SKF 96365 were employed to confirm the involvement of those channels in FK70-induced relaxation. Addition of FK70 to the tissues incubated with either SKF 96365 or nifedipine displayed further relaxation suggesting that other channels than L-type Ca^{2+} channels and TPRC channels are involved in FK70 mediated relaxation, assuming that the concentration of known antagonists used blocked 90-100% of the respective receptors. Furthermore, FK70 caused a nearly complete relaxation of PE-induced contraction with a relatively rapid onset of action when compared to nifedipine, suggesting that the kinetics of FK70 at the receptor is different from nifedipine. The concentration of SKF 96365 used for incubation for this particular experiment block ~90 % of the TRPC channels, which was determined from the previous experiment in this study. The concentration of SKF 96365 used was in line with past studies involving SKF 96365 for incubation before PE addition in SHR aortae which had been reported to be within (10-30 µM) (Gomart *et al*., 2017; Mendez *et al.,* 2017; Ding *et al*., 2019). Nevertheless, it remains to be seen whether the same results could be obtained with FK70 in the presence of SKF 96365 (100 µM). Therefore, it does not allow us to conclude that the mechanism of FK70 is independent of TPRC channels.

In conclusion, this study managed to show that the relaxation effect of FK70 is comparable to that of classical calcium channel blockers in SHR. Notwithstanding the relatively limited amount of SHR tissues, this work offers valuable insights into FK70 pharmacological effects on SHRs. The inhibitory effect of FK70 in SHR was endothelium-independent and it seems that the most critical mechanism involved in FK70-induced relaxation is inhibition of extracellular Ca^{2+} influx. The exact mechanism for this remains to be determined. More work is needed to explore the potential therapeutic utility of FK70 in SHR fully. Given the compelling data including enhanced relaxation effect induced by FK70 when combined with nifedipine or SKF 96365, makes it a suitable lead compound with the prospect of desired antihypertensive properties and we believe that more research on FK70 in SHR would be worthwhile pursuing.

Chapter 7: Effect of FK70 on feeding and lifespan of *Caenorhabditis elegans*

7.1 Introduction

Caenorhabditis elegans (*C. elegans*) is a tiny, free-living nematode found worldwide. *Caenorhabditis* and other nematodes belong to the phylum Nematoda, which is part of a larger group of the clade Ecdysozoa. The *Caenorhabditis* genus is included in the order of Rhabditida, part of the larger subclass, Chromadoria. The name *Caenorhabditis elegans* is a blend of Greek *caeno*- (recent), *rhabditis* (rod-like) and Latin *elegans* (elegant). The newly hatched larvae are 0.25 mm long and the adults are 1 mm long. The *C. elegans* has a rapid life cycle (3 days at 25°C from egg to egg-laying adult) and exists primarily as a self-fertilizing hermaphrodite, and rarely males which arise at a frequency of < 0.2 % (Corsi *et al*.,2015). The worms increase in size throughout four larval stages known as the L1, L2, L3, L4 but individual sexes are not easily distinguished until the L4 stage (see **Figure 7.1**). The basic anatomy of *C. elegans* includes a mouth, pharynx, intestine, gonad, and collagenous cuticle. Like all nematodes, they have neither a circulatory nor a respiratory system. They are usually observed with either dissecting microscopes, which generally allow up to 100X magnification, or compound microscopes, which allow up to 1000X magnification (Wormbook.ed).

A little over 50 years ago, Sydney Brenner, a biologist, and a geneticist had the foresight to develop the nematode (roundworm) *Caenorhabditis elegans* as a genetic model for understanding questions of developmental biology and neurobiology. Over time, research on *C. elegans* has expanded to explore a wealth of diverse areas in modern biology, including studies of the essential functions and interactions of eukaryotic cells, host-parasite interactions, and evolution. *C. elegans* have also become a vital organism to study processes that go awry in human diseases. At least 38% of the *C. elegans* protein-coding genes have predicted orthologues in human genome (Shaye and Greenwald, 2011), 60-80 % of human genes have an orthologue in the *C. elegans* genome (Kaletta and Hengartner, 2006), and 40 % of genes known to be associated with human diseases have clear orthologues in the *C. elegans* genome (Culetto and Satelle, 2000). The *C. elegans* is the first multicellular organism with a complete genome sequence (*C. elegans* Sequencing Consortium, 1998), which has led to the molecular identification of many key genes in developmental and cell biological processes. Besides, they make an outstanding experimental system due to their small size, relatively predictable and short lifespan, transparency, and well-annotated genome.

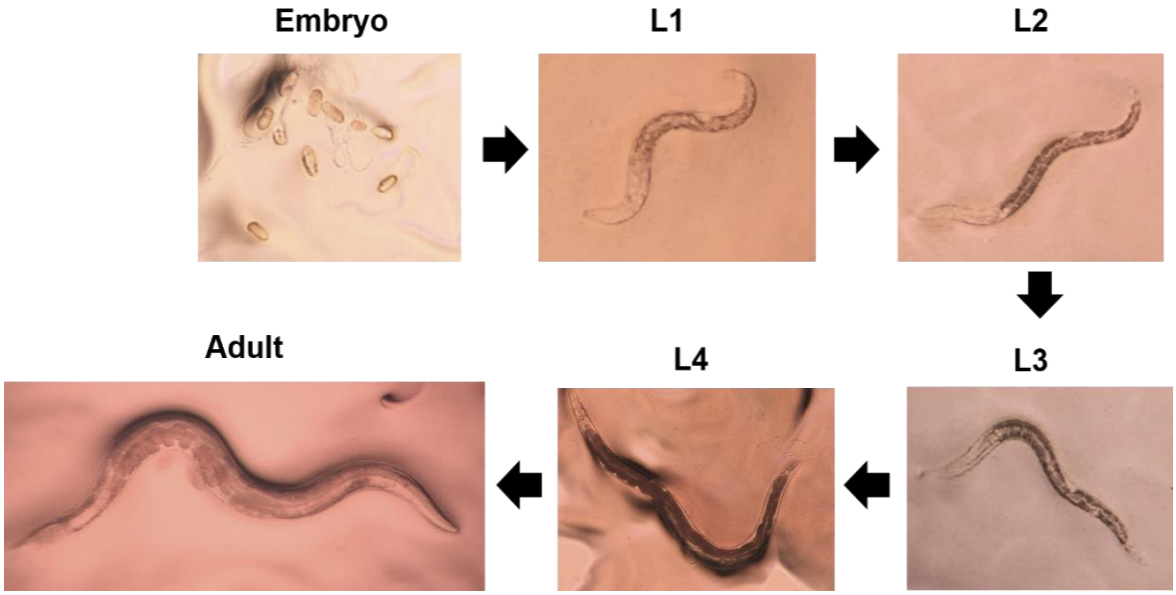


Figure 7.1: Life cycle of *C. elegans* from embryo to adult. The worms increase in size throughout the four larval stages. Photographs were taken on petri dishes (note the bacterial lawns in all).

In the laboratory, the C. *elegans* are typically grown on agar plates containing a lawn of the bacterium *Escherichia coli* (*E.coli*) at temperatures ranging from 12°C to 25°C. They feed on bacteria and once the bacteria is depleted, they utilize their fat supply. Without food, the development of young larval stage worms is arrested. As a result of entering this stasis, they can survive for at least a month. The starved plates can be usefully kept for up to six months at 15°C and as stocks, where they do not require constant feeding. The worms can also be frozen for years and revived when needed. In order to resume their development, a piece of agar from the old plate can be transferred to a new plate with bacteria. Growth at different temperatures makes it possible to control the rate of animal development and assists in the isolation and use of temperature-sensitive mutants. However, continual growth above 25° is not possible as the worms become sterile. Prior to experimental use, the worm population can be synchronized by isolating newly hatched larvae or by treating gravid adults with bleach to isolate eggs, that are resistant to bleach treatment. These features greatly facilitate the maintenance of *C. elegans* stocks and their experimental use. Besides, they also require less expensive equipment and tools for maintenance.

The wild-type *C. elegans* has two sexual forms: self-fertilizing hermaphrodites and males. The gonad of hermaphrodites forms an ovotestis that first produces haploid amoeboid sperm that are stored in the spermatheca in the L4 stage and then near adulthood the germline switches to produce oocytes. Mostly hermaphrodites are females whose gonads temporarily produce sperm before they produce oocytes. Hermaphrodites can produce up to 300 self-progeny that are fertilized by the stored sperm; however, if mated with males, the hermaphrodites are capable of producing ~1000 offspring. Both sexes are diploid with five pairs of autosomal chromosomes; however, the hermaphrodites have two X chromosomes, while the males have a single X chromosome. The *C. elegans* have no Y chromosome as other vertebrates, but the genotype of males is referred to as XO (Zarkower, 2006). The majority of offspring produced by self-fertilization are hermaphrodites; only 0.1-0.2 % of the progeny are males due to rare meiotic non-disjunction of the X chromosome. The self-fertilizing (often referred to as selfing) hermaphrodites offer several advantages for genetic analysis. First, selfing simplifies stocks maintaining as a single animal is adequate to give rise to an entire population. Second, the animals are driven to homozygosity because hermaphrodites cannot mate with other hermaphrodites. Therefore, the strains that are mutagenized are isogenic. Third, selfing follows standard Mendelian rules of segregation to produce 25% homozygous progenies for the mutant allele. This greatly reduces the effort needed to find mutants and can be maintained in the laboratory.

The hermaphrodite embryo hatches with 558 nuclei and becomes a first stage (L1) larva. The animals begin to eat and develop through four larval stages (L1-L4). The L1 stage is ~16 hr long; the other stages are ~12 hr long. Each stage ends with a sleep-like period of inactivity called lethargus (Raizen *et al.*,2008) in which a new cuticle (outer collagenous layer) is made. Lethargus ends with the molting of the old cuticle. Approximately 12 hr after the L4 molt, adult hermaphrodites begin producing progeny for 2-3 days until they have utilized all of their self-produced sperm. Additional progeny can be generated if the sperm-depleted hermaphrodite mates with a male. After the reproductive period, hermaphrodites can live several more weeks before dying of senescence.

7.2 *C. elegans* anatomy

The worm is often described as a series of concentric tubes (see **Figure 7.2**). *C. elegans* is unsegmented, vermiform, and bilaterally symmetrical. It has a cuticle (a tough outer covering, which acts as an exoskeleton), four main epidermal cords, and a fluid-filled pseudocoelom (body cavity).

The outer layer of cells, the epidermis is also known as the hypodermis, encloses a pseudocoelomic fluid-filled cavity housing the main organ systems. Interior to the epidermis are the bands of muscle, which control the movement of the organism, as well as the ventral and dorsal nerve cords that innervate the muscles. The interior of the neuro-muscular region consists of digestive, excretory, and reproductive systems. Besides that, *C. elegans* have six cells in the pseudocoelomic cavity, called coelomocytes, which act as scavengers in the body cavity (Grant and Sato, 2006). These cells behave similarly to vertebrate macrophages as they are highly active in endocytosis, and filter material in the pseudocoelomic cavity of the worms.

The epidermis, the outer layer of embryo undergoes a series of cell fusions to make large multinucleate, or syncytial, epidermal cells. These cells secrete the cuticle, a protective layer of specialized extracellular matrix (ECM) consisting primarily of collagen, lipids, and glycoproteins (Chisholm and Hardin,2005; Page and Johnstone,2007). The cuticle determines the shape of the body and, through a connection from the epidermis to muscle, which provides anchoring points for muscle contraction. At the end of each larval stage, *C. elegans* shed their cuticles and secrete new cuticles to accommodate their growth.

The muscles run along the length of the body of the worms, and the regular contraction and relaxation of the muscle cells lead to the sinusoidal movement. The somatic muscle cells are striated and mononucleated with multiple sarcomeres per cell. Unlike muscle innervations in other vertebrates, the muscle cells in *C. elegans* send extensions to the ventral and dorsal cord to receive synapses from the motor neurons. Besides the body-wall muscle, *C. elegans* also have muscles that control eating (pharyngeal muscles), egg-laying (vulval and uterine muscles, and the contractile gonad sheath), mating (male-specific tail muscles), and defecation (enteric muscles).

A typical adult hermaphrodite has 302 neurons (Sulston and Horvitz,1977; White *et al.*,1986), while the adult male has 383 neurons. The majority of neuronal cell bodies are arranged in a few ganglia in the head, in the ventral cord, and in the tail (the specialized male tail has the majority of the extra neurons). The neurons have a relatively simple structure with one or two neurites (or processes) exiting from the cell body, except FMRF amide-like neuropeptide (FLP) and PVD mechanosensory neurons with a highly branched network (Dong *et al.*,2013). The neurites form synapses in four major areas: the nerve ring (which encircles the pharynx), the ventral nerve cord, the dorsal nerve cord, and the neuropil of the tail. In addition to neurons, *C. elegans* has several glia-like support cells, which are primarily associated with sensory neurons but are not as numerous as invertebrates. The nerve conduction in *C. elegans* appeared to be primarily passive as no sodium-dependent action potentials have been detected in neurons, and no genes for voltage-gated sodium channels (Bargmann, 1998) have been found. However, the neurons express a wide variety of ion channels (Hobert, 2013).

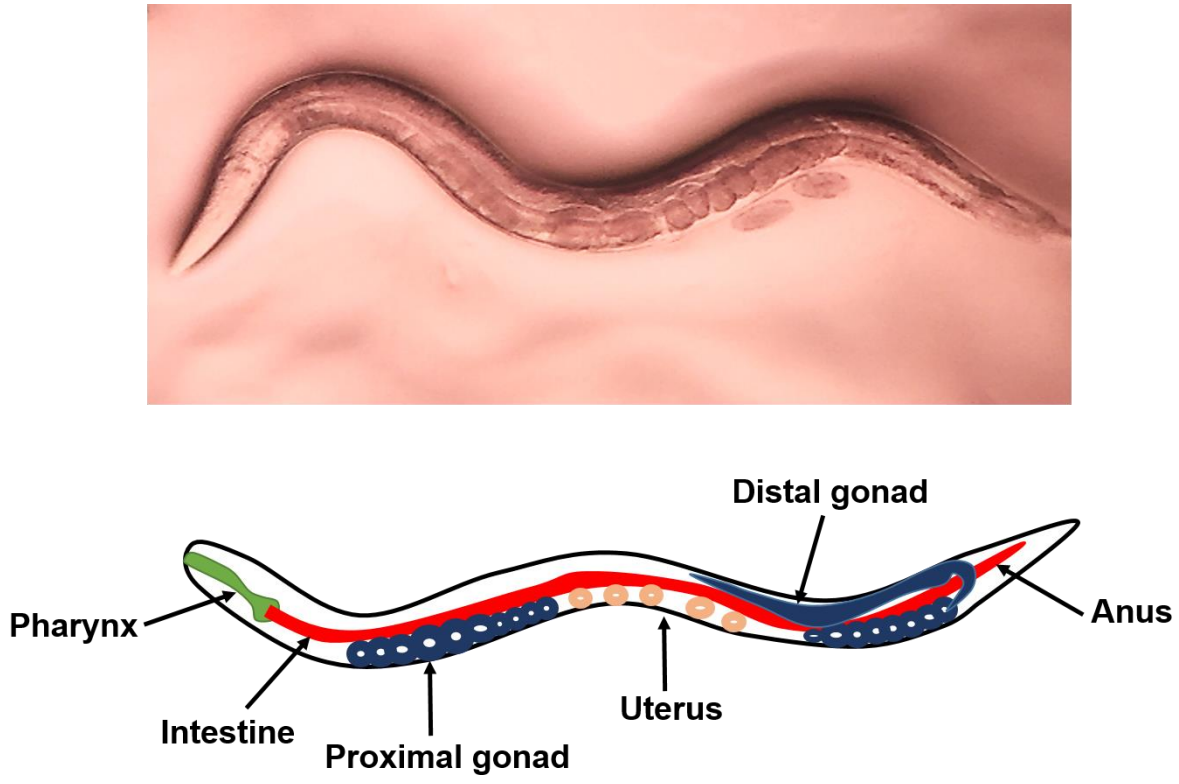


Figure 7.2: Picture of adult *C. elegans* taken under the miscroscope (top) and schematic illustration (bottom) showing the nematode body plan from head to tail.

7.3 Pharyngeal pumping and involvement of calcium

In *C. elegans*, feeding is achieved through a well-characterized behavior, pharyngeal pumping. The pumping is controlled by two pairs of pharyngeal motor neurons (MC and M3). The presence and quality of food regulate the feeding motions in the worm's environment. During feeding, bacteria are taken up by the mouth and transported to the intestine via the pharynx. The investigation of the pharynx and its control is essential in studying feeding behavior as pharyngeal pumping is essential for feeding in *C. elegans. The* pharynx is a muscular pump, 100 µm long with a diameter of 20 µm. It consists of a contractile element of 20 muscle cells, some syncytial, which form eight muscle layers (pm1-8), 20 neurons that comprise the pharyngeal nervous system, 4 glands cells, and 9 marginal cells to strengthen the integrity of the pharyngeal organ and may also play a role in synchronizing its electrical activity (Altun and Hall,2009). One complete cycle of synchronous contraction and relaxation of the corpus and the terminal bulb is called a pump. The rate of pharyngeal pumping reaches a maximum of nearly 300 pumps/min for 2 day-old adults, decreasing with age to about 100 pumps/min by day 8, 10 pumps/min by day 12, and virtually no activity after day 14 (Huang *et al*.,2004).

The muscle cells form three compartments of the pharynx, corpus (divided into pro- and meta), isthmus, and the terminal bulb, which is most posterior (see **Figure 7.3**). In the terminal bulb, there is a cuticular specialization of the muscles, namely grinder, to break up the ingested bacteria before being passed on to the intestine. The pharyngeal pumps and isthmus peristalsis are the two main components of pharyngeal behavior (Avery and You, 2012). Pharyngeal pumping can be divided into three stages. Initially, when the food enters the corpus, the corpus and anterior isthmus contract to initiate pumping, which leads to the opening of the lumen to allow fluid and bacteria into the mouth. During pumping, the posterior isthmus remains closed and isolates the corpus from the terminal bulb. Bacteria are transported to the terminal bulb during isthmus peristalsis, an event that takes place approximately every four pumps (Avery and Horvitz, 1989). This ensures bacteria to be concentrated in the isthmus before they reach the terminal bulb. The terminal bulb contracts and activates the cuticular plates of the grinder to smash the bacteria to be transported to the intestine.

Albeit the pharynx being a myogenic muscular pump, it is mainly modulated by neural circuits. The 20 neurons of the pharyngeal nervous system consist of 14 anatomical types with six bilaterally paired and eight single neurons (Albertson and Thomson, 1976). These neurons are linked to the extrapharyngeal nervous system by a pair of RIP (ring/pharyngeal) interneurons (White *et al*., 1986). RIP neurons interact with the pharyngeal nervous system via a gap junction with a pair of pharyngeal neurons, inner labial (I1). The I1 neurons interact with several pharyngeal neurons, both through chemical synapses and gap junctions, to activate rapid pumping. Motor neuron MC, M2, and M4 control the pharyngeal pumping via nicotinic and muscarinic receptors (Trojanowski *et al*.,2014) (see **Figure 7.3**). The MC neurons entrain the pharyngeal muscle and override the myogenic rhythm to cause rapid neurogenic pumping. When MC activity or postsynaptic response to MC activity is decreased, the myogenic rhythm sets the pumping rate. However, in the absence or ablation of the nervous system function, the pharyngeal pumping ceases completely. The electrically coupled muscle cells of the pharynx respond to a variety of neurotransmitters and neuromodulators and rely on T- and L-type Ca^{2+} channels for action potential (Avery and Horvitz, 1990; Shtonda and Avery, 2005).

The *C. elegans* pharynx contraction is due to repetitive Ca^{2+} transients induced by rhythmic action potentials, which qualitatively bear a resemblance to vertebrate cardiac action potentials. The voltage-gated calcium channels (VOCC) couple changes in membrane potential to numerous biological processes regulated by calcium, such as muscle contraction, neurotransmitter release, and gene regulation. The VOCCs are composed of a pore-forming α_1 subunit associated with up to three auxiliary subunits, α2/δ, β, and γ. The *C. elegans* genome contains five genes encoding putative α_1 subunits, *egl-19, cca-1, unc-2, nca-1,* and *nca-2* (Bargmann, 1998). Sequence analysis had shown that *egl-19, unc-2,* and *cca-1* are homologues to vertebrate α_1 subunits conducting L-type, P/Q/R/N-type, and T-type currents, respectively (Lee *et al*.,1997). Both *egl-19* and the T-type *cca-1* are known to drive currents in pharyngeal muscles. The *cca-1* channels appeared to be activated in response to the small membrane depolarisations (EPSPs) resulting from MC motor neuron stimulation of nicotinic receptors on the pharyngeal muscle surface. Ca^{2+} influx through *cca-1* channels then lift membrane potential to the threshold for activation of *egl-19*, the L-type calcium channels (Stegar *et al*.,2005) in the plasma membrane of pharynx cells, subsequently increasing the cytosolic $[Ca^{2+}]_c$ by Ca^{2+} release from the sarcoplasmic reticulum through the ryanodine receptor (Liu *et al*.,2011). Activation of voltage-dependent potassium channels triggers a rapid repolarization that closes the plasma membrane Ca^{2+} channels, and the Ca^{2+} pumps in sarcoplasmic reticulum membrane and plasma membrane pump back Ca^{2+} out of the cytosol,

restoring the low resting $[Ca^{2+}]_c$ levels. The corpus, anterior isthmus and terminal bulb synchronously contract with fast Ca^{2+} spikes but the midportion, the posterior isthmus, exhibits a slow movement called peristalsis. The Ca^{2+} dynamics in the posterior isthmus are often decoupled during fast pumping and the excitation–calcium coupling is uniquely modulated at the posterior isthmus at the level of Ca^{2+} channels on the plasma membrane (Shimozono *et al*.,2004).

Besides that, the TRP channels also mediate Ca^{2+} entry following the activation of phospholipase C by cell surface receptors. Genetic analyses in *C. elegans* have implicated TRP channels in a wide spectrum of behavioural and physiological processes, ranging from sensory transduction (e.g. chemosensation, touch sensation, proprioception and osmosensation) to fertilisation, drug dependence, organelle biogenesis, apoptosis, gene expression, and neurotransmitter/hormone release (Xiao and Xu, 2011). Many *C. elegans* TRP channels share similar activation and regulatory mechanisms with their vertebrate counterparts. Therefore, *C. elegans* serves as an excellent genetic model organism for the study of function and regulation of TRP channels *in-vivo.* However*,* many *C. elegans* TRP channels are enriched in the nervous system and play important roles in behavioural control. Among the seven TRP subfamilies nearly every subfamily has members that have been implicated in mechanosensation. These include TRPC (TRPC1, TRPC2, TRPC3, TRPC5,and TRPC6), TRPV (TRPV1, TRPV2, TRPV4), TRPM (TRPM4 and TRPM7), TRPN (TRPN1/TRPN4), TRPA (TRPA1), TRPP (TRPP2) and TRPML as well as the more distantly related Yvc1/TRPY1 (Christensen and Corey, 2007; Gottlieb *et al*., 2008; Sharif-Naeini *et al*., 2008). As TRP proteins often interact to form heteromeric channels, they could potentially encode a large group of mechanotransduction channels with diverse biophysical properties and distinct biological functions in *C. elegans* (Kang *et al*., 2010). The TRPC channels in *C. elegans* are coupled to the G_q-PLCβ pathway, and thus regulated by PIP_2, IP_3, and/or DAG. Based on sequence alignment, all three TRPCs contains a calmodulin (CaM) / IP_3R-binding (CIRB) motif in their C-terminus, a feature shared by all mammalian and *Drosophila* TRPC channels (Zhu, 2005).

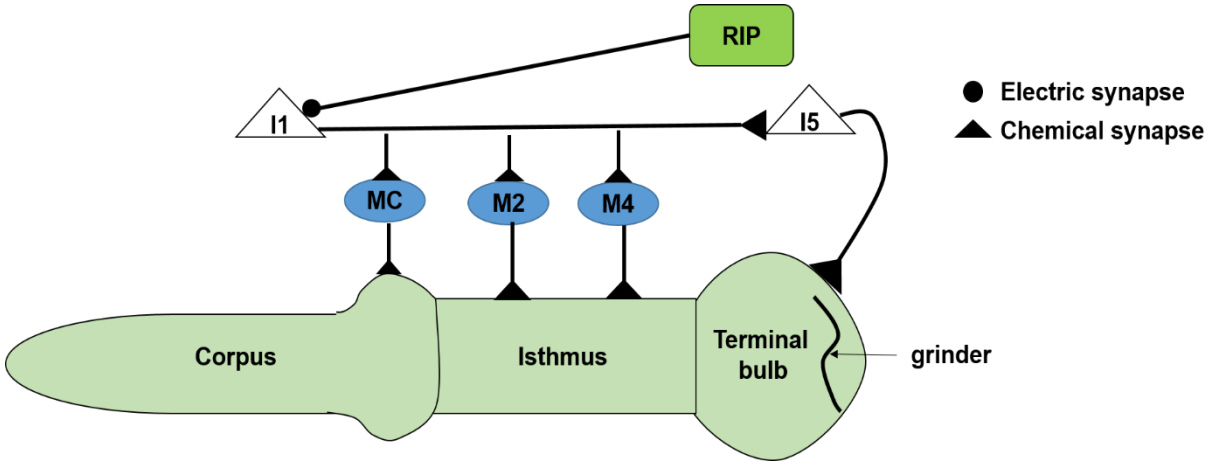


Figure 7.3: Schematic illustration of pharynx. The pharnyx consists of three regions: the corpus is closest to the mouth of the worm, the terminal bulb connects to the intestine, and the two are linked by a slender isthmus. The RIP neurons provide neural connectivity between pharyngeal and extra-pharyngeal nervous system. The pharyngeal neurons release neurotransmitters e.g acethylcholine (Ach), glutamate and 5-HT. The activity of the MC neurons entrains the pharyngeal muscle and overrides the myogenic rhythm to cause rapid neurogenic pumping. When MC activity or postsynaptic response to MC activity is decreased, the myogenic rhythm sets the pumping rate.

7.4 Lifespan assay

The toxicity assessment is a major hurdle in the compound discovery pipeline, which involves large scale animal testing. Therefore an alternative testing platform is highly desired to reduce the use of vertebrates, particularly at the crucial early stages of product development. *C. elegans* has been the first model organism in which the genetic basis of aging was recognized. They are useful for studying different aspects of aging because of their small size, rapid generation time (3 days at 20°C), ease of culturing on inexpensive laboratory media, and short adult lifespan (2 weeks at 25°C). Also, the worms offer unique advantages for aging research due to an invariant lineage, e.g., every adult worm has precisely 959 cells that make up its somatic tissues. These tissues can be easily examined because of their transparent bodies. Moreover, the toxicity ranking screens in *C. elegans* have repeatedly been shown to be as predictive of rat LD_{50} ranking and mouse LD_{50} ranking. Besides that, a similar mode of toxic action has also been noted between *C. elegans* and mammals (Hunt, 2017). The aging signaling pathways are conserved from yeast to worms and mammals, such as mice and human beings. These make a case for the inclusion of *C. elegans* assays in early safety testing and as one component in tiered or integrated toxicity testing strategies. However, nematodes alone cannot replace data from mammals for hazard evaluation. Methods to assess lethality in *C. elegans* range from inexpensive and straightforward for a non-worm lab to set up, to those requiring costly automated motility tracking or microfluidics equipment.

Aging is a fascinating phenomenon that is experienced by most species. Besides the deterioration of cells and tissues, aging is also influenced by genetic pathways. Aging is associated with an increased probability of death, but by itself, the phenomenon is difficult to define or calculate precisely. It covers a spectrum of changes that molecules, cells, organs, and organisms undergo over time, from relatively benign anatomical deterioration to severe functional impairment. Lifespan, on the other hand, is a measured trait signifying the time from birth until death. Although aging and lifespan have distinct connotations, they are interconnected, and the terms have often been used interchangeably (Amrit *et al*.,2014). However, lifespan is a single measurable parameter that defines the amount of time an organism is alive but does not give any indication for how an animal is aging. To date, the measurement of lifespan is the most popular method used to study aging in *C. elegans*.

Aged *C. elegans* display a functional deterioration of neurons and muscle integrity (body wall muscles and pharynx). Studies have revealed several molecular pathways that affect the worm's lifespan, such as the genes involved with the insulin/IGF-1 signaling pathway, caloric restriction, and mitochondrial function. Mitochondrial dysfunction constitutes one of the more distinctive characteristics of aging. In human muscle, the activity of the mitochondrial enzymes responsible for energy production has been shown to decline progressively with age (Marzetti *et al*.,2013). Similar phenomena have been observed in the nematode *C. elegans*. The body wall muscle mitochondria undergo progressive fragmentation with aging, together with a decrease in mitochondrial length and area, and an increase in mitochondrial circularity (Regmi *et al*.,2014). Past and present studies have studied compounds that can affect the worm's lifespan. Some examples include mianserin, an antidepressant target serotonergic signaling, and it tends to extend the lifespan of *C. elegans* by ~30% through dietary restriction mechanisms (Petrascheck *et al*.,2007). Resveratrol, a plant-derived polyphenol, had been shown to activate the catalytic activity of sirtuins, also extends *C. elegans* lifespan (Wood *et al*.,2004). Sirtuins have been shown to mediate some of the beneficial effects of caloric restriction and possess a protective role in brain aging and neurodegeneration (Kim *et al*.,2007). Besides that, alkaloid compound Harmane (2-methyl-β-carboline) has been shown to increase the lifespan of nematodes infected with a human pathogen, the Shiga toxin-producing *Escherichia coli* O157: H7 strain EDL933 and several other bacterial pathogens (Jakobsen *et al*.,2013).

The utilization of *C. elegans* to evaluate the potential toxicity of drugs is gaining attention in experiments. For instance, exposure of heavy metals like chromium, lead, mercury, silver, zinc, and magnesium affected worm growth at all assayed concentrations and induced a severe stress response in the intestine (Shen *et al*.,2009). In another study, *C. elegans* had been used to evaluate the toxicity of synthetic compound 4-organylsulfenyl-7-chloroquinolines. The toxicology screening had enabled to determine the lethal dose 50% (LD_{50}) of the compound and choose a non-lethal concentration which has protective action against the induced damage inflicted by paraquat (Salguiero *et al*., 2014). Recently, *C. elegans* has been used to evaluate the effects of different types of nanomaterials, demonstrating its importance to nanotoxicology (Meyer *et al*., 2010; Ahn *et al*.,2014; Yang *et al*.,2014). To the best of our knowledge, no studies about the toxicity of phenylakylamine have been reported using this *in-vivo* model. Therefore, the results obtained with this model are crucial to predict the FK70 effect in complex animal models.

7.5 Study aim and objectives

In the previous chapters (5 and 6), FK70 appeared to inhibit calcium mobilization in the aorta of SD and SHR. The main aim of this study was to investigate the effects of FK70 in another animal model. The versatile nematode, *C. elegans*, was selected as a model for this study. The *C. elegans*, with its ease of manipulation and storage, short life cycle, and with lower maintenance costs, has long been recognized as one of the premier model organisms for biological research. The *in-vivo* study using this model allows the assessment of both calcium inhibiting property and the toxicity effect of FK70. The choice of this animal model is to replace the need for euthanizing living laboratory animals (referring to Sprague-Dawley rats in this study) in meeting the principle of 'Three Rs' (European Pharmaceutical Industry Update, 2015) in addition to explore the toxicity effect of FK70.

The objectives of this study are to investigate;

i) the effect of FK70 on pharyngeal pumping of *C. elegans*

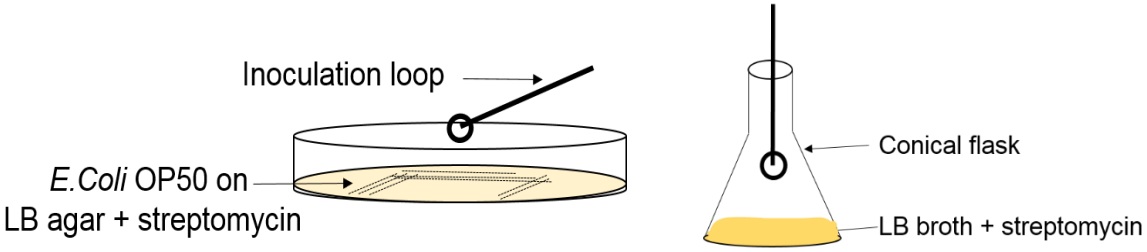
ii) the effect of FK70 on lifespan of *C. elegans.*

7.6 Methods and materials

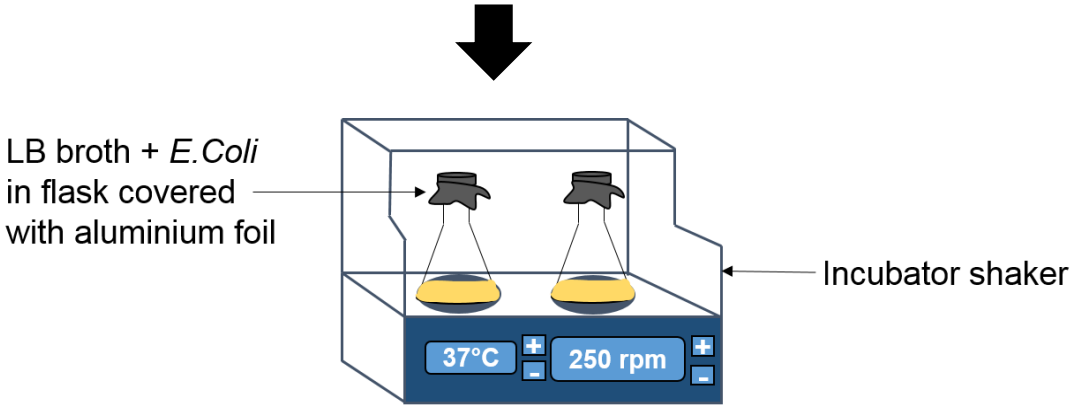
7.6.1 Bacterial and nematode strains and growth conditions

E.coli strain OP50 was cultured on Luria Bertani (LB) (Friendemann Schimdt, USA) supplemented with streptomycin (100µg/ml). All bacterial cultures were grown aerobically at 37°C overnight. The *C. elegans* strain used in this study was *glp-4(bn2).* The worms were maintained on nematode growth medium (NGM) agar plates seeded with a lawn of the standard laboratory food source, *E. coli* OP50. The worm strain were obtained from the Tan Laboratory, Stanford University, USA. The *glp-4(bn2)* worms were grown at 16°C until they were used for assay which were then kept at 25°C. The *glp-4* worms have temperature sensitive defect in germ-line proliferation. The defect can be reversed by shifting worms from restrictive (25°C) to permissive (16°C). This strain was chosen to avoid production of progenies during assay.

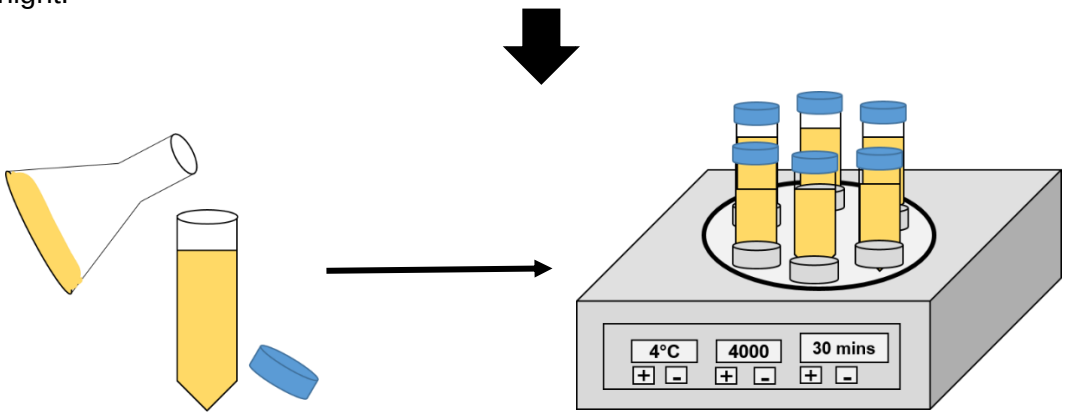
The food source were prepared as represented in **Figure 7.4.** All experiments were carried out at 25°C. Heat killed bacteria was prepared by heating the concentrated culture at 80°C for 30 minutes before adding the bacteria into the solid medium. The heat killed bacteria were used for assay plates.



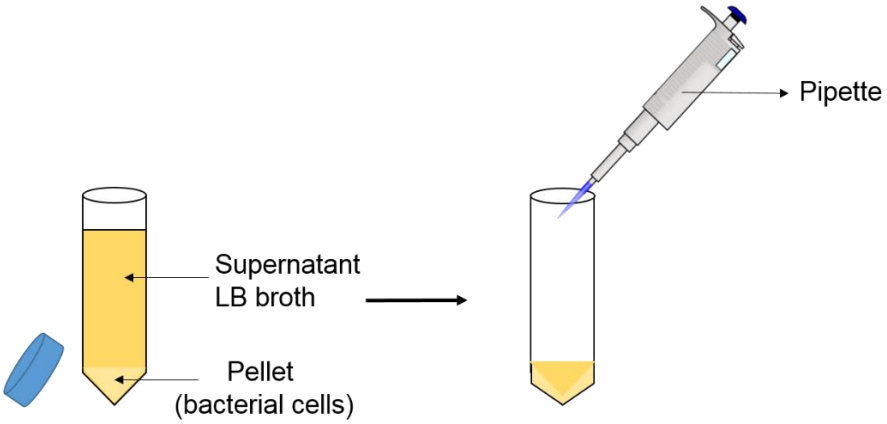
The *E.coli* were grown on LB agar plates using streak plate method. A single colony of bacteria was inoculated into LB broth with streptomycin in a conical flask. In order to ensure good aeration for the bacterial culture, the broth were filled not exceeding ¼ of the size of the flask.



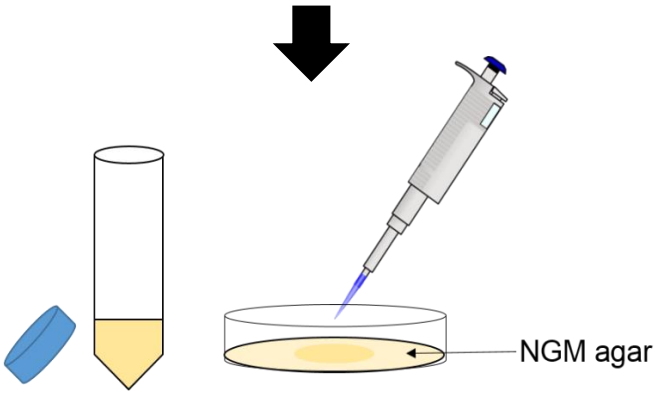
After inoculation, the flasks containing *E.coli* were incubated at 37°C with shaking for overnight.



The next morning, the *E.coli* culture were transferred into falcon tubes and centrifuged at 4000 rpm for 30 minutes at 4°C to precipitate the bacterial cells.



After centrifugation, the supernatant were discarded. Then, the bacterial pellets were made concentrated to 25 fold by adding 2ml LB broth. The mixture were suspended using a vortex mixer until the bacterial cells are fully suspended in the LB broth.



The concentrated *E.coli* were then seeded onto NGM agar plates. The plates were then dried in the laminar flow and kept in the -4°C refrigerator for future use. The amount of bacteria seeded onto the plates depended on the size of the agar plates used. For 6cm plates, about 300-400 µL of bacteria were seeded while for 3.5cm plates 50 µL of bacteria were seeded.

Figure 7.4: Schematic illustration of steps involved in the preparation of *E.coli* OP50 for worms

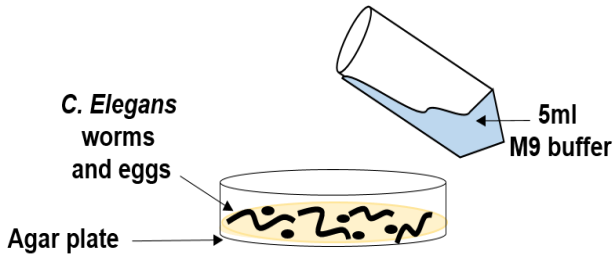
7.6.2 NGM agar preparation

NaCl (3g), bacterial agar (20g), bacterial peptone (2.5g), cholesterol (1ml) were dissolved in 1 litre of distilled water and autoclaved. After autoclaving, the sterile solution of 1M $MgSO_4$ (1 ml), 1M $CaCl_2$ (1 ml), and 1M KH_2PO_4 (25 ml) were added to the mixture.

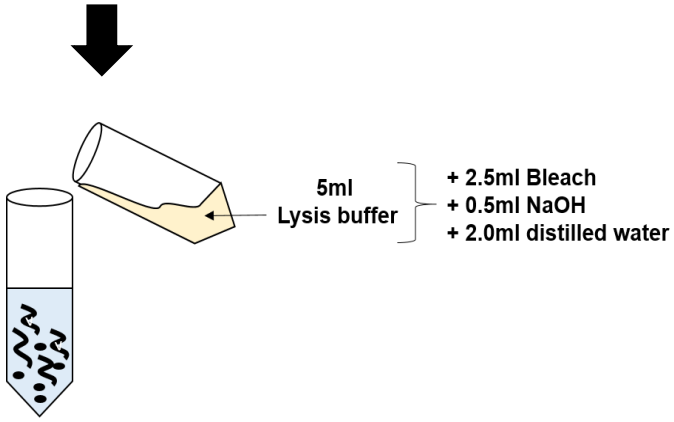
The 1M KH_2PO_4 (pH 6) was prepared by dissolving 136.1g K_2HPO_4 in about 800 ml dH_2O, then adjusted to pH 6.0 with solid KOH (approx 15 g) before bringing up to volume.
Finally, streptomycin (100mg/ml) was added to the mixture.

7.6.3 Worm M9 Buffer and egg preparation

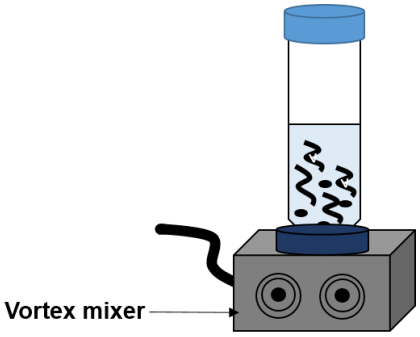
The M9 buffer was prepared by dissolving KH_2PO_4 (3g), Na_2HPO_4 (6g), NaCl (5g) in 1 litre distilled water. The solution was then sterilised by autoclaving. After the solution cools down, the autoclaved 1 M $MgSO_4$ (1ml) was added. Age-synchronization of the worms was conducted by the hypochlorite (bleach) treatment of gravid worms (see **Figure 7.5**).



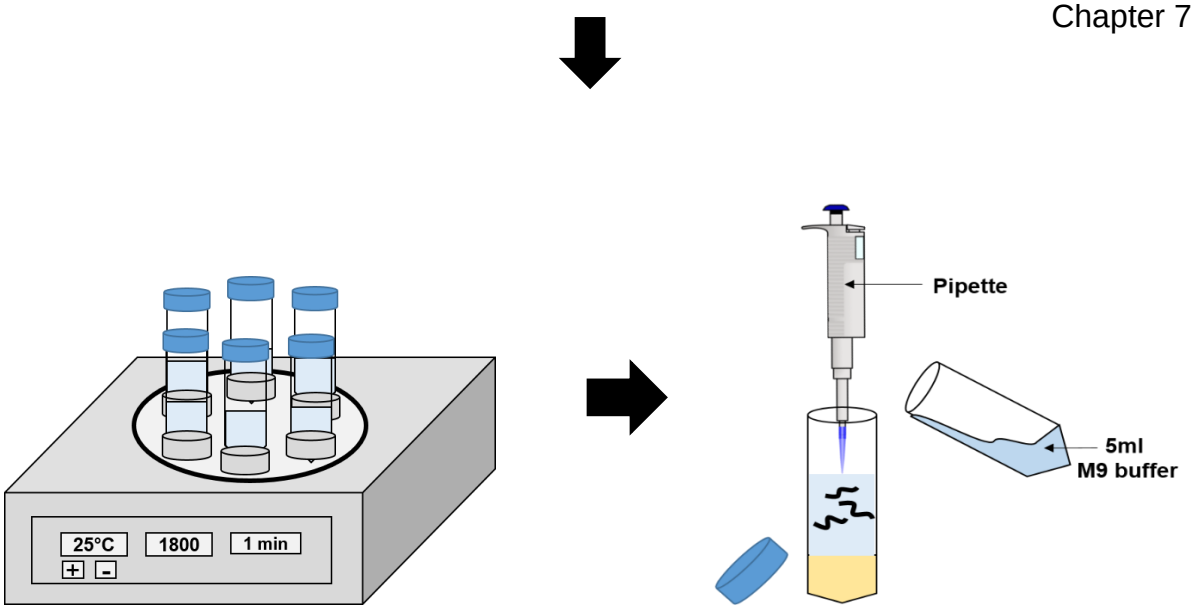
Plates with mainly gravid worms with eggs in the uterus and a lot of laid eggs were chosen for egg preparation. 5ml M9 buffer solution was used to wash the plates containing eggs. The washed mixture was then transferred into falcon tubes.



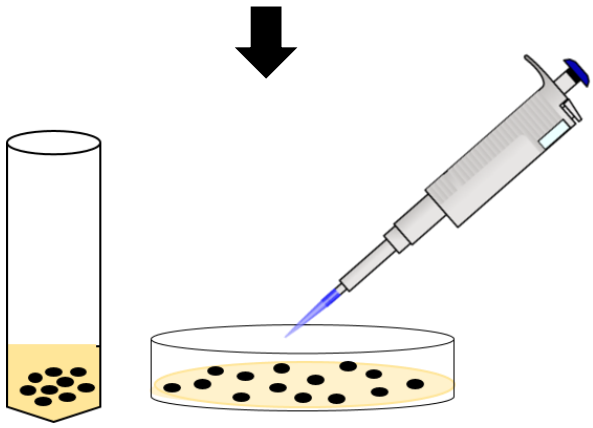
5ml lysis buffer containing bleach, sodium hydroxide (NaOH), and distilled water were added into the mixture containing eggs and gravid worms.



The mixture was then vortexed thoroughly for 5 minutes to break the cuticles of gravid worms to release the eggs. The eggs are covered by chitin shell and are resistant to bleach.



The mixtures were then centrifuged to pellet the eggs. The tubes were span at 1800 rpm for 1 minute at 25°C. The supernatant were discarded and 5ml of M9 buffer was re-added. Then, the tubes were spun again at 1800 rpm for 1 minute at 25°C.



The supernatant were discarded and the remaining eggs in the pellet were pipetted onto fresh NGM plates. The eggs were seeded around the *E. Coli* OP50 lawn. The presence of eggs and the correct placement of eggs were checked under a microscope. The egg droplets were allowed to dry and kept at either 16°C or 25°C.

Figure 7.5: Schematic illustration of steps involved in the egg preparation process

7.6.4 Preparation of compounds and drugs

FK70, Nemadipine A (Sigma, US), nemadipine B (Sigma, UK), and nifedipine (Nacalei Tesque, Japan) were used. All drugs were dissolved in dimethyl sulfoxide (DMSO) before subsequent dilutions in distilled water. These drugs were first dissolved in 100% DMSO to achieve 0.1 M stock concentration, followed by ten-fold dilutions with descending DMSO content from 75 % (v/v), 50 % (v/v), 25 % (v/v) to pure distilled water. Then the compounds were filtered through a 0.2 µM membrane filter (Sartorius Stedim, Germany) followed by storage at -20°C in a desiccated storage container.

7.6.5 Feeding assay

Worms were prepared and exposed to *E.coli* in the presence of FK70 (10 µM, 100 µM and 1 mM). Heat-killed *E.coli* was used to avoid any possible effect of compound/dissolving solvent on the live bacterial cells which may in turn affect the pumping rate of the worms when they feed on these bacteria. The heat killed bacteria *E. coli* contain only dead bacterial cells that will not contribute any unnecessary variables to the experiment.

The pharyngeal pumping of 5 individual worms was counted for 5 seconds under a microscope (Olympus X21FS1). A pharyngeal pump was strictly defined as a complete backward movement of the terminal bulb grinder (Keane and Avery, 2003). The pharyngeal pumping rates were counted for each plate at 4^{th}, 24^{th}, 36^{th} and 72^{nd} hour after transferring the worms into assay plates under a 10X magnification compound microscope.

The average number of pharyngeal pumping was calculated and used for statistical analysis. The experiments were ran in 3 replicates. The same procedure was repeated with DMSO (10 %), nemadipine A (10 µM and 20 µM) and nemadipine B (10 µM and 20 µM) which served as vehicle control and positive control, respectively.

7.6.6 Lifespan assay

The *C. elegans* lifespan was conducted in solid agar medium. FK70 (100µM and 1mM) was added to the heat killed bacteria before adding to the solid medium. Age synchronised young adult nematodes were transferred manually to the plates and incubated in 25°C. The worms were scored every other day as alive or dead by gentle prodding with a platinum wire and gentle tapping of the plate. The experiments were conducted using heat-killed bacteria. The experiments were run in 3 replicates. The same procedure was repeated with DMSO (7.5 %), nifedipine (10µM and 100µM) which served as vehicle control and positive control, respectively.

7.6.7 Data analysis

For all experiments, 3 replicates for each concentration were carried out (with a total of 90 worms for lifespan assay and 15 worms for feeding assay) for statistical purposes. Nematodes were classified as dead when they failed to respond to touch and no pharyngeal pumping was observed. Worms that died due to bursting vulva and crawled out of the plates were censored from further analysis. Differences in survival and lifespan of treatment group and control group were assessed by Log-Rank (Mantel-Cox) significance test using StatView version 5.0.1 (SAS Institute, Inc.).

For feeding assay, data were analysed and compared by using the Two-way ANOVA. The data were analysed and graphs were drawn using PRISM version 7.0 (GraphPad software). Two-way ANOVA was used to compare the statistical differences from two experimental groups: one from a control condition while another from a treatment condition [PRISM Version 7 (Graph Pad Software)]. For ANOVA the data were subjected to post-hoc analysis (Bonferonni). Results were considered statistically significant if $p < 0.05$.

7.7 Results

7.7.1 FK70 reduced pharyngeal pumping of the worms

In order to investigate the effect of FK70 on *C. elegans*, the pharyngeal pumping rates were observed. Nematodes treated with DMSO 10 % served as control group in this experiment. DMSO did not seem to affect the pharyngeal pumping with pumping rate ranging between 19-23 pumps in 5 seconds. No significant difference in pharyngeal pumping was observed between DMSO and blank control treated nematodes (p = >0.99) across time (4-72 hours) (see **Figure 7.6A**).

In order to confirm the involvement of calcium ion on pharyngeal pumping, nemadipine A and nemadipine B (known calcium channel blockers) were employed to validate the investigation. The positive response of the calcium channel blockers were compared with the effect of FK70. Nematodes treated with FK70 (10 µM), (100 µM) and (1 mM) showed a time-dependent decline in pumping rates. The lowest concentration of FK70 showed slower onset of action as compared to the highest concentration where significant decline in pharyngeal were observed at the 4th hour of FK70 exposure (p = <0.0001) (see **Table 7.1** and **Figure 7.6B**).

As expected, Nemadipine A and B treatment reduced the pharyngeal pumping of the nematodes considerably (see **Table 7.2 and Figure 7.6C**). However, nemadipine B (20 µM) seemed to be more potent than nemadipine A (20 µM), as nemadipine B reduced the pumping rate significantly earlier which was after 4th hour of exposure (see **Table 7.3 and Figure 7.6D**).

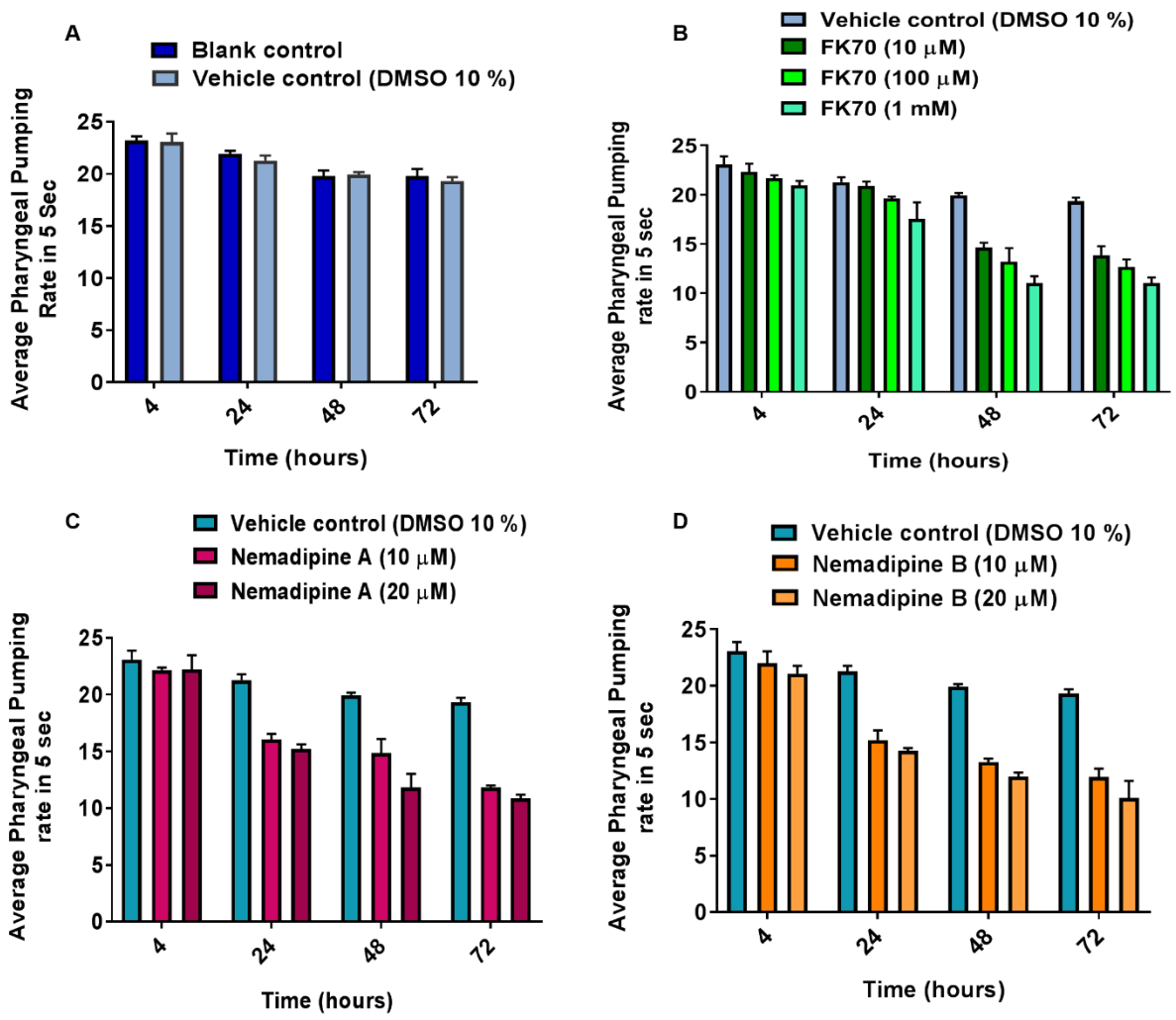


Figure 7.6: Effects of **(A)** blank control and vehicle control (DMSO 10%) **(B)** FK70 (10 µM, 100 µM, and 1 mM) **(C)** nemadipine A (10 µM and 20 µM) **(D)** nemadipine B (10 µM and 20 µM) on the average pharyngeal pumping rate in 5 seconds. The bars correspond to mean ± SD of the pharyngeal contraction in 5 seconds of three replicates.

Table 7.1: Summary of effect of vehicle control (DMSO) and different concentrations of FK70 (10 µM, 100 µM and 1 mM) on the pharyngeal pumping of *C. elegans* (*glp-4*) in 5 seconds at 4, 24, 48 and 72 hours. The values indicate Bonferonni adjusted *p* values when compared to vehicle control. (*A significant difference between control and FK70 treated group).

Treatment	Time (hour)			
	4	24	48	72
Control				
FK70 (10 µM)	0.76	>0.99	<0.0001****	<0.0001****
FK70 (100 µM)	0.1	0.04*	<0.0001****	<0.0001****
FK70 (1 mM)	0.005**	<0.0001****	<0.0001****	<0.0001****

Table 7.2: Summary of effect of vehicle control (DMSO) and different concentrations of nemadipine A (10 µM and 20 µM) on the pharyngeal pumping of *C. elegans* (*glp-4*) in 5 seconds at 4, 24, 48 and 72 hours. The values indicate Bonferonni adjusted *p* values when compared to vehicle control. (*A significant difference between control and nemadipine A treated group).

Treatment	Time (hour)			
	4	24	48	72
Control				
Nemadipine A (10 µM)	0.25	<0.0001****	<0.0001****	<0.0001****
Nemadipine A (20 µM)	0.3	<0.0001****	<0.0001****	<0.0001****

Table 7.3: Summary of effect of vehicle control (DMSO) and different concentrations of nemadipine B (10 µM and 20 µM) on the pharyngeal pumping of *C. elegans* (*glp-4*) in 5 seconds at 4, 24, 48 and 72 hours. The values indicate Bonferonni adjusted *p* values when compared to vehicle control. (*A significant difference between control and nemadipine B treated group).

Treatment	Time (hour)			
	4	24	48	72
Control				
Nemadipine B (10 µM)	0.17	<0.0001****	<0.0001****	<0.0001****
Nemadipine B (20 µM)	0.005**	<0.0001****	<0.0001****	<0.0001****

7.7.2 FK70 did not affect the basal lifespan of the worms

Next, in order to investigate the effect of FK70 on basal lifespan of the worms, different concentrations of FK70 (100 µM and 1 mM) were used. Worms treated with DMSO 7.5 % served as vehicle control. For this experiment, nifedipine (a known calcium channel blocker) used for hypertension in humans were used. The treated worms (FK70 and nifedipine) had lower mean lifespan than the untreated worms. Treatment with highest concentration of FK70 seemed to affect the lifespan of the worms (see **Figure 7.7**) with a shorter time to death mean (TD_{mean}) than control (see **Table 7.4**). Nematodes treated with nifedipine had reduced the TD_{mean} considerably ($p < 0.05$).

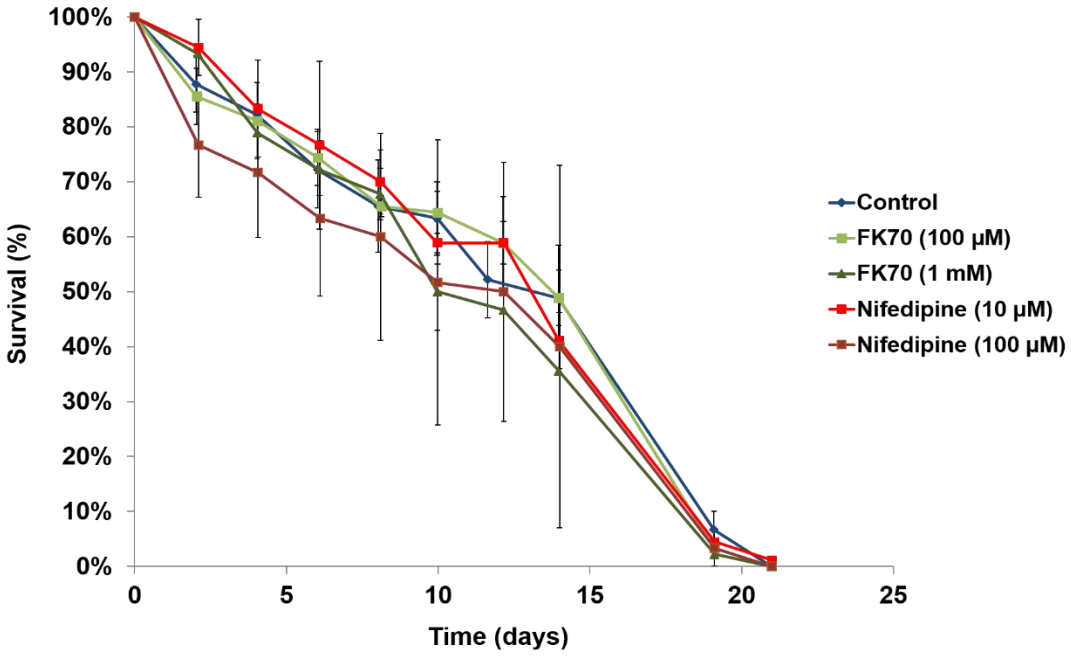


Figure 7.7: Effects of control, different concentrations of FK70 and nifedipine on basal lifespan of the worms. FK70 (1 mM) and nifedipine (10 µM and 100 µM) reduced the lifespan of the worms. The graph represents the mean ± SD of 3 replicates (150 nematodes/ replicate) from a representative of three independent assays.

Table 7.4: Summary of Kaplan-Meier survival statistics for vehicle control, FK70 and nifedipine and Log-Rank (Mantel-Cox) test *p* values for FK70 and nifedipine treated vs control treated nematodes.

Treatment	**TD_{mean} (Mean time to death) (Mean ± SD) (hours)**	***P* value (Log-rank Mantel-cox test)**
Vehicle control (DMSO 7.5 %)	316.7 ± 16.9	
FK70 (100 µM)	316.3 ± 17.1	0.47
FK70 (1 mM)	299.2 ± 18.7	0.04
Nifedipine (10 µM)	310.9 ± 15.7	0.025
Nifedipine (100 µM)	278.7 ± 22.3	0.0004

7.8 Discussion

FK70 appeared to influence calcium-dependent responses in isolated rat aorta of SD and SHR (Chapters 5 and 6). Taking advantage of the tractability and simplicity of *C. elegans* as an animal model, the calcium blocking and toxicity effect of FK70 was explored in the whole organism.

The worm's pharynx has been compared to the human heart due to its similar electrical properties, and similar homolog genes which control the development. Understanding the logic of modulatory neural circuits requires an understanding at the cellular level, which can be challenging to achieve in the vertebrates. Therefore *C. elegans* is well suited to provide insights into the calcium mechanisms involved. Ca^{2+} generate versatile intracellular signals that determine a large variety of functions in virtually every cell type in biological organisms (Berridge *et al*., 2000), including the control of heart muscle cell contraction (Dulhunty, 2006) as well as the regulation of vital aspects of the entire cell cycle, from cell proliferation to cell death. Previous studies have reported calcium activity and calcium signaling in the pharyngeal muscle (Kerr *et al*., 2000; Shimozono *et al*., 2004), vulval muscles (Shyn *et al*., 2003), and body wall muscles.

Treatment with DMSO 10 % did not seem to affect the pharyngeal pumping in *glp-4* mutant worms. This shows that DMSO did not impose any effect on the pharyngeal pumping of the worms. FK70 at different concentrations reduced the pharyngeal pumping at a rate comparable to nemadipine A and nemadipine B. The pharynx is known as the neuromuscular feeding organ, it generates a myogenic rhythm in the pharyngeal muscle in response to stimulation of pharyngeal nervous system (Trojanowski *et al*.,2016). The activity of the MC neurons entrains the pharyngeal muscle and overrides the myogenic rhythm to cause rapid neurogenic pumping. The muscle cells respond to a variety of neurotransmitters and rely on T- and L-type Ca^{2+} channels for its action potential. Besides that, the *C. elegans* does not appear to have voltage-gated sodium channels, suggesting that any active propagation of current in worms occurs via calcium spikes. The L-type Ca^{2+} channels are also expressed in the neurons (Lee *et al*., 1997; Fawcett *et al*.,2006). Based on these facts, it can be hypothesized that FK70 might have disrupted Ca^{2+} activity at either pharyngeal muscle or at the neuromuscular junction to reduce the pharyngeal pumping rate. The influence of FK70 on Ca^{2+} mediated responses in *C.elegans* is consistent and relatable to our previous findings on FK70 in rat tissues. Moreover, the pumping rate in worms treated with FK70 is similar to those worms treated with dihydropyridine (DHP) analogs nemadipine A and B, known calcium channel blockers. Nemadipine A and B are bioactive compounds that resemble a class of

widely prescribed anti-hypertension drugs called the 1,4-dihydropyridines (DHPs) that antagonize the α_1-subunit of L-type Ca^{2+} channels. They target the *egl-19*, gene that encodes the only L-type Ca^{2+} channel α_1-subunit in the *C. elegans* genome (Kwok *et al*.,2006). Nemadipine-A can also antagonize vertebrate L-type Ca^{2+} channels, demonstrating that worms and vertebrates share the orthologous protein target. The Ca_{v1} channels typically exist in three states: a resting closed state, an open state that is triggered by membrane depolarization, followed by a non-conducting inactivated state that is triggered by the influx of Ca^{2+}, and a rapid change in voltage. The DHP analogs alter the conformation of the channel by locking the Ca^{2+} in the pore, thereby blocking the channel conductance (Kwok *et al.,* 2008). This results further suggest the influence of FK70 on calcium-mediated responses in the pharynx.

It is also noteworthy to mention the role of transient receptor potentials channels (TRP) in *C. elegans*. The *C. elegans* TRPC subfamily includes three members: *trp-1*, *trp-2* and *trp-3* (Xiao and Xu, 2012). Many *C. elegans* TRP channels are well correlated with their vertebrate homologues in both physiological functions and regulatory mechanisms. For instance, when the *trp* genes are expressed in HEK293 cells (in-vitro), the *trp-2* and *trp-3* genes promoted receptor-operated Ca^{2+} entry that depends on the G_q-PLCβ pathway (Feng *et al.,*2006). Members of TRPC (*trp-1* and *trp-2*) are expressed in motor neurons, interneurons, a few sensory neurons, and muscles (Feng *et al*., 2006). Besides that, *cup-5*, the sole TRPML channel in *C. elegans* are expressed in many cell types, including sensory neurons, pharynx, reproductive system, and muscle (Fares and Greenwald, 2001). Many *C. elegans* TRP channels are enriched in the nervous system; however, the expression of TRP channels in pharyngeal muscle has not been widely studied. Therefore, it is hard to concretely propose that FK70 might have possibly acted on these channels to modulate Ca^{2+}, but it's worth considering as a possible mechanism of FK70. Besides that, previous studies investigating amines e.g., octopamine and tyramine, had shown to reduce the pumping rate in *C. elegans* (Packham *et al.,* 2010; Li *et al*.,2012). The amines were acting as neurohormones to disrupt the physiological function of the pharynx. Thus, prompting speculation that FK70 being an amine could have exerted a similar effect as amines in reducing pharyngeal pumping. However, the exact mechanism is yet to be found.

The use of *C. elegans* has added value not only in terms of animal replacement but also in the ability to predict the toxicity effect of a compound that would facilitate the rational design of a compound. *C. elegans* whole animal model allows for early and direct assessment of *in-vivo* drug efficacy and provides predictive power of a chemical's toxicity towards humans. The predictive toxicology would be useful in the elimination of high-risk compounds early in the product development and enables to determine the non-lethal dose. The highest concentration of FK70 and nifedipine reduced the basal lifespan of *C. elegans* when compared to control, suggesting the toxic effect of the compounds on adult worms. This shows that FK70 at a higher concentration is slightly toxic, and concentration re-adjustment is necessary for determining the optimal concentration to maximize the pharmacological outcomes (efficacy) while minimizing adverse side effects (toxicity). Nifedipine, on the other hand, reduced basal lifespan considerably, although concentrations of nifedipine used in this protocol are lower than that of FK70. No studies were carried out using nifedipine in *C.elegans*; therefore, the exact mechanism involved in reducing the basal lifespan of *C. elegans* is unknown. The general notion of calcium inhibition in cells prevents or delays aging; however, in this experiment, the worms treated with nifedipine deceased earlier than the control group. One possible interpretation from this finding is that nifedipine being a potent calcium channel blocker, would've perturbed the calcium homeostasis in this particular mutant. For instance, *C. elegans* intestinal epithelial cells generate rhythmic inositol 1,4,5-trisphosphate (IP_3)–dependent Ca^{2+} oscillations that control posterior body wall muscle contraction. The store-independent outwardly rectifying Ca^{2+} (ORCa) channels comprise a major Ca^{2+} entry pathway in intestinal epithelial cells, and they function to regulate IP_3 receptor activity and refill ER Ca^{2+} stores (Xing *et al*.,2008). Thus, dysregulation of Ca^{2+} by nifedipine and the higher concentration of FK70 might have limited the *glp-4* worm life span. It is also important to take note that shortened life span could also be associated with the worms being sick upon exposure to foreign substances, rather than aging more quickly.

Besides that, it is also noteworthy to consider the concentration of FK70 and nifedipine used in this study. Perhaps the concentration of FK70 and nifedipine used were too high for *C. elegans*. The lethal dose for nifedipine in rats is LD_{50}=1022 mg/kg (orally in rats) (DrugBank database, April 2019*).* Considering the fact that the toxicity ranking screens in *C. elegans* is as predictive of rat LD50 ranking and possess a similar mode of toxic action as mammals (Hunt, 2017), the concentration of nifedipine (100 µM) used to incubate in this experiment is an overdose and thereby explaining, the reduction of worm lifespan. However, the exact LD_{50} ranking for nifedipine in *C.elegans* is unknown and yet to be found.

Taken together, FK70 reduced pharyngeal pumping and did not affect the basal lifespan of the worms. The reduction of pharyngeal pumping suggests the calcium blocking effect of FK70. In terms of toxicity, FK70 at a lower concentration did not seem to affect the basal lifespan, but at a higher concentration, it reduced the lifespan of the worms. FK70 at 10 µM seems to reduce pharyngeal pumping efficiently and also have a better safety profile. Although it was not envisaged that *C. elegans* would completely replace vertebrates in toxicity testing, it served as a preliminary and straightforward model in identifying drugs (FK70 and nifedipine) that caused overt toxicity within a short period. It was useful in identifying and eliminating concentrations that are lethal before drug development. That said, although the presence of toxicity in one species may sometimes add evidential weight for risk of toxicity in another, the likelihood is extremely inconsistent, varying substantially for different classes of drugs. Rigorous pre-clinical evaluation should be conducted in at least two animal species in order to determine the toxicity profile and establish the general safety of FK70. Therefore, the toxicity test done on *C. elegans* cannot be considered robust for FK70 and deserves more investigation in the future to facilitate FK70 development without comprising its useful properties.

Chapter 8: General discussion

8.1 Introduction

Hypertension is estimated to cause 7.5 million deaths globally, about 12.8% of the total of all deaths (World Health Organisation, 2018). Either individually or combined with other metabolic diseases, high blood pressure increases the risk of cardiovascular diseases. Albeit the panoply of antihypertensive drugs, the problem of insufficient management of this condition persists. Around 140-160 million people across the world suffer from resistant hypertension (Achelrod *et al*.,2015). The current situation necessitates a new effective pharmacological treatment to improve the long-term clinical management of this disorder.

Malaysia is well endowed with a rich selection of floral species where at least a quarter of tree flora are not found elsewhere in the world. Moreover, Malaysia is also home to many unique floral species. The exploration into the botanical sources has led to a discovery of an alkaloidal composition from the leaves of a previously un-investigated species, e.g., *Ficus schwarzii*. The leaves extract of *Ficus schwarzii* yielded two novel tri-nor-sesquilignan alkaloids, schwarzinicines A and B. According to a recent study, Schwarzinicine A (Sch A) has the potential to be developed into a new antihypertensive therapy (Loong *et al*., 2016 unpublished data). N-phenethyl-1-phenyl-pentan-3-amine, (FK70) is a novel compound synthesised based on Sch A. In evaluating ‘hit' compounds, high throughput screening or reliable screening assays are essential to further work towards identifying clinically useful candidates. The investigation of mechanistic underpinnings of the FK70 was conducted using *in-vitro* animal tests (techniques of organ bath pharmacology) followed by *in-vivo* study (feeding and toxicity assay).

8.2 Functional bioassay directed pharmacological evaluation of FK70

The main aim of this study is to find the pharmacological action of FK70. This is the first study exploring the effects of FK70 on different bioassays. By using the Lipinski rule of five predictions, FK70 exhibited a similar degree of vasorelaxant effect as Sch A and was shown to have better physicochemical properties or drug-like properties than Sch A in rat aorta. The present finding showed that FK70 behaves like Sch A in the aorta and is a reliable alternative compound for further characterisations.

This study had effectively optimised organ bath assay in testing the effect of FK70 on i) rat isolated smooth muscle tissues (aorta, trachea, and bladder), and smooth muscle from different animal species ii) porcine coronary artery iii) guinea-pig isolated ileum. The study served as a prelude for the following studies in evaluating the vascular effects and mechanism of action of FK70.

The structural similarity of FK70 was ascribed to known vascular drugs, dobutamine and (β-adrenoceptor agonist) and verapamil, a calcium channel blocker. The structure-activity relationship (SAR) of dobutamine and verapamil were referred to as a guide to determine the putative bioactivity of FK70. The efficacy of FK70 in rat aorta was comparable to known verapamil, a calcium channel blocker. Verapamil, a member of the phenylalkylamine subclass of calcium-channel blockers, was the first calcium-channel blocker to be discovered and is the only member of this subclass to be widely used in the treatment of hypertension (Sica *et al.,*2007; Hughes 2018). The blood pressure-lowering of verapamil is attributable to a reduction in systemic vascular resistance (Lund-Johansen *et al.,*1987). Verapamil elicits relaxation effect in the aorta by inhibiting calcium entry via the voltage-gated calcium channels. The vasorelaxant profile of FK70 resembled more that of verapamil, which posed a question of whether FK70 is involved in calcium inhibitory action.

In rat aorta, it has been suggested that the initial transient contraction induced by phenylephrine (PE) is the result of the release of intracellular calcium, while the sustained contraction induced by calcium (Ca^{2+}) influx (Lee *at al.,*2001). The PE-induced contraction in rat aorta is caused by α-adrenoceptors, mainly via activation of α_1-adrenoceptors (Iwanaga *et al*.,1998). Moreover, the aortic smooth muscle cells also appear to contain more α-adrenoceptors in the smooth muscle of blood vessels (Barnes and Basbaum, 1983). Therefore, the relaxation response of FK70 in aorta pre-contracted with PE suggested the involvement of α-adrenoceptors. The higher efficacy of FK70 in rat aortic smooth muscle prompted us to conduct further experiments on other types of vascular smooth muscle tissue (in this case, the porcine coronary artery). The anatomic and structural resemblance of swine vascularisation renders the PCA as a possible source for human vascularisation, which makes it an excellent choice for future translation studies onto human vascular tissues. Both coronary artery and aorta belong to the large arteries and share similar structure and functions. However, the porcine coronary artery smooth muscle is β-adrenoceptors sdominant, and activation results in an increase in cAMP and reduction of cytosolic Ca^{2+} (Shogakiuchi *et al*.,1991). This recommends the evaluation of the effect of FK70 on both α and β-adrenoceptors. Besides the adrenoceptors, the relaxation effect in both vascular smooth muscle tested was also attributable to endothelium factors.

In addition to aortic tissues, FK70 also relaxed airway smooth muscle tissue (trachea) and bladder however FK70 reduced carbachol-induced contraction more rapidly in the trachea than in the bladder. The discrepancies in the results could be due to tissue types, and receptors expressed in trachea and bladder are different, and selective effect of FK70 on specific receptors. Besides that, FK70 at a higher concentration managed to reduce spontaneous contraction in the bladder. This phenomenon is related to calcium oscillations in the bladder. Based on the data from different types of smooth muscle involved in this study, FK70 managed to relax the pre-contracted and electrically evoked smooth muscle tissues. The common mechanism that seemed to be involved in the contractile responses of all the tested tissues was the mobilisation of extracellular and intracellular Ca^{2+}. Therefore this prompted further investigations to the role of Ca^{2+} in FK70-induced relaxation.

8.3 Pharmacological evaluation of FK70 in rat aorta

The pharmacological investigation of FK70 had revealed that FK70 exhibited relaxation response in vascular smooth muscle tissues and all the other tissues tested. These exciting results prompted us to investigate the mechanism of action of FK70. The possible sites of action of drug studied in this study were based on the following categories (1) receptors (α- and β-adrenoceptors) (2) involvement of endothelium and endothelium-derived factors (3) potassium channels (4) second messengers (e.g. cyclic AMP and cyclic GMP) (5) enzyme that terminate the responses (e.g. phosphodiesterases). Further investigations from this study confirmed that FK70-induced relaxation was not mediated by either α- and β-adrenoceptors, endothelium-derived relaxing factors, potassium channels, or via cAMP / PDEs pathways. FK70's relaxant response have ruled out the above-proposed mechanisms.

To address the question, we evaluated whether calcium is involved in FK70 mediated response. As expected, FK70 had shown to affect calcium entry in the calcium-free Krebs protocol. Furthermore, FK70 also managed to reduce contractions induced by the pre-contractile agents, PE, KCl and U46619. KCl causes contraction via depolarisation of smooth muscle cells through activation of voltage-operated calcium channels (VOCCs). PE, on the other hand, causes contraction via α_1-adrenoceptors, which is coupled to the G_q-protein. Because PE, U46619, KCl-induced contraction involves calcium modulation, it seemed appropriate to propose a hypothesis that FK70 may impose an effect on calcium modulation as these contractile agents play a significant role in calcium signalling pathways in the aorta.

8.4 Role of calcium in contractile agents-induced contraction in rat aorta

To further explore the calcium-dependent mechanism of FK70, we thought it is crucial to first understand the regulation of agonist-induced Ca^{2+} oscillations in vascular smooth muscle. To explore the role of extracellular and intracellular Ca^{2+} on contractile agent-induced contraction, we used phenylephrine (PE), KCl, and 5-hydroxytryptamine (5-HT). Both PE and 5-HT are G_q-protein- coupled receptor agonists, while KCl causes contraction through the activation VOCC by depolarising the cells. This particular study aimed to investigate the role of extracellular and stored Ca^{2+} in contractile agents-induced contraction in rat aorta.

KCl causes contraction by stimulating an enhanced uptake of calcium through voltage-dependent Ca^{2+} channels which leads to an increase in the cytoplasmic Ca^{2+} concentration (Ratz *et al.* 2005). KCl also activates additional Ca^{2+} entry pathways via TRP channels to permit strong force-maintenance in tonic vascular smooth muscle (Ratz *et al.,* 2006). PE and 5-HT, on the other hand, are selective α_1-adrenoceptor agonist and serotonin receptor agonist, respectively. Both α_1-adrenoceptor and serotonin receptors are coupled to the G_q-protein. In the most common signalling pathway, activation of a receptor coupled to G_q-protein results in the activation of phospholipase C (PLC), which leads to the generation of inositol triphosphate (IP_3) and diacylglycerol (DAG), hence releasing Ca^{2+} from intracellular and extracellular milieu, respectively (Abdel-Latif, 2001). The G_q signalling can also activate Rho/Rho-kinase (Rho/ROCK) signalling pathway via Rho-guanine nucleotide exchange factors (Rho-GEF) (Kamato *et al.*, 2015).

The KCl and 5-HT displayed a slower onset of action when compared to PE in the rat aorta. The KCl contraction was observed at the highest concentration and abolished in the absence of extracellular Ca^{2+}, and a similar observation was recorded for 5-HT. By contrast, PE-induced contraction occurred rapidly, and the contraction could still be observed and measurable in the calcium-free medium. This result suggests that KCl, 5-HT, and PE- induce calcium oscillations in the aorta but maybe at a different frequency. The differences in the sensitivity to 5-HT could also be due to differences in the type(s) of 5-HT receptors expressed in the aorta.

Moreover, the KCl and 5-HT contraction could not be sustained while PE contraction was sustained in both calcium medium. While the influx of Ca^{2+} and phosphorylation of MLC are required for the initiation of smooth muscle contraction, it is not clearly understood how the contractile force is sustained over time. The small amount of Ca^{2+} in the cytoplasm is thought to be maintained by a balance of Ca^{2+} influx through Ca^{2+} channels and efflux via Ca^{2+}-ATPases and Na^{+}/Ca^{2+} exchangers in the plasma membrane (Nazer and van Breemen, 1998; Thornloe and Nelson, 2005). Besides, the L-type Ca^{2+} channel appeared to be particularly important in maintaining vascular smooth muscle tone (Moosmang *et al.,* 2003; Cobine *et al.,* 2007). In the past, it was suggested that in the presence of external calcium, PE induce a transient phasic contraction followed by maintained tonic contraction. The initial phase of PE contraction is due to Ca^{2+} release from intracellular calcium, whereas the sustained contraction is due to increased Ca^{2+} influx via ROCC (Van Breeman, 1969; Lee *et al.,*2001). However, our present finding indicated that PE contraction could be sustained even in the absence of calcium influx. This suggests that the tonic phase of PE contraction has two components; 1) major component (70 %) due to stimulated Ca^{2+} influx from the extracellular space and 2) minor component (30 %) due to activation of protein C-kinase (Khalil *et al.,* 1988). Our findings, therefore, indicate the importance of Ca^{2+} influx and suggest only a minor role of intracellular Ca^{2+} in maintaining the tonic contraction of the contractile agents in the vascular smooth muscle.

The three major findings from this study were; (1) KCl-induced contraction depends mostly on extracellular Ca^{2+} (2) 5-HT contraction depends mostly on extracellular Ca^{2+} (3) PE-induced contraction depends on both extracellular and intracellular Ca^{2+} in rat aortic smooth muscle. The present study had shown that that the external and stored Ca^{2+} synergistically promote contraction in the aorta, but the removal of either source still enables a tone of the tissue to be maintained. This information was useful to further the investigation on calcium-dependent mechanism underlying FK70-induced relaxation.

8.5 Role of FK70 in calcium-mediated vascular responses in normotensive rats and spontaneously hypertensive rats (SHR)

The focus was after that diverted to investigate the role of FK70 on calcium-mediated mechanisms in normotensive rats and spontaneously hypertensive rats (SHR).

In this study, FK70 managed to reduce the PE, 5-HT, and KCl-induced contraction, suggesting the inhibitory action of FK70 on their signalling pathways. FK70 was also found to inhibit PE-induced contraction irreversibly. The common main calcium channels involved upon stimulation of these agonists are L-type Ca^{2+} channels and TRPC channels. Therefore in this study, the involvement of L-type Ca^{2+} channels and TRPC channels was hypothesised, and further experiments were conducted to validate the hypothesis. As a first step, the FK70 CRC was compared with known calcium channel blockers, nifedipine (an L-type Ca^{2+} channel blocker) and SKF 96365 (a non-selective TRPC channel blockers). FK70 displayed relaxation effect comparable to the known calcium channel blockers and similar relaxation responses as a putative TRPC channel blocker (SKF 96365) confirming FK70's blocking effect on Ca^{2+}-dependent contraction.

Next, the role of L-type Ca^{2+} channel on FK70 was evaluated in the presence of nifedipine and FK70. The results of the investigation showed that combined treatment with FK70 and nifedipine further reduced the PE-induced contraction. A similar observation was recorded in the presence of verapamil (a T-type Ca^{2+} channel blocker). Based on our previous findings, the TRPC channels seem to play a crucial role in the initial influx of Ca^{2+} before the second phase of Ca^{2+} entry via the L-type Ca^{2+} channels. The role of TRPC on FK70 was investigated using SKF 96365. However, our present findings confirmed that FK70 induced relaxation is not mediated by the TRPC channels either. Collectively our findings show that the combined treatment of FK70 with either nifedipine/ verapamil/ SKF 96365 further reduced the PE-induced contraction. This suggests that FK70-induced relaxation may not involve modulation of L / T-type Ca^{2+} channels or the TRPC channels.

Besides L-type Ca^{2+} and TRPC channels, the role of FK70 on intracellular Ca^{2+} pathway had also been investigated. FK70 did not seem to affect the phasic contraction in calcium-free Krebs; however, the contraction could not be sustained. The inability of FK70 treated aortic rings to sustain PE phasic contraction could be due to the inhibition of the residual contraction, which underlay the phasic contraction (Khalil and Van Breeman, 1988). As a note, the proposal warrants further studies.

The role of FK70 in inhibiting Ca^{2+}-mediated contraction better than the known calcium channel blockers should prove to be particularly valuable in hypertensive studies. Therefore, to further elucidate the calcium-mediated mechanism underlying FK70, spontaneously hypertensive rats (SHRs) were employed. One of the critical mechanisms of antihypertensive drugs is to lower the vascular resistance by directly dilating the blood vessels (Sun *et al*., 2011). FK70 could relax the SHR aorta pre-contracted by PE, thereby displaying potential antihypertensive properties. Our results from this study showed that the intact aortic tissues from SHRs are comparable to that of the denuded aorta in normotensive rats. This indicates that the SHR aortic tissues have compromised endothelium activity. Albeit the endothelial dysfunction in SHR, the relaxation response of FK70 was not affected, and the magnitude was comparable to that of the normotensive rats. Thus, we could confidently conclude that FK70-induced relaxation is endothelium-independent.

In a separate experiment involving dual treatment of FK70 and (nifedipine or SKF 96365) on PE-induced contraction, the results in SHR aortic tissues have demonstrated to be corollaries to those of normotensive SD rats. FK70 in the presence of either nifedipine or SKF 96365 showed a further reduction in PE-induced contraction, suggesting that Ca^{2+} channel other than L-type Ca^{2+} channels and TRPC channels might be involved in FK70-induced relaxation. The function and enhanced expression of multiple calcium channels subtype e.g. L/T type Ca^{2+} channels, and TRPC channels in pathophysiological hypertensive disease highlight their importance as potential targets for pharmacological intervention. The ability of FK70 in relaxing SHR tissues in the presence of classical calcium channel blockers may offer additional benefits as a therapeutic strategy of hypertension. In summary, FK70 displayed relaxation effect in both SD and SHR aortic tissues, which led to the conclusion that FK70 might possibly affect the Ca^{2+}-dependent contractions.

8.6 *In-vivo* investigation of FK70 on *Caenorhabditis elegans*

8.6.1 Effect of FK70 on pharyngeal pumping

As FK70 had shown calcium blocking effect in the *in-vitro* model, we decided to conduct further investigation on FK70 calcium-mediated mechanisms using an *in-vivo* model. Therefore, we took advantage of *C. elegans* as a tractable, experimental model system to assess FK70 induced effect on pharyngeal pumping of the worms.

The *C. elegans* pharynx contraction is due to repetitive Ca^{2+} transients induced by rhythmic action potentials. The motoneuron stimulation triggers cell depolarization, which induces Ca^{2+} entry through two types of voltage-dependent Ca^{2+} channels in the plasma membrane of pharynx cells and the subsequent cytosolic Ca^{2+} increase is amplified by Ca^{2+} release from the sarcoplasmic reticulum through the ryanodine receptor (Liu *et al*.,2011). The pharyngeal action potential is regulated by three major voltage-gated currents, conducted by a T-type Ca^{2+} channel (*cca-1*), an L-type Ca^{2+} channel (*egl-19*) and a potassium channel (*exp-2*) (Shtonda, 2005).

The calcium ion is known broadly as a signalling molecule, but it also has several specialized functions in the nervous system, e.g., in synaptic vesicle release, in modulation of ion channel activity and as an ion that is involved in generating currents across excitable membranes in neurons (Avery and Horvitz, 1990; Shtonda and Avery, 2005). The role of Ca^{2+} in *C. elegans* is imperative since they do not generate sodium-based action potentials (Bargmann, 1998; Goodman *et al.,*1998). The sources of Ca^{2+} influx in C. *elegans* are from nicotinic acetylcholine receptors (nAChR), transient receptor potential type C channels (TRPC3) and voltage-gated Ca^{2+} channels (VGCC). The Ca^{2+} efflux, on the hand, is mediated by plasma membrane calcium ATPase (PMCA), sodium-calcium exchanger (NCX) and sarco-endoplasmic reticulum calcium ATPase (SERCA) (Grienberger and Konnerth, 2012). The Ca^{2+} release from internal stores is mediated by inositol trisphosphate receptors (IP_3R) and ryanodine receptors (RyR). The inositol phosphate can be generated by metabotropic glutamate receptors (mGluR), and G_q-protein GPCRs. Besides that, the mitochondria also play an essential role in neuronal Ca^{2+} homeostasis e.g., calcium uniporter encoded by *mcu-1* gene.

The *C. elegans* feeding depends on the action of the pharynx, a neuromuscular pump that joins the mouth to the intestine. The pumping rate is determined most directly by the motor neuron MC, and its firing initiates the pharyngeal muscle contraction. Technically, two neural circuits control the pumping rate, the pharyngeal and extra-pharyngeal system (Bhatla and Horvitz, 2015; Dalliere *et al.*, 2016). Two factors control the MC firing, first, via the food sensed (Avery and Horvitz, 1989; Raizen *et al*., 1995) and serotonin (Avery and Thomas, 1997; Hobson *et al*., 2006). When food is present, the mechanosensation and serotonin signal redundantly on MC to cause fast pumping. The presence of food also facilitates pumping through several transmitters that act at the level of the pharynx, e.g. ACh, glutamate, 5-HT, neuropeptides or via neurohormonal mediated signalling e.g. GABA, 5-HT and neuropeptides. Two pharyngeal 5-HT receptors have been identified; *ser-1* in the pharyngeal muscle (Tsalik *et al*., 2003) and *ser-7* in MC. The 5-HT activates two feeding motions mainly by activating two separate neural pathways in response to food. For pumping activation, the *ser-7* serotonin receptor in the MC motor neurons in the feeding organ activate cholinergic transmission from MC to the pharyngeal muscles by activating the $G_s\alpha$ signalling pathway. While for isthmus peristalsis activation, the *ser-7* in the M4 (and possibly M2) motor neuron in the feeding organ activates the $G_{12}\alpha$ signalling pathway in a cell-autonomous manner, which presumably activates neurotransmission from M4 to the pharyngeal muscles.

Our pharyngeal pumping assessment on *C. elegans* has revealed that FK70 reduced pharyngeal pumping of the worms at a rate comparable to known calcium channel blockers, nemadipine A and B. Nemadipine A and B are bioactive compounds which resemble a class of widely prescribed anti-hypertension drugs called the 1,4-dihydropyridines (DHPs) that antagonize the α_1-subunit of L-type Ca^{2+} channels (Kwok *et al*.,2006). Nemadipine-A also antagonize vertebrate L-type Ca^{2+} channels, demonstrating that worms and vertebrates share the orthologous protein target. Besides the Ca^{2+} channels on the pharyngeal muscle, the neurons also express Ca^{2+} channels such as VGCC, e.g. Ca_{v2}-type channels are localized to the presynaptic active zone, where they function in vesicle exocytosis (Catteral, 2000), and many *C. elegans* TRP channels are enriched in the nervous system e.g. (TRPC, TRPML, TRPP). Because FK70 was shown to be involved in calcium-mediated pathways in vertebrates, further experiments on *C.elegans* strengthened our hypothesis that FK70 might have affected calcium-mediated contraction in the pharynx, to cause a reduction in the pumping rate. Moreover, FK70 showed relaxation response in electrically evoked isolated guinea pig ileum, which involves neurotransmission from neurones to smooth muscle.

Therefore it seems logical to suggest that FK70 might also have acted on pharyngeal neurones possibly by altering Ca^{2+} signalling, thus reducing neurotransmitter release at the neuromuscular junction. The past study had also shown that *tph-1* mutants that are deficient in 5-HT synthesis showed a reduction in pumping rate in the presence of food (Sze *et al.,* 2000). It is also noteworthy that FK70 had reduced 5-HT response in the rat tissues; therefore, it seems reasonable to hypothesise that FK70 might have also blocked the 5-HT response in the worms thus reducing the pumping rate. However, more research is needed to verify the proposed hypothesis.

8.6.2 Toxicity assay of FK70

Measuring toxicity is one of the main steps in drug development. Hence, there is a high demand for large scale animal testing to predict the toxicity. Considering the fact, search for alternative testing platforms is, therefore, an important priority. In concurrence, for this study, we have developed a convenient, low-cost assay utilising the nematode *C. elegans*, to assess the toxicity of FK70 rapidly.

The principal aim of this study was to predict the toxicity of FK70. The effect of FK70 on *C. elegans* lifespan was measured *in-vivo*. We exposed the worms to different concentrations of FK70 and nifedipine, a known L-type calcium channel, a widely used antihypertensive that is known to be safe in mammals (Striessnig *et al*.,2015). The higher concentration of FK70 (1 mM) caused acute toxicity with a shorter time to death mean (TD_{mean}) while FK70 at a lower concentration (100 µM) managed to inhibit the calcium-dependent pharyngeal pumping and displayed better safety profile. This shows that FK70 at a higher concentration is slightly toxic, and concentration re-adjustment is necessary for determining the optimal concentration to maximize the pharmacological outcomes (efficacy) while minimizing adverse side effects (toxicity). Exposure to nifedipine reduced basal lifespan considerably despite the concentrations of nifedipine used in this protocol is lower than that of FK70. The exact reason for this observation is unknown and yet to be found. Perhaps nifedipine and FK70 at higher concentration would have perturbed calcium signalling, e.g., reduced pharyngeal pumping leading to the death of the worms.

8.7 Summary and findings

Taken together, FK70 has shown to reduce the efficacy and potency of PE, KCl, and 5-HT-induced response and the calcium entry by $CaCl_2$ in the calcium-free medium. It seems that the most critical mechanism involved in FK70-induced relaxation is inhibition of calcium-dependent contraction maybe via inhibition of extracellular Ca^{2+} influx in all the animal models studied. Further experiments on SD and SHR showed that FK70-induced relaxation involves neither the L-type Ca^{2+} channels nor the TRPC channels. The activation of TRP channels by PE, KCl and 5-HT in rat tissues and expression of *trp* genes in the nervous system and pharyngeal muscle of *C. elegans* have raised the interest of investigating the role of FK70 on TRP channels in detail in the future. Many TRP subfamilies beside TRPC are expressed in different vascular tissues and *C. elegans*, e.g., TRPV and TRPM. Besides that, the function of NCX and the opening of TRP channels are closely linked, e.g., Na^{+} entry through TRP channels is a driving force for Ca^{2+} entry caused by the reverse-mode NCX. Therefore these channels warrant further consideration as a potential target in the future.

8.8 Limitations of the study

The present study successfully met the aims of optimising a functional bioassay methodology to investigate the pharmacological effects of a novel synthetic compound using an organ bath technique and *C. elegans*. Despite the success, this study is not without limitations.

Investigation of FK70 on various animal models, rat aorta (SD and SHR) and *C. elegans* displayed calcium inhibiting effect. Even with the presence of similar calcium channels in these tissues, the expression of some calcium channel subtypes must be different and yet to be investigated. Therefore, to find the exact mechanism and specific calcium channel involved in FK70 mediated mechanism, further investigation is warranted.

Another limitation to this study was the variation of tissue responsiveness to drugs within different batches of rats (Sprague-Dawley male and Wistar-Kyoto male laboratory rats in the case of the present study). Tissue segments with low drug response (< 0.4g stimulated tension force) were discarded and replaced with a new tissue segment which consequently resulted in some wastage of tissues. A few outliers were omitted when some tissues produced a more potent drug response than usual. The variation in responses was observed despite keeping the rats the controlled laboratory environment (with regards to habitat temperature, habitat lighting, animal food, and disease control).

Even though similar calcium inhibiting effect of FK70 was observed in SHR and SD, the responses, however, could not be compared directly due to different strain of rats used. The Sprague Dawley rats are outbred stock while the Wistar-Kyoto rats are an inbred strain. The genetic material is highly constant in time in inbred strains. However, this is not the case for outbred populations. The relative genetic distance between these two strains is unknown as no genetic mapping studies were conducted. That said, in the present study, the PE-contractile response in SHR has no significant difference when compared to SD rats. The observation shows that the aortic smooth muscle from SHR is not hyper-reactive to PE thus the α-receptor in aortic smooth muscle from the SHR does not appear to be abnormal than in the SD rats. FK70 and other known calcium channel blockers showed elevated relaxation response in SHR than SD, thus suggesting the increased reactivity could be part of a compensatory mechanism brought about by hypertension and the genes responsible for this mechanism is not the same as in SD.

Nevertheless, the principal aim of this study was to investigate the ability of FK70 to relax SHR aortic rings, and the results showed the calcium inhibiting effect of FK70 in these tissues. Even then, the calcium mechanism underlying FK70 is still in its infancy and warrants further research.

Besides that, we did not investigate differences in L-type Ca^{2+} channel and TRP channel isoform expression in aortic smooth muscle between SD and SHR. The test to identify the role of FK70 on TRPC channel and L-type Ca^{2+} channel in SHR suggest channels other than TRPC and L-type Ca^{2+} channel may be involved in FK70-induced relaxation. Despite using known concentrations of known calcium channel blockers, nifedipine and SKF 96365 for incubation were pre-determined from previous experiments in this study, it remains unknown if our findings are due to differential expression of TRPC and L-type Ca^{2+} channels in SHR and SD, and hence cannot be ruled out as a possible cause for the differences seen between SD and SHR. Further experiments, e.g. Western blot, is required to investigate the differences in calcium channel expressions in both SHR and SD.

Furthermore, there is often insufficient consideration as to whether the other criteria, especially the mimicry of human disease, are satisfied with the choice of cardiovascular disease rat models. For example, cardiovascular diseases such as hypertension and heart failure in humans are usually slowly developing with wide-ranging neuro-humoral adaptations. The SHR however, have the uniform polygenetic disposition and excitatory factors which produce a uniform change in the indirect and direct effects on the cardiovascular system within each colony (Lindpaintner *et al*.,1992). Although this lack of inter-individual variation is considered as one of the major advantages of the SHR, it also means that the SHR can only model one of many possible causes of human hypertension. Secondly, cardiovascular disease is uncommon in young humans but markedly increases with age. The SHR differs from human hypertension in that SHRs reproducibly develop hypertension in young adulthood rather than in middle age as in humans. The normal life spans of SHRs are quite short, between 1.5–2.5 years (Doggrell *et al.,*1998). Therefore, in the present study, we used more than > 4 months old SHR rats to reduce age-dependent variabilities and increase the relevance to human hypertension. Despite the disadvantages listed, the advantages preponderate over the disadvantages. The male SHR rats have been used for decades and considered the best model to define hypertension-induced changes in signalling mechanisms (Doggrell *et al.,*1998). They are a stable model which produces symptoms that are predictable, controllable, and avoid strenuous or life-threatening technical interventions.

Besides SHRs are used widely to test medicines for their effectiveness in lowering blood pressure, and to study the mechanisms of established hypertension, for example, felodipine (Lund-Johansen, 1990).

Although it was not envisaged that *C. elegans* would completely replace vertebrates in toxicity testing, it served as a preliminary and straightforward model in identifying and eliminating concentrations that are lethal before the drug development. One disadvantage is that, although the presence of toxicity in one species may sometimes add evidential weight for risk of toxicity in another, the likelihood is extremely inconsistent, varying substantially for different classes of drugs. Rigorous pre-clinical evaluation should be conducted in at least two animal species in order to determine the toxicity profile and establish general safety of FK70.

Unavoidable wastage of tissues and other resources did occur during this study. In the present study, we utilised fresh aortic smooth muscles for experiments to avoid any potential influence of storage on the relaxant effect of FK70. Initially, the extra aortic rings were discarded, but to overcome wastage, we maximised the use of all fresh tissues from each sacrificed tissues by running a second round of experiments on the same day. Additionally, more organ baths were installed to accommodate the use of all fresh aortic tissues and avoid the storage of tissues. For *C. elegans*, the plates did get contaminated at times, usually by bacteria, fungus, or mites. In this case, we cleaned the worms either by frequent passaging to new plates or by bleach treatment. We had also transferred worms using a worm pick. Such transfers led to unnecessary delay in the experiment and also contributed to plastic waste. To minimise this occurrence, we incorporated the aseptic and sterile technique throughout the experiment. Plastic petri dishes containing bacteria were sterilized before disposal, regardless of whether or not they have been used to culture bacteria. The plates were then sterilized and recycled.

8.9 Future studies

The calcium-inhibiting effect of FK70 has urged further investigations to be carried out to find the exact mechanism underlying FK70-induced relaxation. With that, several future experiments can be carried out as stated below;

i) Calcium imaging assay can be employed for FK70 screening. This technique would directly measure the dynamic calcium flux within the neurones /fresh aortic strips and how these neurones/ aortic fresh strips process signals from the extracellular space in response to FK70.

ii) The whole-cell patch-clamp technique (voltage- and current-clamp configurations) can be applied to both SD and SHR aortic smooth muscle cells to examine the ion channels involved in response to FK70. The whole-cell patch-clamp experiments would provide direct evidence for the inhibitory action of FK70 on any specific Ca^{2+} channel current in vascular smooth muscle cells.

iii) Assessment of western blot and immunohistochemistry study on normotensive SD rats and SHR. It will be useful in assessing the extracellular signal-regulated kinase (ERK) / mitogen-activated protein kinase (MAP) activation. The activation of ERK/MAP kinase is responsible for the Ca^{2+}-independent component of agonist-evoked contraction. With a recently developed antibody directed against caldesmon phosphorylated at the MAP kinase site, this approach would allow us to assess the involvement of FK70 on MAP kinase activation and Ca^{2+}-independent smooth muscle contraction. Besides that, it will also be helpful to find out the specific protein e.g. TRP channels involved in the aorta. This identification would provide a rational basis for designing antihypertensive therapies to normalize particular channel expression and the responsible channels in the development of anomalous vascular tone in hypertensive pathologies.

iv) Future studies investigating the inhibitory role of FK70 on TRP channels are warranted. The emerging role of TRP channels in mediating vascular smooth muscle contraction has provided a new therapeutic site for hypertension treatments. Even when the present study ruled out the involvement of TRPC channel, comparative studies using selective TRPC channel inhibitors e.g 1-[4-[(2,3,3-Trichloro-1-oxo-2-propen-1-yl)amino]phenyl]-5-(trifluoromethyl)-1*H*-pyrazole-4-carboxylic acid (Pyr3), a selective (TPRC3) inhibitor and larixyl acetate, a selective (TPRC6) inhibitor can be carried out. Besides TRPC channels, other TRP channels are also worth investigating as the aortic smooth muscle express range of TRP channels (refer **Table 8.1**).

FK70 appears to share similarity to TRP antagonists (refer to **Figure 8.1**); therefore, selective TRP channel inhibitors are the best candidates to be used in the comparative studies.

Table 8.1: Summary of TRP channels in aortic smooth muscle (reviewed by Earley and Brayden 2015)

TRP	Function	Tissue
TRPC 1	ROCE, SOCE	Aorta and lung
TRPC 3	GPCR mediated constriction	Aorta and lung
TRPC 6	GPCR mediated vasoconstriction, myogenic tone	Aorta
TRPC 4	SOCE	Aorta
TRPV 1	Vasoconstriction	Aorta
TRPV 2	Mechanosensitive Ca^{2+}	Aorta
TRPV 3	Mechanism unknown	Aorta
TRPM 8	Vasoconstriction	Aorta

v) As FK70 showed inhibition of calcium entry in general, the research can be broadened to look at other channels than L/T-type Ca^{2+} and TRP channels in PE-induced contraction such as the reverse Na^{+}/Ca^{2+} exchanger (NCX) channels. Functional studies have demonstrated that plasma membrane NCX channels are also involved in the regulation of vascular Ca^{2+} homeostasis by contributing to SOCE (Arnon *et al*.,2000). As NCX are involved in SOCE, NCX has been shown to mediate Ca^{2+} influx during activation of G-protein linked receptors. Functional coupling between the reverse-mode NCX and the canonical transient receptor potential channels (TRPCs) has also been proposed to mediate Ca^{2+} influx in HEK-293 cells overexpressing TRPC3 (Lemos *et al.*, 2007) and similar functional coupling of NCX was observed in endogenously expressed TRPC6 in rat aorta smooth muscle cells. There is an increasing amount of evidence that NCX and TRP channels are either functionally or physically linked (Rosker *et al*.,2004). Besides rat tissues, the NCX genes are also found to be expressed in *C. elegans*, in diverse cell types including primary sensory neurons, interneurons, and motor neurons, as well as various pharyngeal, body-wall, and vulval muscle cells (Sharma *et al*.,2013). Therefore, comparative studies using KB-R7943, a selective inhibitor of reverse-mode NCX can be considered in the future. Furthermore, FK70 also appears to share similarity to reverse-mode NCX antagonists (refer to **Figure 8.2**).

vi) As discussed earlier, many *C. elegans* ion channels share similar activation and regulatory mechanisms with vertebrate counterparts, including humans. Therefore *C. elegans* represents an excellent genetic model organism for the study of function and regulation of ion channels *in-vivo* (Xiao & Xu 2012). The genetic potential of *C. elegans* can be exploited further to provide detailed information of the pathways affected by FK70, e.g. specific L/T-type Ca^{2+} channels mutant, TRP-mutant or NCX-mutant worms can be tested with FK70 to study the changes in sensory behaviour and the complex behaviour of the worms in response to the novel compound.

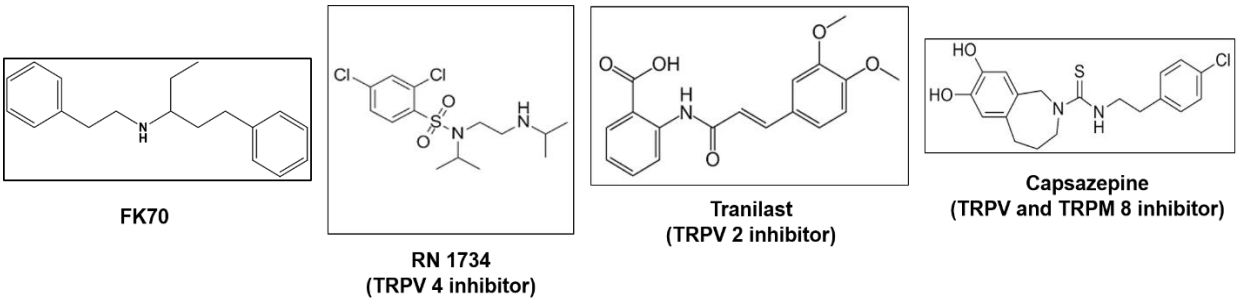


Figure 8.1: The chemical structures of (from left to right), FK70 and TRP channel inhibitors, RN-1734, tranilast and capsazepine (obtained from PubChem database).

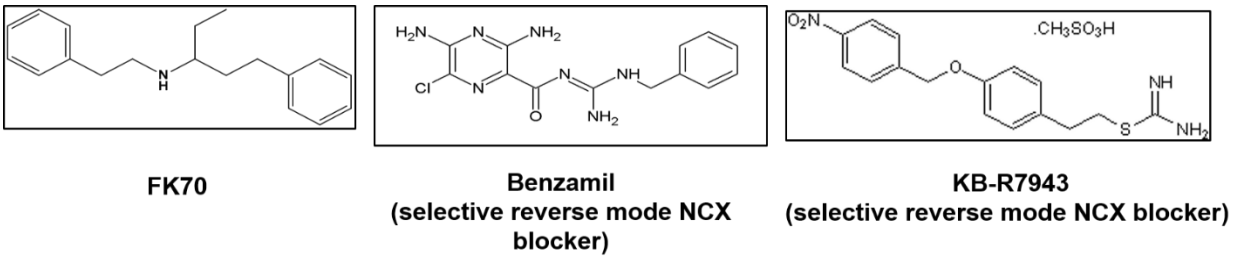


Figure 8.2: The chemical structures of (from left to right), FK70 and selective reverse mode NCX inhibitors, Benzamil and KB-R7943 (obtained from PubChem database).

8.10 Conclusion

The source for many new drugs and active ingredients of medicines are derived from natural products due to the chemical diversity and versatility of plants. Plant phytochemicals are often the target candidate for new drug discovery, but despite that, rarely they are directly used in clinical applications. Cost constraints, lengthy undertakings, and limited source of plant supply are prominent reasons. Not to mention the many structures of natural products exist as redundant atoms, which do not participate in the binding to target and convey disadvantageous effects on the physicochemical, pharmacokinetic, and biopharmaceutical properties. Furthermore, drug discovery and eventual commercialization might lead to undesirable environmental concerns. Therefore, after new lead discovery, chemical synthesis can be opted to address these issues.

Sch A, a novel compound derived from the plant *Ficus Schwarzii* has been previously shown to possess vasorelaxation effect in rat and porcine tissues. Given the structural similarity between FK70 and Sch A, this study was created with the objectives (refer to Chapter 1) to explore the mechanism of action of FK70. The present study has successfully met the objectives and can be concluded as the following;

(i) A functional bioassay methodology using organ bath technique was used to determine the pharmacological activity of FK70 in different rat smooth muscles (aorta, trachea, and bladder), porcine coronary artery smooth muscle and guinea pig isolated ileum segments. The relaxant effect of FK70 was revealed in these tissues.

(ii) Using the same functional bioassay, the relaxation mechanisms by FK70 in rat aortic tissues was demonstrated to be independent of the influences of endothelium and nitric oxide while not mediated by α / β-adrenoceptors, potassium channels, and cAMP/cGMP. FK70 showed extracellular Ca^{2+} inhibiting effect in rat aorta.

(iii) The role of stored and extracellular Ca^{2+} upon activation of contractile agents in rat aorta was investigated. The study has revealed that that the external and stored Ca^{2+} synergistically promote contraction in the aorta, but the removal of either source still enables a tone of the tissue to be maintained. The KCl and 5-HT-induced contraction depend mostly on extracellular Ca^{2+} while PE-induced contraction depends on both extracellular and intracellular Ca^{2+} in rat aortic smooth muscle.

iv) From the gathered results on rat aortic tissues (SD and SHR) and pharyngeal pumping of *C. elegans,* the relaxation effect of FK70 appears to be via inhibition of extracellular Ca^{2+} influx. However, the exact pathway remains undiscovered. Future studies will focus on investigating the effect of FK70 on NCX reverse mode channels and specific TRP channels, as proposed in **Section 8.9**.

v) The toxicity study done on FK70 had revealed that FK70 at a lower concentration did not seem to affect the basal lifespan, but at a higher concentration, it reduced the lifespan of the worms. FK70 at 100 µM was found to possess a potent relaxation effect on the pharyngeal pumping and also have a better safety profile.

Appendix

Animal ethics approval:

For Sprague Dawley rats;

Obtained from University of Nottingham's Animal Welfare Ethics Committee (UNMC#2kn and UNMC 12)

For Spontaneously hypertensive rats;

Obtained from University of Nottingham's Animal Welfare Ethics Committee (UNMC_00006_KNT and UNMC18)

The University of Nottingham

Animal Welfare and Ethical Review Body

Cover Form applicable to the use of animals in non-regulated procedures

Title of Study: **Investigation on the effect of natural product extracts and compounds on the isolated rat smooth muscles**

Name of Applicant: **Kang Nee TING**

School: **Life Sciences (Malaysia Campus)**

AWERB REF: UNMC12

1. Requests to use animals in non-regulated procedures must incorporate the information required below in the format requested for project licence applicants. The completed form should be returned electronically to the bsu@nottingham.ac.uk. When approved a protocol reference number will be provided to the applicant and must be used when ordering animals for use in the non-regulated study.

Its purpose is to highlight the key points in the proposed programme of work and to provide an executive summary in non-technical language accessible to the lay members of the Animal Welfare and Ethical Review Body.

Your application must be accompanied by this summary written in non-technical terms. You should complete this summary template. We expect that, for all but the most complex of studies, you will be able to provide a satisfactory summary using 500 to 1,000 words.

Study Title (max. 50 characters)	In-vitro organ bath experiments investigating effects of natural product extracts and isolated compounds on rat smooth muscles.		
Key Words (max. 5 words)	Isolated tissues; aorta; bladder; airway		
Expected duration of the study (yrs)	4 years (this is formed part of PhD projects, BSc Biomedical Sciences, MPharm year 4 research projects and a grant application to be submitted to the Malaysia government)		
Purpose of the study	Basic research	**Yes**	
	Translational and applied research		No
	Regulatory use and routine production		No
	Protection of the natural environment in the interests of the health or welfare of humans or animals		No
	Preservation of species		No
	Higher education or training	**Yes**	
	Forensic enquiries		No
	Maintenance of colonies of genetically altered animals[1]		No

1

The University of Nottingham

Describe the objectives of the study (e.g. the scientific unknowns or scientific/clinical needs being addressed)	This application is divided into two main objectives: 1. MPharm year 3 and BSc Biomedical Sciences research projects: To enable students to further understand the pharmacology of smooth muscles including understanding the process of research in natural product drug discovery. 2. PhD projects: This forms part of the research skills training. In vitro organ bath technique will be one of the methodology to be included in the thesis. There are currently 3 students under my supervision using this technique of experiments
What are the potential benefits likely to derive from this study (how science could be advanced or humans or animals could benefit from the project)?	1. Students will gain a better understanding of the pharmacology of the smooth muscles in different vascular beds where technical skills will be gained as part of their training in undergraduate or postgraduate level. 2. These experiments will provide new information on the effect of active extracts and compounds isolated from the natural flora on isolated smooth muscles from the airways, bladder and aorta. We are moving to test new compounds synthesize based on the skeletal structure of novel compounds isolated from local plant species. 3. In Malaysia, the skills on isolated organ bath are limited and this forms part of an important technique for drug discovery.
What species and approximate numbers of animals do you expect to use over what period of time?	Adult rats will be utilised for these experiments. With the current set up, it is estimated about 3 to 4 animals will be used weekly.
What procedures will be conducted?	All animals will be sacrificed by an experienced animal technician according to the methods described in Schedule 1 of the UK Animals (Scientific Procedures) Act 1986.
In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?	No treatments will be carried out on living animals. After humane killing, the tissues will be removed for isolated organ bath experiments.

Application of the 3Rs	
1. Replacement State why you need to use animals and why you cannot use non-animal alternatives	The study of some of the pharmacology can only be meaningful when carried out in complex integrated tissues from animals. We have tried carrying out some experiments on primary smooth muscle cell lines and to date this has not been successful. Cell lines only provide limited information on the effects of the compounds and extracts that we have been working with. Answering a direct question, for example whether a compound would dilate the airway is only possible from investigations on the isolated smooth muscle rings.
2. Reduction Explain how you will assure the use of minimum numbers of animals	The maximum number of different organs from each animal will be utilised. For example, the aorta, bladder and airways (bronchus and trachea) will be used from each rat.
3. Refinement Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.	At present no other sources of animal tissues are available but, for future planning, sourcing animal tissues from sheep obtained from local slaughter houses will be considered to minimise the use of rats in these experiments.
4. Housing and Care Please confirm that animals will be housed and cared for according to the requirements of the UK Animals (Scientific Procedures) Act 1986 draft Code of Practice for the care and accommodation of animals (February 2013) **or** provide details and justification for alternative housing and care standards.	Animals will be purchased from the animal facilities in University Putra Malaysia (UPM) and University Kebangsaan Malaysia (UKM) Both of these animal facilities are governed by their own institutional animal care and use committee (IACUC). They adhere to the Malaysian code of practice for the care and use of animals for scientific purposes which are consistent with the UK Home Office regulations. Information on the facility can be found here http://www.vet.upm.edu.my/eiacuc.html (UPM) http://www.kkl.ukm.my:8080/laru/wp-content/uploads/2013/03/UKMAEC-GUIDELINE-2007.pdf (UKM)

5. Humane Killing Please confirm that animals will be humanely killed using an appropriate method of humane killing detailed in Schedule 1 of the UK Animals (Scientific Procedures) Act 1986 **or** provide details and justification for alternative methods of killing.	Animals will be killed by concussion of the brain and/or exposure to carbon dioxide gas. Death is confirmed by dislocation of the neck.
For Office Use Only	
Will the study be subject to Retrospective Assessment?	Yes | No | Date due:

Committee use only

Comments by NACWO:

The proposed project appears appropriate with no welfare issues providing animals are humanely killed by trained and competent staff.
Neil Yates
05.09.13

Amendment 07/12/16
The request to source animals from an additional University is reasonable and confirmation has been received that both animal providers adhere to the Malaysian Code of Practice. However additional information on actual standards of animal care has been requested as the standards in place are not clear from the web links provided.
Neil Yates
07/12/16

Confirmation received - OK to proceed.
NY 13/03/17

Comments by NVS:

This would appear to be satisfactory, although an approximation for the total number of rats to be used per year or over the lifetime of the project should be given. Users should be trained to a level of competency that will ensure humane euthanasia is achieved at all times.

Ewan McNeill 05-09-13

I am happy with the information provided.
E. McNeil 13/3/17

We have contacted the animal house at UPM. The technician confirms that the rats are kept between 18 and 20 C. Reply from Kang Nee Ting 15/2/17

The University of Nottingham

Dear Annemarie

Please find attached the requested information on benefits accrued from this work.

From our record we have used 318 rats between September 2014 until now. Many thanks.
Best wishes
Kang Nee

Update on research output following the approval of UNMC2kn

Publication list

PhD thesis of Bi Juin Loong 2016: Development of a functional bioassay to study the mechanism of action of a novel phenylethylamine alkaloid, schwarzinicine

BJ Loong, KH Lim, Y Mbaki and KN Ting: Calcium mobilisation and relaxant properties of a novel phenyethylamine alkaloid, schwarzinicine A (In preparation)

Mei-Kee Lee, Kuan-Hon Lim, Chon-Seng Tan, Szu-Ting Ng, Sue-Mian Then, Suresh Kumar Mohankumar, Kang-Nee Ting: Bronchodilator effects of *Lignosus rhinocerotis* extract on rat isolated airways is linked to the blockage of calcium entry (Submitted to Journal of Phytomedicine).

Jun-Lee Lim, Kae-Shin Sim, Kien-Thai Yong, Bi-Juin Loong, Kang-Nee Ting, Siew-Huah Lim, Yun-Yee Low, Toh-Seok Kam: Biologically active vallesamine, strychnan, and rhazinilam alkaloids from Alstonia: Pneumatophorine, a nor-secovallesamine with unusual incorporation of a 3-ethylpyridine moiety. *Phytochemistry* 09/2015; 117.

Suet-Pick Wong, Chew-Yan Gan, Kuan-Hon Lim, Kang-Nee Ting, Yun-Yee Low, Toh-Seok Kam: Arboridinine, a Pentacyclic Indole Alkaloid with a New Cage Carbon–Nitrogen Skeleton Derived from a Pericine Precursor. *Organic Letters* 07/2015; 17(14):3628-3631.

B J Loong, J H Tan, K H Lim, Y Mbaki, K N Ting: Contractile function of smooth muscle retained after overnight storage. *Naunyn-Schmiedeberg's Archives of Pharmacology* 06/2015; 388(10):1061-1067.

Veronica Alicia Yap, Bi-Juin Loong, Kang-Nee Ting, Sandy Hwei-San Loh, Kien-Thai Yong, Yun-Yee Low, Toh-Seok Kam, Kuan-Hon Lim: Hispidacine, an unusual 8,4'-oxyneolignan-alkaloid with vasorelaxant activity, and hispiloscine, an antiproliferative phenanthroindolizidine alkaloid, from Ficus hispida Linn. *Phytochemistry* 01/2015; 109:96-102.

Choy-Eng Nge, Chew-Yan Gan, Kuan-Hon Lim, Kang-Nee Ting, Yun-Yee Low, Toh-Seok Kam: ChemInform Abstract: Criofolinine and Vernavosine, New Pentacyclic Indole Alkaloids Incorporating Pyrroloazepine and Pyridopyrimidine Moieties Derived from a Common Yohimbine Precursor. *Organic Letters* 12/2014; 46(21).

Students trained

Bi Juin Loong PhD, graduated 2016
Mei Kee Lee, 3rd year PhD
Kayatri Govindaraju, 2nd year PhD
Fong Kai Lee, 1st year PhD
Undergraduates (final year projects for MPharm and BSc Biomedical Sciences & summer placements) > 10

Justification for extension of the work

At present, we have 3 active PhD students (see the list above) using the post mortem tissues for their organ bath experiments. We have also completed a grant from the Ministry of Higher Education entitled "Mechanism of action of Lignosus rhinocerus (Tiger Milk Mushroom) on airway smooth muscle contractility and mast cells degranulation". We are currently preparing two proposals to be submitted to Newton Senior Fellowship and Wellcome Fellowship for the extension of the Tiger Milk Mushroom work. In addition, we have just submitted two grant

applications to the Ministry of Higher Education to study a series of novel compounds with vasorelaxation effects.
These are our justification for requesting extension of the ethics application for another 4 years from Sept 2017 to August 2021.

Comments by primary reader for the Committee (if applicable):

Comments by Lay Person (If applicable):

Committee decision: Approved

Communicated to applicant (date): 13/3/17

The University of Nottingham

Animal Welfare and Ethical Review Body

Cover Form applicable to the use of animals in non-regulated procedures

Title of Study: **Investigation on Schwarzinicine A (Sch A), a novel pheylethylamine alkaloid, and its derivatives on their effects in isolated tissues obtained from spontaneous hypertensive rats (SHR)**

Name of Applicant: **Kang Nee TING**

School: **Life Sciences (Malaysia Campus)**

AWERB REF: UNMC18

1. Requests to use animals in non-regulated procedures must incorporate the information required below in the format requested for project licence applicants. The completed form should be returned electronically to the bsu@nottingham.ac.uk. When approved a protocol reference number will be provided to the applicant and must be used when ordering animals for use in the non-regulated study.

Its purpose is to highlight the key points in the proposed programme of work and to provide an executive summary in non-technical language accessible to the lay members of the Animal Welfare and Ethical Review Body.

Your application must be accompanied by this summary written in non-technical terms. You should complete this summary template. We expect that, for all but the most complex of studies, you will be able to provide a satisfactory summary using 500 to 1,000 words.

<table>
<tr><td>Study Title (max. 50 characters)</td><td colspan="3">Investigation on Schwarzinicine A (Sch A), a novel pheylethylamine alkaloid, and its derivatives on their effects in isolated tissues obtained from spontaneous hypertensive rats (SHR)</td></tr>
<tr><td>Key Words (max. 5 words)</td><td colspan="3">Isolated tissues; aorta; bladder; airway</td></tr>
<tr><td>Expected duration of the study (yrs)</td><td colspan="3">3 years (this forms part of three/four PhD projects and three research proposals awarded in 2017 by the Ministry of Higher Education and Toray Foundation)</td></tr>
<tr><td>Purpose of the study</td><td colspan="3"><table>
<tr><td>Basic research</td><td>Yes</td><td></td></tr>
<tr><td>Translational and applied research</td><td></td><td>No</td></tr>
<tr><td>Regulatory use and routine production</td><td></td><td>No</td></tr>
<tr><td>Protection of the natural environment in the interests of the health or welfare of humans or animals</td><td></td><td>No</td></tr>
<tr><td>Preservation of species</td><td></td><td>No</td></tr>
<tr><td>Higher education or training</td><td>Yes</td><td></td></tr>
<tr><td>Forensic enquiries</td><td></td><td>No</td></tr>
</table></td></tr>
</table>

The University of Nottingham

	Maintenance of colonies of genetically altered animals[1]		No
Describe the objectives of the study (e.g. the scientific unknowns or scientific/clinical needs being addressed)	The objectives of the study are: 1. To compare the effectiveness and the potency of a series of newly synthesised compounds based on the phamacophore of Sch A in the isolated aorta of SHRs. We have some data from previous work to show that these compounds are more efficacious in SHR when compared to normotensive rats (see Appendix 1). 2. To further understand the mechanism of action of Sch A (and derivatives) in SHR. We have data to support the role of calcium entry and and interference of Gq-coupled activation of receptor are affected by Sch A (see Appendix 2). 3. To study the selectivity of Sch A (and derivatives) on other smooth muscle tissues such as bladder and trachea. All the experiments will be carried out in organ bath experiments set up.		
What are the potential benefits likely to derive from this study (how science could be advanced or humans or animals could benefit from the project)?	1. Students will gain a better understanding of the pharmacology of the smooth muscles in different rat species where technical skills will be gained as part of their PhD training. 2. These experiments will provide new information on the effect of these active compounds on isolated smooth muscles from the airways, bladder and aorta in SHR. 3. In Malaysia, the skills on isolated organ bath are limited and this forms part of an important technique for drug discovery. 4. We are able to understand the mechanism of action of Sch A (and derivatives) potentially file a patent of their applications for treatment of hypertension. We have some data to support the action via TRPC channels. This would be a novel target for treatment of hypertension.		
What species and approximate numbers of	Adult rats will be utilised for these experiments. With the current set up, it is estimated about 4		

animals do you expect to use over what period of time?	animals will be used for a period of 5 to 6 weeks in a year. An estimate of not more than 80 SHRs to be used for the next 3 years.
What procedures will be conducted?	All animals will be sacrificed by an experienced animal technician according to the methods described in Schedule 1 of the UK Animals (Scientific Procedures) Act 1986.
In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?	No treatments will be carried out on living animals. After humane killing at the University of Monash (Malaysia campus) animal facility, the tissues will be removed for isolated organ bath experiments.
Application of the 3Rs	
1. Replacement State why you need to use animals and why you cannot use non-animal alternatives	The study of some of the pharmacology can only be meaningful when carried out in complex integrated tissues from animals. We have tried carrying out some experiments on primary smooth muscle cell lines and to date this has not been successful. Cell lines only provide limited information on the effects of the compounds and extracts that we have been working with. Answering a direct question, for example whether a compound would dilate the airway is only possible from investigations on the isolated smooth muscle rings. We have a series of data on the functional responses in normotensive rats and we would like to investigate if the SHRs will be more sensitive to our compounds with smooth muscle relaxant activities.
2. Reduction Explain how you will assure the use of minimum numbers of animals	The maximum number of different organs from each animal will be utilised. For example, the aorta airways (bronchus and trachea) will be used from each rat for two different projects. The extra tissues will be frozen for staining and protein expression work subsequently.
3. Refinement Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to	At present no other sources of animal tissues are available but, for future planning, sourcing animal tissues from sheep obtained from local slaughter houses will be considered to minimise the use of rats in these experiments.

minimise welfare costs (harms) to the animals.	
4. Housing and Care Please confirm that animals will be housed and cared for according to the requirements of the UK Animals (Scientific Procedures) Act 1986 draft Code of Practice for the care and accommodation of animals (February 2013) **or** provide details and justification for alternative housing and care standards.	Animals will be purchased from the animal facilities in a local university (University of Monash Malaysia) This animal facility is governed by its own institutional animal care and use committee (IACUC). They adhere to the guidelines prepared by the Expert Working Group of the Animal Welfare Committee of the National Health and Medical Research Council, Australia (see attached guidelines).
5. Humane Killing Please confirm that animals will be humanely killed using an appropriate method of humane killing detailed in Schedule 1 of the UK Animals (Scientific Procedures) Act 1986 **or** provide details and justification for alternative methods of killing.	Animals will be killed by concussion of the brain and/or exposure to carbon dioxide gas. Death is confirmed by dislocation of the neck.
For Office Use Only	
Will the study be subject to Retrospective Assessment?	Yes | No | Date due:

Committee use only

Comments by Experimental Officer:
A relatively small number of SHR rats have been proposed over the 3 yr period. No *in vivo* work will take place. The rats will be culled by a S1 method and a range of tissues removed. A comparable approach have been used in the past involving normotensive and SHR rats (UNMC 2, UNMC6 and UNMC12).

- **Could you provide a brief experimental plan to explain how you will meet the objectives** (e.g. what data will be required, use of controls. NY 10/04/18).
  Response: See Annex 1

- **Please provide the same assurances are in place as before regarding:**
  a) **checking that the rats are hypertensive before culling takes place**
  b) **the competency of the person carrying out the humane killing of the rats and**
  c) **the ability to transport the tissues in a manner which does not affect the viability of the tissues.**

Response: The rats will be obtained and killed at the animal facility of University of Monash which is about 40km away from the Nottingham Campus. The required tissues will be removed immediately at Monash and transported back to Nottingham in ice cold Krebs. This protocol has been used previously and the results obtained did not show any loss of tissue viability. A trained technician in Monash University will take the blood pressure using the tail cuff method a day before the sacrificing of the rats by humane killing. He will supply adult rats that registered systolic blood pressure of between 170mmHg and 210mmHg.

A.Kelly 13/3/18

Comments by NVS:

This would appear to be a reasonable request and the applicant has stated specific aims for the study. Can you confirm;

- **No normal rats are required as controls for comparison studies?**

  Response: We have done a series of work on normal rats as control (Sprague Dawley rats under the approved applications UNMC2kn and UNMC12). The current work will be to compare our observations in SD and SHR tissues.

- **There will be no more than 80 SHRs used over the 3 year period (I was unsure how this links to '4 animals will be used for a period of 5 to 6 weeks in a year').**
  (If presumed that this is 4 rats over a 5 week period giving 10 periods per year = 40 rats per year for 3 years then approximately 120 rats and not 80 rats in total could be used over the 3 years of this study – clarification required. NY 10/04/18)

  Response: We plan to use 4 rats per week for a short period of 6 weeks or 6 rats per week for 5 weeks. For the first phase of work we will order 30 rats. Phase two or three will be carried out with the remaining rats in the next two years if we have newer compounds to test in SHR or to clarify the mechanism of these compounds in SHR tissues. We do not plan to use more than 80 rats in total for a period of 3 years.

Ewan McNeill 09-04-18

Comments by primary reader for the Committee (if applicable):

Comments by Lay Person (If applicable):

Committee decision:

Approved following Clarification – NY 13/04/18

References

Journals and Books:

Abbasi BH, Ali H, Yucesan B, Saeed S, Rehman K, Khan MA (2016). Evaluation of biochemical markers during somatic embryogenesis in Silybum marianum. L3 *Biotech*. 6(1):71

Abernethy DR and Schwartz JB (1999). Calcium-antagonist drugs. *N. Engl. J. Med* 341(19):1447–57.

Abdel-Latif AA (2001). Cross Talk Between Cyclic Nucleotides and Polyphosphoinositide Hydrolysis, Protein Kinases, and Contraction in Smooth Muscle*. Exp. Biol. Med.* SAGE Publications 226(3): 153–163.

Abelson B, Sun D, Que L, Nebel RA, Baker D, Popiel P, Amundsen CL, Chai T, Close C, DiSanto M, Fraser MO, Kielb SJ, Kuchel G, Mueller ER, Palmer MH, Parker-Autry C, Wolfe AJ, Damaser MS (2018). Sex differences in lower urinary tract biology and physiology. *Biol. Sex. Differ* 9(1):45.

Abu Bakar AR, Manaharan T, Merican AF, Mohamad S (2018). Experimental and computational approaches to reveal the potential of Ficus deltoidea leaves extract as α-amylase inhibitor. *Nat. Prod. Res.* 32(4): 473–476.

Abubakar IB, Lim KH, Loh HS (2015). Alkaloid extracts of Ficus species and palm oil-derived tocotrienols synergistically inhibit proliferation of human cancer cells. *Nat. Prod. Res* 29(22): 2137–2140.

Achelrod D, Wenzel U, Frey S (2015). Systematic review and meta-analysis of the prevalence of resistant hypertension in treated hypertensive populations. *Am J Hypertens* 358:355-61

Agarwal SP, Sinha MK, Kumar R (2017). Identification of human genes and its genomics functions via miRNAs of *C. elegans* on bioinformatics platforms. *Next Generat Sequenc & Applic* 4:2

Ahn JM, Eom HJ, Yang X, Meyer JN, Choi J (2014). Comparative toxicity of silver nanoparticles on oxidative stress and DNA damage in the nematode, *Caenorhabditis elegans. Chemosphere*. 108:343–352.

Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P (2002). Molecular Biology of the Cell. 4th edition New York: Garland Science.

Albertson DG and Thomson JN (1976). The pharynx of Caenorhabditis elegans. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *275*(938): 299–325.

Alexander SPH, Christopoulos A, Davenport AP, Kelly E, Marrion NV, Peters JA, Faccenda E, Harding SD, Pawson AJ, Sharman JL, Southan C, Davies JA (2017). The Concise Guide to PHARMACOLOGY 2017/18: G protein-coupled receptors. *Br J Pharmacol.* 174(1): 17-129

Al Disi, SS, Anwar MA, Eid AH. (2016). Anti-hypertensive Herbs and their Mechanisms of Action: Part I. *Frontiers in pharmacology 6:* 323

Ali M, Abbasi BH, Ahmad N, Khan H, Ali GS (2017). Strategies to enhance biologically active-secondary metabolites in cell cultures of Artemisia - current trends. *Crit Rev Biotechnol.* 37(7):833-851

Altun ZF and Hall DH (2009). Alimentary system, pharynx. *WormAtlas* Amberg GC, Navedo MF (2013). Calcium dynamics in vascular smooth muscle. *Microcirculation*. 20(4):281-9.

Amberg GC, Navedo MF (2013). Calcium dynamics in vascular smooth muscle. *Microcirculation.* 20(4): 281-9.

Amrit FRG, Ratnappan R, Keith SA and Ghazi A (2014). The C. elegans lifespan assay toolkit. Methods 68 (3): 465–475.

Andersan ML and Winter LMF (2017). Animal models in biological and biomedical research - experimental and ethical concerns. *Anais da Academia Brasileira de Ciências* . scielo.

Anderson GL, Cole RD, Williams PL (2004). Assessing behavioral toxicity with *Caenorhabditis elegans*. *Environ. Toxicol. Chem*. 23: 1235–1240

Andersen MJ, Hwang SJ, Kave GC, Melenovsky V, Olson TP, Fetterly K, Borlang BA (2015). Enhanced pulmonary vasodilator reserve and abnormal right ventricular. *Circ Heart Fail* 8: 542-550.

Anderson JL, Morran LT, Phillips PC (2010). Outcrossing and the maintenance of males within C. elegans populations. *J Hered*. 101(1):S62-74.

Andersson KE, Arner A (2004). Urinary bladder contraction ad relaxation: physiology and pathophysiology. *Physiol Rev* 84: 935-986

Andersson EK (2017). On the Site and Mechanism of Action of β_3-Adrenoceptor Agonists in the Bladder. *Int Neurourol J.* 21(1): 6–11.

Ann KC, Wightman B, Chalfie M (2015). A Transparent window into biology: A primer on Caenorhabditis elegans Biology Department, The Catholic University of America, Washington, DC 20064; [2]Biology Department, Muhlenberg College, Allentown, PA 18104; Department of Biological Sciences, Columbia University, New York, NY 10027.

Arce C, Vicente D, Segura V, Flacco N, Montó F, Almenar L, Agüero J, Rueda J, Jiménez-Altayó F, Vila E, Noguera MA, D'Ocon P, Ivorra MD (2017). Activation of α_{1A} -adrenoceptors desensitizes the rat aorta response to phenylephrine through a neuronal NOS pathway, a mechanism lost with ageing. *Br J Pharmacol.* 174(13): 2015-2030.

Arii T, Ohyanagi M, Shibuya J, Iwasaki T (1999). Increased function of the voltage-dependent calcium channels, without increase of Ca^{2+} release from the sarcoplasmic reticulum in the arterioles of spontaneous hypertensive rats*. Am. J. Hypertens 12(12): 1236–1242.*

Arnon A, Hamlyn JM, Blaustein MP (2000). Na^{+} entry via store-operated channels modulates Ca^{2+} signaling in arterial myocytes. *Am. J. Physiol., Cell Physiol.* 278(1): 163–73.

Arsyad A and Dobson GP (2016). Lidocaine relaxation in isolated rat aortic rings is enhanced by endothelial removal: possible role of K_v, K_{ATP} channels and A_{2a}-receptor crosstalk. *BMC Anesthesiol* 16(1):121.

Asano M, Nomura Y, Ito K, Uyama Y, Imaizumi Y, Watanabe M (1995). Increased function of voltage-dependent Ca^{2+} channels and Ca^{2+}-activated K channels in resting state of femoral arteries from spontaneously hypertensive rats at prehypertensive stage. *J Pharmacol Exp Ther* 275: 775–783.

Auguet M, Delaflotte S, Pirotzky E, Clostre F, Braquet P (1989). Role of endothelium on phenylephrine-triggered contractile events in aorta of spontaneously hypertensive rats. *Fundam Clin Pharmacol.* 3(6): 655-64.

Avery L and Horvitz HR (1989). Pharyngeal pumping continues after laser killing of the pharyngeal nervous system of *C. elegans*. *Neuron*, *3*(4): 473–485.

Avery L and Horvitz HR (1990). *Effects of starvation and neuroactive drugs on feeding in Caenorhabditis elegans*. *J Exp Zool* 253: 263–270.

Avery L and Thomas JH. (1997). Feeding and defecation. In *C. elegans* II, D. L. Riddle, T. Blumenthal, B. J. Meyer, and J. R. Preiss, eds. (New York, Cold Spring Harbor Press), 679-716.

Avery L and You YJ (2012). *C. elegans* feeding. *WormBook*, 1–23.

Ay B, Prakash YS, Pabelick CM, Sieck GC (2004). Store-operated Ca^{2+} entry in porcine airway smooth muscle. *Am. J. Physiol., Cell Physiol.* 286(5): 909–91

Babu GJ, Loukianov E, Loukianova T, Huke S, and Periasamy M (2001). Loss of SM-B myosin affects muscle shortening velocity and maximal force development. *Nat Cell Biol* 3: 1025-1029

Bacakova L, Travnickova M, Filova E, Matějka R, Stepanovska J, Musilkova J, Zarubova J and Molitor M (2018). The Role of Vascular Smooth Muscle Cells in the Physiology and Pathophysiology of Blood Vessels. *Muscle Cell and Tissue* Chapter 12.

Bacon CR, Cary NRB, Davenport AP (1996). Endothelin peptide and receptors in human atherosclerotic coronary artery and aorta. *Circ. Res.* 79: 794–801.

Bae YM, Kim A, Lee YJ, Lim W, Noh YH, Kim EJ, Kim J, Kim TK, Park SW, Kim B, Cho SI, Kim DK, Ho WK (2007). Enhancement of receptor-operated cation current and TRPC 6 expression in arterial smooth muscle cells of deoxycortico-sterone acetate-salt hypertensive rats. *J Hypertens*. 25: 809–817

Bailey J, Thew M, Balls M (2014). An analysis of the use of animal models in predicting human toxicology and drug safety. Altern *Lab Anim.* 42(3): 181-99.

Bakris GL, Iyengar M, Lukas MA, Ordronneau P, Weber MA (2010). Effect of combining extended-release carvedilol and lisinopril in hypertension: results of the COSMOS study *J Clin Hypertens* 12(9): 678–686.

Ball CJ, Wilson DP, Turner SP, Saint DA, Beltrame JF (2009). Heterogeneity of L- and T-channels in the vasculature: rationale for the efficacy of combined L- and T-blockade. *Hypertens* 53:654–660.

Baradaran A, Nasri H, Rafieian-Kopaei M (2014). Oxidative stress and hypertension: Possibility of hypertension therapy with antioxidants. *J Res Med Sci* 19(4): 358–367.

Bargmann CI (1998). Neurobiology of the *Caenorhabditis elegans* genome. *Science* 282: 2028-2033.

Barnes PJ and Bashaum CB (1983). Mapping of adrenergic receptors in the trachea by autoradiography. *Exp Lung Res* 5: 183

Barnes PJ and LIU SF (1995). Regulation of pulmonary vascular tone. *Pharmacol. Rev.* 47: 87–131.

Baron CB, Cunningham M, Strauss JF and Coburn RF (1984). Pharmacomechanical coupling in smooth muscle may involve phosphatidylinositol metabolism. *Proc. Natl. Acad. Sci. USA* 81: 6899 -6903.

Bauer V and Kuriyama H (1982). Evidence for non-cholinergic, non-adrenergic transmission in the guinea-pig ileum. *J Physiol* 330: 95-110.

Beanan MJ, Strome S (1992). Characterization of a germ-line proliferation mutation in *C. elegans. Development.* 116(3): 755-66.

Beevers G, Lip GY, O'Brien E (2001). ABC of hypertension: The pathophysiology of hypertension. *BMJ (Clinical research ed.)* 322(7291): 912–916.

Begum N, Duddy N, Sandu O, Reinzie J, Ragolia L (2000). Regulation of myosin bound protein phosphatase by insulin in vascular smooth muscle cells. Evaluation of the role of Rho kinase and PI3-kinase dependent signalling pathways. *Mol Endocrinol* 14: 1365–1376.

Bello Idris, Mohammad Zaini Asmawi, Usman Salisu Nasiba, Roziahanim Mahmud and Kabiru Abubakar (2015). Antihypertensive and Vasorelaxant Effect of *Alstonia scholaris* Stem Bark Extracts and Fractions. *Int. J. Pharmacol.* 11: 327-334.

Ben-Harari RR, Dalton BA, Osman R, and Maayani S (1991). Kinetic characterization of 5-hydrowytryptamine receptor desensitization in isolated guinea pig trachea and rabbit aorta. *J. Phamacol. Ewp.* Ther. 257: 416-424.

Berg CC and Culmsee H (2011). *Ficus schwarzii* redefined and two new species of *Ficus* (Moraceae) from Sulawesi (Indonesia) described. *Blumea* 56: 265–269

Bergson P, Lipkind G, Lee SP, Duban ME, Hanck DA (2011). Verapamil block of T-type calcium channels. *Mol Pharmacol*. 79(3): 411-9.

Bernatova I (2014). Endothelial Dysfunction in Experimental Models of Arterial Hypertension: Cause or Consequence? *Biomed Res Int*, 1–14.

Berridge MJ, Lipp P, and Bootman MD (2000). The versatility and universality of calcium signalling. *Nat. Rev. Mol. Cell Biol*. 1: 11–21.

Berry C, Hamilton CA, Brosnan MJ, Magill FG, Berg GA, McMurray JJ, and Dominiczak AF (2000). Investigations into the sources of superoxide in human blood vessels: angiotensin II increases superoxide production in human internal mammary arteries. *Circulation* 101: 2206–2212.

Bezie Y, Lacolley P, Laurent S and Gabella G (1998). Connection of Smooth Muscle Cells to Elastic Lamellae in Aorta of Spontaneously Hypertensive Rats. *Hypertens*. 32: 166–169.

Bézie Y, Lamazière JM, Laurent S, Challande P, Cunha RS, Bonnet J and Lacolley P (1998). Fibronectin expression and aortic wall elastic modulus in spontaneously hypertensive rats. *Arterioscler Thromb Vasc Biol.* 18(7): 1027-34.

Bhatla N, Droste R, Sando SR, Huang A, and Horvitz, HR (2015). Distinct neural circuits control rhythm inhibition and spitting by the myogenic pharynx of C. elegans. *Curr Biol* 25(16): 2075-2089.

Billington CK and Penn RB (2003). Signaling and regulation of G protein-coupled receptors in airway smooth muscle. *Respir Res* 4(1): 2

Billington CK, Ojo OO, Penn RB, Ito S (2013). cAMP regulation of airway smooth muscle function. *Pulm Pharmacol Ther* 26(1): 112–120.

Blaustein MP (1977). Sodium ions, calcium ions, blood pressure regulation, and hypertension: a reassessment and a hypothesis. *Am J Physiol*. 232: C165–73

Blaustein MP, Lederer WJ (1999). Sodium/calcium exchange: its physiological implications. *Physiol Rev* 79(3): 763–854.

Boie Y, Rushmore TH, Darmon-Goodwin A, Grygorczyk R, Slipetz DM, Metters KM, Abramovitz M (1994). Cloning and expression of a cDNA for the human prostanoid IP receptor. *J. Biol. Chem.* 269: 12173–12178

Bootman MD, Collins TJ, Mackenzie L, Roderick HL, Berridge MJ, Peppiatt CM (2002). 2-Aminoethoxydiphenyl borate (2-APB) is a reliable blocker of store-operated Ca^{2+} entry but an inconsistent inhibitor of $InsP_3$-induced Ca^{2+} release. *The FASEB Journal* 16(10): 1145–1150.

Boulanger CM, Morrison KJ, Vanhoutte PM (1994). Mediation by M_3-muscarinic receptors of both endothelium-dependent contraction ad relaxation to acetylcholine in the aorta of the spontaneously hypertensive rats. *Br J Pharmacol* 112(2): 519-524.

Boyd WA, McBride SJ, Rice JR, Snyder DW, Freedman JH (2010). A high-throughput method for assessing chemical toxicity using a *Caenorhabditis elegans* reproduction assay. *Toxicol Appl Pharmacol*. 245(2):153–159.

Braam, B, Sandra J, Taler, Rahman M, Fillaus JA, Greco BA, Forman JP, Reisin E, Cohen DL, Saklayen MG, Hedayati SS (2017). Recognition and Management of Resistant Hypertension. *Clin J Am Soc Nephrol* 12(3): 524-535.

Brahmachari G (2012). Bioactive natural products: Opportunities and challenges in medicinal chemistry. *Natural products in drug discovery* 1–199.

Brass LF, Newma DK, Wannermacher KM, Zhu L, Stalker TJ (2013). Signal Transduction During Platelet Plug Formation. *Platelets 367–398.*

Brenner S (1974). The genetics of Caenorhabditis elegans. *Genetics*. 77(1): 71-94.

British Joint Formulary- Joint Formulary Committee (2018). BNF 76 September 2018, London Pharmaceutical Press

Bruschi G, Spaggiari M, TacinneUi L, Bruschi ME, Caroppo M, Cavatorta A (1984). Regulation of arterial adrenoceptors during hypertension development in spontaneously hypertensive rats. *Hypertens* 2(3): 89-93

Burka JF, Blair RMJ, Chong C, Hogan JE (1990). Effects of calcium channel blockers on pharmacologically induced contractions of rainbow trout (Oncorhynchus mykiss) intestine. *Fish Physiol. Biochem.* 8(6): 521-527.

Butler MG (2010). Genetics of hypertension. Current status. *The Lebanese medical journal*. Lebanon, 58(3): 175–178.

Cai H and Harrison DG (2000). Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ. Res*. 87: 840–4.

Caldicott IM, Larsen PL, Riddle DL (1994). In: *Cell biology: a laboratory handbook*. (San Diego: Academic Press), 389.

Carey RM, Calhou DA, Bakri GL, Brook RD, Daugherty SL, Dennison-Himmelfarb CR (2018). Resistant Hypertension: Detection, Evaluation, and Management: A Scientific Statement From the American Heart Association. *Hypertens* 72(5).

Carrier GO and White RE (1985). Enhancement of alpha-1 and alpha-2 adrenergic agonist-induced vasoconstriction by removal of endothelium in rat aorta. *J. Pharmacol. Exp. Ther.* 232: 682–687.

Catterall WA (2000). Structure and regulation of voltage-gated Ca^{2+} channels. *Annu. Rev. Cell Dev. Biol.* 16: 521–555.

Catterall WA (2011). Voltage-Gated Calcium Channels. *Cold Spring Harb Perspect Biol.* 3(8): 1–24.

Catterall WA, Swanson TM (2015). Structural basis for pharmacology of voltage-gated sodium and calcium channels. *Mol Pharmacol* 88(1): 141-150.

C. elegans Sequencing Consortium. Genome sequence of the nematode C. elegans: a platform for investigating biology. *Science.* 282(5396):2012-8.

Cernes R, Zimlichman R, Shargorodsky M (2008). Arterial Elasticity in Cardiovascular Disease: Focus on Hypertension, Metabolic Syndrome and Diabetes*. Advances in Cardiology* (45): 65–81.

Chauhan VM, Orsi G, Brown A, Pritchard DI, Aylott JW (2013). Mapping the pharyngeal and intestinal pH of *Caenorhabditis elegans* and real-time luminal pH oscillations using extended dynamic range pH-sensitive nanosensors. *ACS Nano* 7: 5577–5587.

Cheng KT, Ong HL, Liu X, Ambudkar IS (2013). Contribution and Regulation of TRPC Channels in Store-Operated Ca^{2+} Entry. *Curr Top Membr.* 71:149-79.

Chen X, Yang D, Ma S, He H, Luo Z, Feng X, Cao T, Ma L, Yan Z, Liu D, Tepel M and Zhu Z (2010). Increased rhythmicity in hypertensive arterial smooth muscle is linked to transient receptor potential canonical channels. *J Cell Mol Med* 14: 2483–2494.

Chess-Williams R (2002). Muscarinic receptors of the urinary bladder: detrusor, urothelial and prejunctional. *Auton Autacoid Pharmacol* 22(3): 133-45.

Chevalier, M, Lory P, Mironneau C, Macrez N, and Quignard JF (2006). T-type Cav3.3 calcium channels produce spontaneous low-threshold action potentials and intracellular calcium oscillations. *Eur. J. Neurosci.* 23(9): 2321–2329.

Chisholm AD and Hardin J (2005). Epidermal morphogenesis (December 01, 2005), *WormBook*, ed. The *C. elegans* Research Community, WormBook

Chisholm AD, Hsiao TI (2012). The Caenorhabditis elegans epidermis as a model skin. I: development, patterning, and growth. *Wiley Interdiscip Rev Dev Biol*. 1(6): 861-78.

Chitra Devi R, Sim SM, and Ismail R (2012). Effect of Cymbopogon citratus and Citral on Vascular Smooth Muscle of the Isolated Thoracic Rat Aorta. *Evidence-Based Complementary and Alternative Medicine*, 8: 2012

Christensen AP and Corey DP (2007). TRP channels in mechanosensation: direct or indirect activation? *Nat. Rev*. 8: 510-521.

Chruscinski A, Brede ME, Meinel L, Lohse MJ, Kobilka BK, Hein L (2001). Differential distribution of beta-adrenergic receptor subtypes in blood vessels of knockout mice lacking beta(1)-or beta(2) adrenergic receptors. *Mol.Pharmacol* 60: 955-962

Chopra S, Baby C, Jacob JJ (2011). Neuro-endocrine regulation of blood pressure. *Indian J Endocrinol Metab* 4(4): 281–288.

Chung S, Funakoshi T, CivelliOrphan O (2008). GPCR research. *Br J. Pharmacol.* 153 (1): 339-346

Clark DE (1999). Rapid calculation of polar molecular surface area and its application to the prediction of transport phenomenal. Prediction of intestinal absorption. *J.Pharm Sci* 88: 807-814.

Clause BT (1998). The Wistar Institute Archives: Rats (Not Mice) and History, *Mendel Newsletter* February

Cobine C, Callaghan B, Keef K. (2007). Role of L-type calcium channels and PKC in active tone development in rabbit coronary artery. *Am. J. Physiol. Heart Circ. Physiol.* 292 H3079–H3088.

Cogolludo AL, Perez VF, Fajardo S, Ibarra M, Tamargo J (1999). Effects of nicorandil as compared to mixtures of sodium nitroprusside and levcromakalim in isolated rat aorta. *Br. J. Pharmacol*. 126: 1025–1033.

Cohn JN (1996). Is there a role for endothelin in the natural history of heart failure. *Circulation*. 94: 604–606.

Cousins HM, Edwards FR, Hirst GDS (1995). Neuronally released and applied acetylcholine on the longitudinal muscle of the guinea-pig ileum. *Neuroscience* 65(1): 193-207

Cox RH, Lozinskaya I, Dietz NJ (2003). Calcium exerts a larger regulatory effect on potassium^{+} channels in small mesenteric artery mycoytes from spontaneously hypertensive rats compared to Wistar-Kyoto rats. *Am. J. Hypertens.* 16(1): 21–27

Cribbs LL (2006). T-type Ca^{2+} channels in vascular smooth muscle: multiple functions. *Cell Calcium* 40(2): 221-230.

Culetto E and Sattelle DB (2000). A role for *Caenorhabditis elegans* in understanding the function and interactions of human disease genes. *Hum. Mol. Genet*. 9: 869-877.

Dalliere N, Bhatla N, Luedthe Z, Ma DK, Woolman J, Walker RJ, Holden-Dye L, O'Connor V (2016). Multiple excitatory and inhibitory neural signals converge to fine tune *Caenorhabditis elegans* feeding to food availability. *FASEB Journal*, 30(2): 836–848.

Daugherty SL, Powers D, Magid DJ, Tavel HM, Masoudi FA, Margolis KL, O'Connor PJ, Selby JV, Ho PM (2012). Incidence and prognosis of resistant hypertension in hypertension patients. *Circulation* 125(13): 1635-1642.

Davis JP, Tikunova, SB and Janssen PML (2019). Mechanisms of Muscle Contraction and Relaxation. *Muscle and Exercise Physiology,* 39–50.

Deleu D, Hanssens Y, Northway MG (2004). Subcutaneous apomorphine: an evidence-based review of its use in Parkinson's disease. *Drugs Aging* 21: 687–709.

De Ley P (2006). A quick tour of nematode diversity and the backbone of nematode phylogeny. In: WormBook: The Online Review of C. elegans Biology Pasadena (CA): WormBook; 2005-2018.

Dharmashankar K, Widlansky ME (2010). Vascular endothelial function and hypertension: insights and directions. *Curr Hypertens Rep*. 12(6): 448-55.

Dhawan R, Dusenbery D.B, Williams PL (1999). Comparison of lethality, reproduction, and behavior as toxicological endpoints in the nematode *Caenorhabditis elegans*. *J. Toxicol. Environ. Health A.* 58(7): 451–462.

Dias DA, Urban S, Roessner U (2012). A historical overview of natural products in drug discovery. *Metabolites*. 2:303–336.

Dietrich A, Kalwa H, Gudermann T (2010). TRPC channels in vascular cell function. *Thromb Haemost.* 103: 262–270.

DiNicolantonio JJ, Fares H, Niazi AK, Chatterjee S, D'Ascenzo F, Cerrato E, O'Keefe JH (2015). β-Blockers in hypertension, diabetes, heart failure and acute myocardial infarction: a review of the literature. *Open Heart* 2(1): 230.

Ding L, Jia C, Zhang Y, Wang W, Zhu W, Chen Y, and Zhang T (2019). Baicalin relaxes vascular smooth muscle and lowers blood pressure in spontaneously hypertensive rats. *Biomed Pharmacother,* 111: 325–330.

Doggrell S(1998). Rat models of hypertension, cardiac hypertrophy and failure. *Cardiovasc. Res* 39(1): 89–105.

Dong X, Liu OW, Howell AS, and Shen K (2013). An extracellular adhesion molecule complex patterns dendritic branching and morphogenesis. *Cell* 155: 296-307.

Dougall IG and Unitt J (2015). Evaluation of the Biological Activity of Compounds. *The Practice of Medicinal Chemistry* 15–43*.*

Dora KA, Hinton JM, Walker SD, Garland CJ (2000). An indirect influence of phenylephrine on the release of endothelium-derived vasodilators in rat small mesenteric artery. *Br J Pharmacol*. 129(2): 381-7

Doris PA (2017). Genetics of hypertension: an assessment of progress in the spontaneously hypertensive rat. *Physiol Genomics.* 49(11): 601–617.

Dulhunty AF (2006). Excitation-contraction coupling from the 1950s into the new millennium. *Clin. Exp. Pharmacol. Physiol*. 33: 763–772.

Durand MJ and Gutterman DD (2013). Diversity in mechanisms of endothelium-dependent vasodilation in health and disease. *Microcirculation* 20(3): 239–247.

Earley S and Brayden JE (2015). Transient Receptor Potential Channels in the Vasculature. *Physiol Rev* 95(2): 645–690.

Eglen RM, Hedge SS, Watson N (1996). Muscarinic receptor subtypes and smooth muscle function. 48(4): *Pharmacol Rev* 531-565

Elahi MM, Kong YX, Matata BM (2009). Oxidative stress as a mediator of cardiovascular disease. *Oxid Med Cell Longev*. 2(5): 259-69.

Emoto N, Yanagisawa M (1995). Endothelin-converting enzyme-2 is a membrane-bound, phosphoramidon-sensitive metalloprotease with acidic pH optimum. J*. Biol. Chem.* 270: 15262–15268

England SK, Wooldridge TA, Stekiel WJ, Rusch NJ (1993). Enhanced single-channel K^+ current in arterial membranes from genetically hypertensive rats. *Am J Physiol*. 264: 1337–1345.

Epstein M (2007). Resistant hypertension: prevalence and evolving concepts. *J Clin Hypertens* 9(1): 2-6.

Exton JH (1985). Mechanisms involved in alpha-adrenergic phenomena. *Am J Physiol.* 248(1): 633-47.

Fares H and Greenwald I (2001). Regulation of endocytosis by CUP-5, the Caenorhabditis elegans mucolipin-1 homolog. *Nat Genet.* 28(1): 64-8.

Fawcett GL, Santi CM, Butler A, Harris T, Covarubius M, Salkoff L (2006). Mutant analysis of the Shal (Kv4) voltage-gated fast transient K^+ channel in *Caenorhabditis elegans*. *J Biol Chem* 281: 30725–30735.

Feher J (2012). Smooth Muscle. *Boston: Academic Press* 283–293.

Feher J (2017). Smooth Muscle. Quantitative Human Physiology (2nd ed). *Elsevier* 351-361

Feletou M and Vanhoutte PM (2006). Endothelial dysfunction: a multifaceted disorder. *Am J Physiol Heart Circ Physiol* 291: 985-1002.

Félétou M, Vanhoutte PM (2007). Endothelium-dependent hyperolarizations: past beliefs and present facts. *Annals of Med.* 39:495–516.

Feletou M, Vanhoutte PM (2009). EDHF: an update. *Clin Sci (Lond)* 117:139–155.

Félétou M, Köhler R, Vanhoutte PM (2010). Endothelium-derived vasoactive factors and hypertension: possible roles in pathogenesis and as treatment targets. *Curr Hypertens Rep*. 12(4): 267-75.

Feng Z, Li W, Ward A, Piggott BJ, Larkspur ER, Sternberg PW, Xu XZ (2006). A *C. elegans* model of nicotine-dependent behavior: regulation by TRP-family channels. *Cell*. 127(3): 621-33.

Fitz F, Sartori M, Girão MJ, Castro R (2017). Pelvic floor muscle training for overactive bladder symptoms - A prospective study. *Rev Assoc Med Bras* 63(12): 1032-1038.

Folkow B (1982). Physiological aspects of primary hypertension. *PhysM Rev* 62:347-504

Folkow B (1993). Early structural changes in hypertension: pathophysiology and clinical consequences. *J. Cardiovasc Pharmacol* 22(1): 1-6

Fordyce CB, Roe MT, Ahmad T, Libby P, Borer JS, Hiatt WR, Bristow MR, Packer M, Wasserman SM, Braunstein N, Pitt B, DeMets DL, Cooper-Arnold K, Armstrong PW, Berkowitz SD, Scott R, Prats J, Galis ZS, Stockbridge N, Peterson ED, Califf RM (2015). Cardiovascular Drug Development *J M Coll Cardiol* 65(15): 1567-1582.

Foskett JK, White C, Cheung KH, Mak DO (2007). Inositol trisphosphate receptor Ca^{2+} release channels. *Physiol Rev*. 87(2): 593-658.

Fransen P, Van Hove C, van Langen J, Bult H (2016). Contraction by Ca^{2+} influx via the L-type Ca^{2+} channel voltage window in mouse aortic segments is modulated by nitric oxide. *Current Basic and Pathological Approaches to the Function of Muscle Cells and Tissues-From molecules to humans,* Chapter 4

Freeze BS, McNulty MM, Hanck DA. (2006) State-dependent verapamil block of the cloned human Ca(v)3.1 T-type Ca^{2+} channel. *Mol Pharmacol* 70: 718–726.

Frieman SM (1983). Vascular reactivity, in Genest J, Kuchel O, Hamet P, Cantin M (eds): Hypertension. New York, McGraw- Hill Book Co 457-473.

Frishman WH, Beravol P, Carosella C (2009). Alternative and complementary medicine for preventing and treating cardiovascular disease. *Dis. Mon.* 55: 121–192.

Fryer AD and Jacoby DB (1998). Muscarinic Receptors and Control of Airway Smooth Muscle. *Am J Respir Crit Care Med* 158(2): 154–160.

Fuchs B, Dietrich A, Gudermann T, Kalwa H, Grimminger F, Weissmann N (2010). The role of classical transient receptor potential channels in the regulation of hypoxic pulmonary vasoconstriction. *Adv Exp Med Biol.* 661: 187–200.

Fukuda T, Ogurusu T, Furukawa KI, Munekazu S (1990). Protein kinase C-dependent phosphorylation of sarcolemmal Ca^{2+}-ATPase isolated from bovine aortic smooth muscle. *J. Biochem. 1*08(4): 629–634.

Gaud A, Simon JM, Witzel T, Carre-Pierrat M, Wermuth CG, Segalat L (2004). Prednisone reduces muscle degeneration in dystrophin-deficient Caenorhabditis elegans. *Neuromuscul. Disord.* 14(6): 365–370.

Ghanemi A (2015). Targeting G protein coupled receptor-related pathways as emerging molecular therapies. *SAUDI PHARM J* 23(2): 115–129.

Gloriam DE, Fredriksson R, Schioth HB (2007). The G protein-coupled receptor subset of the rat genome. *BMC Genomics* 8: 338.

Gokce G and Arun MZ (2014). Ergothioneine produces relaxation in isolated rat aorta by inactivating superoxide anion. *Eur Rev Med Pharmacol Sci* 18: 3339-3345.

Goldblatt H, Lynch J, Hanzal RF, Summerville WW (1934). Studies on experimental: I. the production of persistent elevation of systolic blood pressure by means of renal ischenmia. *J Exp Med.* 9:347–379.

Goldsmith SR (2006). The role of vasopressin in congestive heart failure. *Cleveland Clinic journal of medicine*. 73(3): 19-23.

Gomart S, Gaudreau-Ménard C, Jespers P, Dilek OG, Hupkens E, Hanthazi, A, Naeije R, Melot C, Labranche N, Dewachter L, Mc Entee K. (2017). Leptin-Induced Endothelium-Independent Vasoconstriction in Thoracic Aorta and Pulmonary Artery of Spontaneously Hypertensive Rats: Role of Calcium Channels and Stores. *PLOS ONE* 12(1): e0169205.

Go AS, Bauman MA, Coleman King SM, Fonarow CC, Lawrence W, Williams KA, Sachez E (2014). *Hypertens* 63: 878-885

Goodman MB, Hall DH, Avery L and Lockery SR (1998). Active currents regulate sensitivity and dynamic range in *C. elegans* neurons. *Neuron 20*: 763-772.

Gopal S, Sogaard P, Multhaupt HA, Pataki C, Okina E, Xian X, Pedersen ME, Stevens T, Griesbeck O, Park PW, Pocock R, Couchman JR (2015). Transmembrane proteoglycans control stretch-activated channels to set cytosolic calcium levels. *J Cell Biol*. 210: 1199–1211.

Gordienko DV, Bolton TB (2002). Crosstalk between ryanodine receptors and IP(3) receptors as a factor shaping spontaneous Ca^{2+}- release events in rabbit portal vein myocytes. *J Physiol*. 542(3):743-62

Gosai SJ, Kwak JH, Luke CJ, Long OS, King DE, Kovatch KJ, Pak SC (2010). Automated high-content live animal drug screening using C. elegans expressing the aggregation prone serpin alpha1-antitrypsin Z. *PloS one* 5(11): 15460.

Gottlieb P, Folgering J, Maroto R, Raso A, Wood TG , Kurosky A, Bowman C, Bichet D, Patel A, Sachs F (2008). Revisiting TRPC1 and TRPC6 mechanosensitivity. *Pflugers Arch.* 455 : 1097-1103

Grant BD and Sato M (2006). Intracellular trafficking (January 21, 2006), *WormBook*, ed. The *C. elegans* Research Community, WormBook.

Grienberger C and Konnerth A (2012). Imaging calcium in neurons. *Neuron 73*: 862-885.

Grover AK, Kwan CY, Rangachari PK, Daniel EE (1983). Na^{+}-Ca^{2+} exchange in a smooth muscle plasma membrane-enriched fraction. *Am J Physiol* 244(3): 158–65.

Guan YY, Kwan CY, Daniel EE (1988). The effects of EGTA on vascular smooth muscle contractility in calcium-free medium. *Can J Physiol Pharmacol.* 66(8): 1053-6.

Gurnani N, Mehta D, Gupta M, Mehta BK (2014). Natural products: Source of potential drugs. *Afr J Basic Appl Sci.* 6:171–186.

Haga T (2013). Molecular properties of muscarinic acetylcholine receptors. *Proceedings of the Japan Academy, Series B* 89(6): 226–256.

Hajjar I, Kotchen TA (2003). Trends in prevalence, awareness, treatment, and control of hypertension in the United States. *JAMA* 290(2): 199-206.

Hall DH, Altun ZF (2008). C. elegans Atlas. Cold Spring Harbor Laboratory Press: Cold Spring Harbor.

Hamada H, Damron D, Hong SJ, van Wagoner D, Murray P (1997). Phenylephrine-Induced Ca^{2+} Oscillations in Canine Pulmonary Artery Smooth Muscle Cells. *Circ. Res.* 81:812–823.

Han X, Zhang R, Anderson L, and Rahimian R (2014). Sexual dimorphism in rat aortic endothelial function of streptozotocin-induced diabetes: possible involvement of superoxide and nitric oxide production. *Eur. J. Pharmacol. 723*: 442–450.

Hansch C, Leo A, Hoekman D (1995). In exploring QSAR: hydrophobic, electronic and steric constants. SR Heller (Ed). American Chemical Society, Washington DC

Hansen PB, Poulsen CB, Walter S, Marcussen N, Cribbs LL, Skøtt O, Jensen BL (2011). Functional Importance of L- and P/Q-Type Voltage-Gated Calcium Channels in Human Renal Vasculature. *Hypertens* 58: 464–470.

Harrison DG and Gongora MC (2009). Oxidative stress and hypertension. *Med. Clin. North Am.* 93(3): 621–635.

Haynes WG, Webb DJ (1994). Contribution of endogenous generation of endothelin-1 to basal vascular tone. *Lancet.* 344: 852–854.

Haynes WG, Ferro CE, Webb DJ (1995). Physiologic role of endothelin in maintenance of vascular tone in humans. *J. Cardiovasc. Pharmacol.* 26: S183–S185.

Hays TT, Ma B, Zhou N, Stoll S, Pearce WJ, Qiu H (2018). Vascular smooth muscle cells direct extracellular dysregulation in aortic stiffening of hypertensive rats. *Aging cells* 17: 3

Hazell GG , Hindmarch CC, Pope GR, Roper JA, Lightman SL, Murphy D, O'Carroll AM, Lolait SJ (2012). G protein-coupled receptors in the hypothalamic paraventricular and supraoptic nuclei – serpentine gateways to neuroendocrine homeostasis. *Front. Neuroendocrinol.* 33(1): 45-66

Heilpern K (2008). Pathophysiology of hypertension. *Annals of emergency medicine* 5-6.

Henrik J, Bojer MS , Marinus MG, Xu T, Struve C, Krogfelt KA, Løbner-Olesen A (2013). The Alkaloid Compound Harmane Increases the Lifespan of *Caenorhabditis elegans* during Bacterial Infection, by Modulating the Nematode's Innate Immune Response. *PLoS One*. 8(3).

Herbert A, Cruickshank JK, Laurent S, Boutouyrie P (2014). Establishing reference values for central blood pressure and its amplification in a general healthy population and according to cardiovascular risk factors. *Eur Heart J* 35(44): 3088-3090.

Herbert RA, Janardhan KS, Pandiri AR, Cesta MF and Miller RA (2018). *Nose, Larynx, and Trachea. Boorman's Pathology of the Rat,* 391–435

Hirshman CA, Lande B and Croxton TL (1999). Role of M_2 muscarinic receptors in airway smooth muscle contraction*. Life Sci* 64(6-7): 443–448.

Hobert O (2013). The neuronal genome of *Caenorhabditis elegans* . *WormBook*, ed. The *C. elegans* Research Community, WormBook.

Hobson RJ, Hapiak VM, Xiao H, Buehrer KL, Komuniecki PR, and Komuniecki RW. (2006). SER-7, a *Caenorhabditis elegans* 5-HT7-like receptor, is essential for the 5-HT stimulation of pharyngeal pumping and egg laying. *Genetics 172*, 159-169.

Hofmann T, Obukhov A G, Schaefer M, Harteneck C, Gudermann T, Schultz G (1999). Direct activation of human TRPC6 and TRPC3 channels by diacylglycerol. *Nature* 397: 259–263.

Holloway ET and Bohr DF (1973). Reactivity of vascular smooth muscle in hypertensive rats. *Circ Res* 33: 678- 685

House SJ, Potier M, Bisaillon J, Singer HA, Trebak M (2008). The non-excitable smooth muscle: calcium signalling and phenotypic switching during vascular disease. *Pflüg Arch Eur J Physiol.* 456(5): 769–85.

Hoyer D, Clarke DE, Fozard JR, Hartig PR, Martin GR, Mylecharane EJ, Saxena PR, Humphrey PP (1994). International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (Serotonin). *Pharmacol Rev.* 46(2):157-203.

Huang C, Xiong C, Kornfeld K (2004). Measurements of age-related changes of physiological processes that predict lifespan of Caenorhabditis elegans. *Proc Natl Acad Sci USA*. 101: 8084–8089.

Hughes JP, Rees S, Kalindjian SB, Philphett KL (2011). Principles of early drug discovery. *Br. J. Pharmacol* 162(6).

Hughes A (2018). Calcium Channel Blockers. *Hypertension: A Companion to Braunwald's Heart Disease*, 242–253.

Hulsmann AR, de Jongste JC (1993). Studies of human airways in vitro: a review of the methodology. *J Pharmacol Toxicol Methods.* 30(3): 117-32.

Hunt PR (2017). The C. elegans model in toxicity testing. *J Appl Toxicol* 37(1): 50–59.

Iino M, Kasai H, Yamazawa T (1994). Visualization of neural control of intracellular Ca^{2+} concentration in single vascular smooth muscle cells in situ. *EMBO J* 13: 5026–5031.

Inbred strains and outbred stocks. (1994). *Techniques in the Behavioral and Neural Sciences* 119–153.

Iwaski H, Mori Y, Hara Y, Uchida K, Zhou H, and Mikoshiba K (2001). 2-aminoehtoxydiphenyl borate (2-APB) inhibits capacitative calcium entry independently of the function of inositol 1,4,5-trisphosphate receptors. *Receptors Channel*s 7: 429–439.

Iwamoto T, Kita S, Zhang J, Blaustein MP, Arai Y, Yoshida S, Wakimoto K, Komuro I, Katsuragi T (2004). Salt-sensitive hypertension is triggered by Ca^{2+} entry via Na^{+}/Ca^{2+} exchanger type-1 in vascular smooth muscle. *Nat Med* 10:1193–9.

Iwanaga S, Shibata O, Tsuda A, Hashimoto S, Makita T, Cho S, Sumikawa K (1998). The role of alpha1-adrenoceptors in the clonidine-induced contraction and relaxation of rat aorta. *Res Commun Mol Pathol Pharmacol.* 102(2): 137-47.

Jabeen S, Hanifa MS, Khan MM, Qadri RWK (2014). Natural products sources and their active compounds on disease prevention: A review. *IJCBS* 6:76–83.

Jackson WF (2000). Ion channels and vascular tone. *Hypertens*. 35: 173–178.

Jakobsen H, Bojer MS, Marinus MG, Xu T, Struve C, Krogfelt KA, Lobner-Olesen A (2013). The Alkaloid Compound Harmane Increases the Lifespan of *Caenorhabditis elegans* during Bacterial Infection, by modulating the Nematode's Innate Immune Response. *PLoS ONE* 8(3): e60519.

Jalil J, Lavandero S, Chiong M, Ocaranza MP (2005). Rho/Rho kinase signal transduction pathway in cardiovascular disease and cardiovascular remodelling. *Rev. Esp. Cardiol.* 58(8): 951-961.

James ZY, Middleton FA,and Faraone SV (2013). Genetic architecture of Wistar-Kyoto rat and spontaneously hypertensive rat substrains from different sources. *Physiol Genomics.* 45(13): 528–538.

Jespersen B, Tykocki NR, Watts SW, Cobbett PJ (2015). Measurement of smooth muscle function in the isolated tissue bath-applications to pharmacology research. *J Vis Exp* (95): 52324.

Jin JG, Neya T, Nakayama S (1989). Contractions of the guinea-pig ileum evoked by stimulation of the submucous plexus. *Eur J Pharmacol.* 161(1): 73-8.

Johnson BD, Hockerman GH, Scheuer T, Catterall WA. (1996) Distinct effects of mutations in transmembrane segment IVS6 on block of L-type calcium channels by structurally similar phenylalkylamines. *Mol Pharmacol* 50: 1388–1400.

Johnson M (1998). The β -Adrenoceptor. *American Journal of Respiratory and Critical Care Medicine* 158(2): 146–153.

Joint Formulary Committee (2018) *BNF 76: September 2018.* London: Pharmaceutical Press.

Joseph BK, Thakali KM, Moore CL, Rhee SW (2013). Ion channel remodelling in vascular smooth muscle during hypertension: Implications for novel therapeutic approaches. *Pharmacol Res*. 70(1): 126-38.

Judd E, Calhoun DA (2014).Apparent and true resistant hypertension: definition, prevalence and outcomes. *J Hum Hypertens.* 28(8): 463-8.

Jung SY, Park YJ, Park YJ, Cha SH, Lee MZ, Suh CK (2007). Na^{+}-Ca^{2+} exchanger modulates Ca^{2+} content in intracellular Ca^{2+} stores in rat osteoblasts. *Exp Mol Med* 39(4): 458-468

Kaletta T and Hengartner MO (2006). Finding function in novel targets: *C. elegans* as a model organism. *Nat. Rev. Drug Discov.* 5: 387-398.

Kamato D (2015). Structure, Function, Pharmacology, and Therapeutic Potential of the G Protein, Gα/q,11. *Front. Cardiovasc. Med.* 14.

Kang YM, Ma Y, Zheng JP, Elks C, Sriramula S, Yang ZM, Francis J (2009). Brain nuclear factor-Kappa B activation contributes to neurohumoral excitation in angiotensin II-induced hypertension. *Cardiovasc Res* 82(3): 503-512

Kang LJ, Gao JW, Schafer WR, Xie XZ, Xu S (2010). *C. elegans* TRP Family Protein TRP-4 Is a Pore-Forming Subunit of a Native Mechanotransduction Channel. *Neuron* 67(3): 381-391.

Kannisto P, Batra S, Owman C, Walles B (1987). Extracellular and intracellular calcium sources mediating contractile responses of smooth muscle in bovine ovarian follicle and ovarian artery. *Eur. J. Pharmacol.* 144(3): 299-308

Kashiwabara T, Nakajima S, Izawa T, Fukushima H, and Nishikori K (1991). Characteristics of KRN2391, a novel vasodilator, compared with those of cromakalim, pinacidil and nifedipine in rat aorta. *Eur. J. Pharmacol.* 196(1): 1–7

Katiyar C, Gupta A, Kanjilal S, Katiyar S (2012). Drug discovery from plant sources: An integrated approach. *Ayu*. 33(1): 10-9.

Kauffman RF, Schenck KW, Utterback BG, Crowe VG and Cohen ML (1987). In vitro vascular relaxation by new inotropic agents: relationship to phosphodiesterase inhibition and cyclic nucleotides. *J.Pharmacol. Exp. Ther* 242(3) : 864-872.

Kenakin TP (2012). Drug Antagonism. *Pharmacology in Drug Discovery*, 51–80.

Kerr R, Lev-Ram V, Baird G, Vincent P, Tsien RY, and Schafer WR (2000). Optical imaging of calcium transients in neurons and pharyngeal muscle of *C. elegans*. *Neuron* 26:583–594.

Khalil RA and Van Breeman C (1988). Sustained contraction of vascular smooth muscle: Calcium influx or C-kinase activation. *J. Pharmacol. Exp. Ther.* 244: 537-542.

Khan S, Higdon N, Meisheri K (1998). Coronary vasorelaxation by nitroglycerin: Involvement of plasmalemmal calcium-activated K^+ channels and intracellular Ca^{2+} stores. *J. Pharmacol. Exp. Ther.* 284:838–846.

Kim D, Nguyen MD, Dobbin MM, Fischer A, Sananbenesi F, Rodgers JT, Delalle I, Baur JA, Sui G, Armour SM, Puigserver P, Sinclair DA, Tsai LH (2007). SIRT1 deacetylase protects against neurodegeneration in models for Alzheimer's disease and amyotrophic lateral sclerosis. The *EMBO* journal 26: 3169–3179.

Kirschstein T, Rehberg M, Bajorat R, Tokay T, Porath K, Köhling R (2009). High K+-induced contraction requires depolarization-induced Ca^{2+} release from internal stores in rat gut smooth muscle. *Acta Pharmacol Sin*. 30(8): 1123-31.

Kiselyov K and Patterson RL (2009). The integrative function of TRPC channels. *Front. Biosci.* 14: 45–58

Kobayashi S, Kanaide H, Nakamura M (1985). K^+-depolarization induces a direct release of Ca^{2+} from intracellular storage sites in cultured vascular smooth muscle cells from rat aorta. *Biochem. Biophys. Res. Commun.* 129(3): 877–884.

Kobayashi S, Kitazawa T, Somlyo AV and Somlyo AP (1989). Cytosolic heparin inhibits muscarinic andα -adrenergic Ca^{2+} release in smooth muscle. *J. Biol. Chem.* 264: 17997 -18004.

Klabunde RE (2012). Cardiovascular Physiology Concepts. 2nd Edition. Philadelphia: Lippincott Williams and Wilkins

Komas N, Lugnier C and Stoclet JC (1991). Endothelium-dependent and independent relaxation of the rat aorta by cyclic nucleotide phosphodiesterase inhibitors. *Br. J. Pharmacol.* 104(2): 495–503.

Ko EA, Han J, Jung ID, Park WS (2008). Physiological roles of K^+ channels in vascular smooth muscle cells. *J Smooth Muscle Res.* 44(2): 65–81.

Korovkina VP and England SK (2002). Molecular diversity of vascular potassium channel isoforms. *Clin. Exp. Pharmacol. Physiol.* 29: 317–323.

Kubo T, Taguchi K, and Ueda M (1998). L-Type Calcium Channels in Vascular Smooth Muscle Cells from Spontaneously Hypertensive Rats: Effects of Calcium Agonist and Antagonist. *Hypertens Res.* 21(1): 33–37.

Kuga T, Sadoshima J, Tomoike H, Kanaide H, Akaike , Nakamura M (1990). Actions of Ca^{2+} antagonists on two types of Ca^{2+} channels in rat aorta smooth muscle cells in primary culture. *Circ. Res.* 67: 469–480.

Kullmann A, Daugherty SL, de Groat WC, Birder LA (2014). Bladder smooth muscle strip contractility as a method to evaluate lower urinary tract pharmacology. *J Vis Exp* 90: 51807

Kullmann FA, Downs TA, Artim D, Limberg BJ, Shah M, Contract D, de Groat WC, Rosenbaum JS (2011). Ureothelial beta3 adrenergic receptors in the rat bladder. *Neurol Urodyn* 30(1): 144-150

Kuo IYT, Wölfle SE, and Hill CE (2011). T-type calcium channels and vascular function: the new kid on the block? *J Physiol* 589(4): 783–795.

Kwok TC, Ricker N, Fraser R, Chan AW, Burns A, Stanley EF, Roy PJ (2006). A small-molecule screen in C. elegans yields a new calcium channel antagonist. *Nature* 441(7089): 91–95.

Labdrid C, Rocher I, Guery O (1989). Structure-Activity Relationships as a Response to the Pharmacological Differences in Beta Receptor Ligands. *AJH* 2:245S-251S

Lahlou M (2013). The success of natural products in drug discovery. *J Pharm Pharmacol.* 4(3A):17–31.

Lainé V, Frøkjær-Jensen C, Couchoux H and Jospin M (2011). The α1 Subunit EGL-19, the α2/δ Subunit UNC-36, and the β Subunit CCB-1 Underlie Voltage-dependent Calcium Currents inCaenorhabditis elegansStriated Muscle. *J. Biol. Chem.* 286(42): 36180–36187.

Lee CH, Poburko D, Sahota P, Sandhu J, Ruehlmann RO, and van Breemen C (2001). The mechanism of phenylephrine-mediated $[Ca^{2+}]_i$ oscillations underlying tonic contraction in the rabbit inferior vena cava. *J Physiol.* 534(3): 641–650.

Lee MK, Lim KH, Millns P, Mohankumar SK, Ng ST, Tan CS, Then SM, Mbaki Y, Ting KN (2018). Bronchodilator effects of Lignosus rhinocerotis extract on rat isolated airways is linked to the blockage of calcium entry. *Phytomedicine.* 15(42): 172-179.

Lee RY, Lobel L, Hengartner M, Horvitz HR, Avery L (1997). Mutations in the alpha1 subunit of an L-type voltage-activated Ca^{2+} channel cause myotonia in Caenorhabditis elegans. *EMBO J.* 16: 6066–6076

Leff P and Maflin GR (1988). Differences in agsnist dissociation constant estimates for 5-HT at 5-HT2 receptors: a problem of acute desensitization? *Br J. Pharmacol.* 95: 569 -577.

Lemos VS, Poburko D, Liao CH, Cole WC, Breemen CV (2007). Na^+ entry via TRPC6 causes Ca^{2+} entry via NCX reversal in ATP stimulated smooth muscle cells. *Biochem. Biophys. Res. Commun.* 352(1): 130–134.

Leung MC, Williams PL, Benedetto A, Au C, Helmcke KJ, Aschner M, Meyer JN (2008). Caenorhabditis elegans: an emerging model in biomedical and environmental toxicology. *Toxicol Sci*. 106(1): 5-28.

Levitan ES, Gealy R, Trimmer JS, Takimoto K (1995). Membrane depolarization inhibits Kv1.5 voltage-gated K^+ channel gene transcription and protein expression in pituitary cells. *J Biol Chem.* 270(11): 6036-41.

Li H, Liu X, Cui H, Chen YR, Cardounel AJ, and Zweier JL (2006). Characterization of the mechanism of cytochrome P450 reductase-cytochrome P450-mediated nitric oxide and nitrosothiol generation from organic nitrates. *J Biol Chem* 281: 12546–12554.

Li Z, Li Y, Yi Y, Huang W, Yang S, Niu W, Zhang L, Xu Z, Qu A, Wu Z, Xu T (2012). Dissecting a central flip-flop circuit that integrates contradictory sensory cues in *C. elegans* feeding regulation. *Nat. Commun* 3: 776

Lin HY, Lee YT, Chan YW, Tse G (2016). Animal models for the study of primary and secondary hypertension in humans. *Biomedical reports*. 5(6): 653–659.

Lin YC, Lin JW, Wu MS, Chen KC, Peng CC, Kang YN (2017). Effects of calcium channel blockers comparing to angiotensin-converting enzyme inhibitors and angiotensin receptor blockers in patients with hypertension and chronic kidney disease stage 3 to 5 and dialysis: A systematic review and meta-analysis. *PLOS ONE* 12(12): 0188975.

Lincoln TM, Dey N, Sellak H (2001). Invited Review: cGMP-dependent protein kinase signaling mechanisms in smooth muscle: from the regulation of tone to gene expression. *J Appl Physiol* 91(3): 1421–1430.

Lindpaintner K, Kreutz R, Ganten D (1992). Genetic variation in hypertensive and 'control' strains. What are we controlling for anyway? *Hypertens* 19: 428-430.

Linpinski CA, Lombardo F, Dominy BW, Freeney PJ (1997). Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv.Drug Deliv.Rev* 23: 3-25

Lipinski CA, Lombardo F, Dominy BW, Feeney PJ (2001). Experiment and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv.Drug Deliv.Rev* 46(1-3): 3-26

Liu D, Scholze A, Zhu Z, Kreutz R, Wehland-von-Trebra M, Zidek W, and Tepel M (2006). Increased Transient Receptor Potential Channel TRPC3 Expression in Spontaneously Hypertensive Rats. *AJH* 18: 1503–1507.

Liu D, Yang D, He H, Chen X, Cao T, Feng X, Ma L, Luo Z, Wang L, Yan Z, Zhu Z and Tepel M (2009). Increased transient receptor potential canonical type 3 channels in vasculature from hypertensive rats. *Hypertens.* 53: 70–76.

Liu D, Xiong S and Zhu Z (2014). Imbalance and dysfunction of transient receptor potential channels contribute to the pathogenesis of hypertension. *Sci China Life Sci* 57: 818–825.

Liu P, Ge Q, Chen B, Salkoff L, Kotlikoff MI, Wang ZW (2011). Genetic dissection of ion currents underlying all-or-none action potentials in C. elegans body-wall muscle cells. *J Physiol.* 589(1): 101–117.

Llorens S, Mancini A, Serrano-Díaz J, D'Alessandro A, Nava E, Alonso G, and Carmona M. (2015). Effects of Crocetin Esters and Crocetin from Crocus sativus L. on Aortic Contractility in Rat Genetic Hypertension. *Molecules,* 20(9):17570–17584.

Lockette W, Otsuka Y, and Carretero O (1986). The loss of endothelium-dependent vascular relaxation in hypertension. *Hypertens.* 8: II61– II66.

Loirand G, Guérin P, Pacaud P (2006). Rho Kinases in Cardiovascular Physiology and Pathophysiology. *Circ Res* 98(3): 322-334.

Loong BJ, Mbaki Y, Lim KH, KN Ting (2015). Contractile function of smooth muscle retained after overnight storage', *Naunyn-Schmiedeberg's Archives of Pharmacology*. *Naunyn Schmiedebergs Arch. Pharmacol* 388(10): 1061–1067.

Low AM, Kwan CY AND Daniel EE (1992). Evidence for two types of internal Ca^{2+} stores in canine mesenteric artery with different refilling mechanisms. *Am. J. Physiol.* 262: H31–H37.

Lu R, Alioua A, Kumar Y, Kundu P, Eghbali M, Weisstaub NV, Gingrich JA, Stefani E, Toro L (2008). c-Src tyrosine kinase, a critical component for 5-HT2A receptor-mediated contraction in rat aorta. *J Physiol* 586(16): 3855–3869.

Lund-Johansen P, Omvik P (1987). Central hemodynamic changes of calcium antagonists at rest and during exercise in essential hypertension. *J Cardiovasc Pharmacol.* 10(1): S139-148

Lund-Johansen P (1990). Hemodynamic effects of felodipine in hypertension: a review. *J Cardiovasc Pharmacol* 15(4): 34-39.

Lurie KG, Tsujimoto G and Hoffman BB (1985). Desensitization of alpha-1 adrenergic receptor-mediated vascular smooth muscle contraction. *J Pharmacol Exp Ther* 234 (1): 147-152.

Lüscher TF, Vanhoutte PM (1986). Endothelium-dependent contractions to acetylcholine in the aorta of the spontaneously hypertensive rat. *Hypertens.*8: 344–348.

Lüscher TF, Tanner FC, Tschudi MR, Noll G (1993). Endothelial dysfunction in coronary artery disease. *Annu Rev Med.* 44: 395–418

Ma B, Shatsky M, Wolfson HJ, Nussinov R (2002). Multiple diverse ligands binding at a single protein site: a matter of pre-existing populations. *Protein Sci.* 11(2): 184-197

Ma W, Dong JY, Ma D, Wells JW (2011). Cleavage-resistant fusion proteins of the M(2) muscarinic receptor and Galpha(i1). Homotropic and heterotropic effects in the binding of ligands. *Biochim. Biophys. Acta.* 1810(6): 592-602.

Maione F, Cicala C, Musciacco G, De Feo V, Amat AG, Ialenti A, Mascolo N (2013). Phenols, alkaloids and terpenes from medicinal plants with antihypertensive and vasorelaxant activities. A review of natural products as leads to potential therapeutic agents. *Nat. Prod. Commun.* 8(4): 539-544.

Mann SJ (2017). Redefining beta-blocker use in hypertension: selecting the right beta-blocker and the right patient. *J Am Soc Hypertens* 11(1): 54–65.

Manrique C, Lastra G, Gardner M, Sowers JR (2009). The renin angiotensin aldosterone system in hypertension: roles of insulin resistance and oxidative stress. *Med. Clin. North Am.* 93(3): 569–582.

Marinissen MJ, Gutkind JS (2001). G-protein-coupled receptors and signaling networks: emerging paradigms. *Trends Pharmacol Sci* 22: 368–376.

Marzetti E, Calvani R, Cesari M, Buford TW, Lorenzi M, Behnke BJ, Leeuwenburgh C (2013). Mitochondrial dysfunction and sarcopenia of aging: from signalling pathways to clinical trials. *Int J Biochem Cell Biol.* 45: 2288–2301.

Mashiko S, Onda T, Hamano M, Tomita I, Ikeda M, Tomita T (1995). Reduced spontaneous relaxation following maximum contraction by Angiotensin II in endothelium-denuded aorta from SHRSP. *Jpn Heart J* 36(4): 528.

Masson J, Emerit MB, Hamon M, Darmon M (2012). Serotonergic signaling: multiple effectors and pleiotropic effects. *Wiley Interdisciplinary Reviews: Membrane Transport and Signaling* 1(6): 685–713.

Materson BJ (1995). Calcium channel blockers: Is it time to split the lump? *Am. J. Hypertens.* 8(3): 325–329

Matsuda JJ, Volk KA, Shibata EF (1990). Calcium currents in isolated rabbit coronary arterial smooth muscle myocytes. *J. Physiol*. 427: 657–680.

Maurice D. H, Ke H, Ahmad F, Wang Y, Chung J, Manganiello VC (2014). Advances in targeting cyclic nucleotide phosphodiesterases. *Nat. rev* 13(4): 290–314.

McFadzean I and Gibson A (2002). The developing relationship between receptor-operated and store-operated calcium channels in smooth muscle. *Br J Pharmacol* 135(1): 1–13.

McGill JB, Haffner S, Rees TJ, Sowers JR, Tershakorce AM, Weber M (2009). Progress and controversies: Treating obesity and insulin resistance in the context of hypertension. *J. Clin Hypertens* (Greenwich) 22:36-41

McKay JP, Raizen DM, Gottschalk A, Schafer WR, and Avery L (2004). *eat-2* and *eat-18* are required for nicotinic neurotransmission in the *Caenorhabditis elegans* pharynx. *Genetics* 166*:* 161-169.

Méndez-López I, Bomfim GHS, Musial DC, Arranz-Tagarro JA, Velasco-Martín JP, Regadera J, Padín JF (2017). Altered mitochondrial function, capacitative calcium entry and contractions in the aorta of hypertensive rats. *J Hypertens.* 35(8): 1594–1608.

Messerli FH, Bangalore S, Bavishi C, Rimoldi SF (2018). Angiotensin-Converting Enzyme Inhibitors in Hypertension: To Use or Not to Use? *J. Am. Coll. Cardiol.* 71(13): 1474–1482.

Meyer JN, Lord CA, Yang XY (2010). Intracellular uptake and associated toxicity of silver nanoparticles in Caenorhabditis elegans. *Aquat Toxicol*. 100: 140–150.

Michel MC, Brodde OE, and Insel AP (1990). Peripheral Adrenergic Receptors in Hypertension. *Hypertens* 16(2): 107-116

Mita M, Ito K, Taira K, Nakagawa J, Walsh MP, Shoji M (2010). Attenuation of store-operated Ca^{2+} entry and enhanced expression of TRPC channels in caudal artery smooth muscle from Type 2 diabetic Goto-Kakizaki rats. *Clin Exp Pharmacol Physiol* 37: 670–678.

Mizuno N and Itoh H (2009). Functions and Regulatory Mechanisms of G_q-Signaling Pathways. *Neurosignals* 17(1): 42–54.

Moerman DG, and Fire A (1997). Muscle Structure, Function and Development. In *C. elegans* II, D. L. Riddle, T. Blumenthal, B. J. Meyer, and J. R. Priess, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), 417-470.

Molnár T, Yarishkin O, Iuso A, Barabas P, Jones B, Marc RE, Phuong TT and Križa D (2016). Store-Operated Calcium Entry in Müller Glia Is Controlled by Synergistic Activation of TRPC and Orai Channels. *J Neurosci* 36(11): 3184-3198.

Montezano AC, Touyz RM (2012). Molecular mechanism of hypertension. Reactive oxygen species and antioxidants. A basic science update for the clinician. *Can J Cardiol* 28: 288-295

Montgomery BS, Fry CH (1992). The action potential and net membrane currents in isolated human detrusor smooth muscle cells. *J Urol* 147: 176-84

Moore KL, Dalley AF, Agur AM (2013). *Clinically Oriented Anatomy, 7th ed*. Lippincott Williams & Wilkins. 241–246.

Moosmang S, Schulla V, Welling A, Feil R, Feil S, Wegener J (2003). Dominant role of smooth muscle L-type channel $Ca_{v1.2}$ for blood pressure regulation. *EMBO* 22 6027–6034.

Morris SJ and Shore AC (1996). Skin blood flow responses to the iontophoresis of acetylcholine and sodium nitroprusside in man: possible mechanisms. *J Physiol*. 496(2): 531-42

Mostaghim R, Maddox YT, Ramwell PW (1986). Endothelial potentiation of relaxation response to beta adrenoceptor blocking agents. *J Pharmacol Exp Ther* 239(3): 797-801.

Musini VM, Rezapour P, Wright JM, Bassett K, Jauca CD (2015). Blood pressure-lowering efficacy of loop diuretics for primary hypertension. *The Cochrane database of systematic reviews* (5).

Mushtaq S, Abbasi BH, Uzair B, Abbasi R (2018). Natural products as reservoirs of novel therapeutic agents. *EXCLI J* 17: 420-451.

Muthusamy VV (2016). BR 08-3 Management of dyslipidemia in hypertension. *J Hypertens* 34.

Nakashima M and Vanhoutte PM (1995). Isoproterenol causes hyperpolarization through opening of ATP-sensitive potassium channels in vascular smooth muscle of the canine saphenous vein. *J. Pharmacol. Exp. Ther.* 272: 379–384.

Nansseu JRN, Noubiap JJN, Mengnjo MK, Aminde LN, Essouma M, Jingi AM, Bigna JJR (2016). The highly neglected burden of resistant hypertension in Africa: a systematic review and meta-analysis. *BMJ Open* 6(9): e011452.

Navia MA, Chaturvedi PR (1996). Design principles for orally bioavailable drugs. *Drug Discov. Today* 1(5):179-189.

Nazer M, van Breemen C. (1998). Functional linkage of Na^+-Ca^{2+} exchange and sarcoplasmic reticulum Ca^{2+} release mediates Ca^{2+} cycling in vascular smooth muscle. *Cell Calcium* 24 275–283.

Nelson MT, Huang Y, Brayden JE, Hescheler J and Standen NB (1990). Arterial dilations in response to calcitonin gene-related peptide involve activation of K^+ channels. *Nature* 344: 770– 773.

Nelson MT, Cheng H, Rubart M, Santana LF, Bonev AD, Knot HJ (1995). Relaxation of arterial smooth muscle by calcium sparks. *Science* 270: 633-7.

Nelson MT, Patlak JB, Worley JF, Standen NB (1990). Calcium channels, potassium channels, and voltage dependence of arterial smooth muscle tone. *Am. J. Physiol*. 259: 3–18

Nelson MT and Quayle JM (1995). Physiological roles and properties of potassium channels in arterial smooth muscle. *Am. J. Physiol.* 268: 799–822.

Niazmand S, Fereidouni E, Mahmoudabady M, Mousavi SM (2014). Endothelium-independent vasorelaxant effects of hydroalcoholic extract from *Nigella sativa* seed in rat aorta: the roles of Ca^{2+} and K^{+} channels. *Biomed Res. Int.* 2014:247054.

Nilius B and Szallasi A (2014). Transient Receptor Potential Channels as Drug Targets: From the Science of Basic Research to the Art of Medicine. *Pharmacol. Rev.* 66(3): 676-814.

Noorani MM, Noel RC, Marrelli SP (2011). Upregulated TRPC 3 and downregulated TRPC 1 channel expression during hypertension is associated with increased vascular contractility in rat. *Front. Physiol.* 2.

Nordqvist C (2018). Coronary Heart Disease. What you need to know. Medical news today article

Okada T, Shimizu S, Wakamori M, Maeda A, Kurosaki T, Takada N (1998). Molecular cloning and functional characterization of a novel receptor-activated TRP Ca^{2+} channel from mouse brain. *J Biol Chem*. 273: 10279–10287.

Okamoto K and Aoki K (1963). Development of a strain of spontaneously hypertensive rats. *Jpn Circ J.* 27: 282-93.

Ohya Y, Tsuchihashi T, Kagiyama S, Abe I, Fujishima M (1998). Single L-type calcium channels in smooth muscle cells from resistance arteries of spontaneously hypertensive rats. *Hypertens* 31(5): 1125-9

Oikonomou G and Shaham S (2011). The glia of *Caenorhabditis elegans*. *Glia* 59: 1253–1263.

O'Reilly LP, Luke CJ, Perlmutter DH, Silverman GA, Pak SC (2014). *C. elegans* in high-throughput drug discovery. *Adv. Drug Deliv. Rev.* 69(70): 247–253.

Oshima T, Ozono R, Yano Y, Higashi Y, Teragawa H, Miho N, Ishida T, Ishida M, Yoshizumi M, Kambe M (2005). Beneficial Effect of T-Type Calcium Channel Blockers on Endothelial Function in Patients with Essential Hypertension. *Hypertens Res* (28): 889–894.

Otsuka T, Takada H, Nishiyama Y, Kodani E, Saiki Y, Kato K, Kawada T (2016). Dyslipidemia and the Risk of Developing Hypertension in a Working-Age Male Population. *J. Am. Heart. Assoc.* 5(3): 3053–3053.

Ozkor MA and Quyyumi AA (2011). Endothelium-Derived Hyperpolarizing Factor and Vascular Function. *Cardiol Res Pract* 2011: 1–12.

Packham R, Walker RJ, and Holden-Dye L. (2010). The effect of a selective octopamine antagonist, epinastine, on pharyngeal pumping in *Caenorhabditis* elegans. *Invert. Neurosci.* 10(1): 47–52.

Page AP and Johnstone IL (2007). The cuticle (March 19, 2007), *WormBook*, ed. The *C. elegans* Research Community.

Panthiya L, Pantan R, Tocharus J, Nakaew A, Suksamrarn A, Tocharus C (2019). Endothelium-dependent and endothelium-independent vasorelaxant effects of tiliacorinine 12'-O-acetate and mechanisms on isolated rat aorta. *Biomed Pharmacother.* 109: 2090-2099.

Paoletti R and Govoni S (1987). Classification of calcium antagonists: proposal of the WHO committee Pharmacological Research Communications 19(3): 195–208.

Paton WDM (1955). The response of the guine-pig ileum to electrical stimulation by coxial electrodes. *J. Physiol London* 127: 40- 41.

Paul M, Poyan Mehr A, Kreutz R (2006). Physiology of local renin-angiotensin systems. *Physiol Rev.* 86:747–803.

Paul D (2015). Quantitative Parameters of Drug Action. *Reference Module in Biomedical Sciences, Elsevier.*

Pelaia G, Renda T, Gallelli L, Vatrella A, Busceti MT, Agati S, Caputi M, Cazzola M, Maselli R, Marsico SA (2008). Molecular mechanisms underlying airway smooth muscle contraction and proliferation: Implications for asthma. *Respir Med* 102(8): 1173–1181.

Petrascheck M, Ye X, Buck LB (2007). An antidepressant that extends lifespan in adult *Caenorhabditis elegans*. *Nature.* 450: 553–556.

Pesic A, Madden JA, Pesic M, Rusch NJ (2004). High blood pressure upregulates arterial L-type Ca^{2+} channels: is membrane depolarization the signal? *Circ Res*. 94(10): 97-104.

Phillipson JD (2001). Phytochemistry and medicinal plants. *Phytochemistry.* 56:237–243.

Popovi Z, Mati R, Bojovi S, Stefanovi M, Vidakovi V (2016) Ethnobotany and herbal medicine in modern complementary and alternative medicine: An overview of publications in the field of I&C medicine 2001-2013. *J Ethnopharmacol* 181: 182-192.

Poruthukaren KJ, Palatty PL, Baliga MS, Suresh S (2014). Clinical evaluation of *Viscum album* mother tincture as an antihypertensive: a pilot study. *J Evid Based Complementary Altern Med.* 19:31–5.

Poulter NR, Prabhakaran D, Caulfield M (2015). Hypertension. *Hypertens.* 386(9995): 801–812.

Potje SR, Grando MD, Chignalia AZ, Antoniali C and Bendhack LM (2019). Reduced caveolae density in arteries of SHR contributes to endothelial dysfunction and ROS production. *Scientific Reports* 9: 6696.

Pravenec M , Klír P, Kren V, Zicha J, and Kunes J (1989). An analysis of spontaneous hypertension in spontaneously hypertensive rats by means of new recombinant inbred strains. *J Hypertens.* 7: 217–221.

Quayle JM, Nelson MT and Standen NB (1997). ATP-sensitive and inwardly rectifying potassium channels in smooth muscle. *Physiol. Rev.* 77: 1165–1232.

Quignard JF, Harricane MC, Menard C, Lory P, Nargeot J, Capron L, Mornet D, Richard ST (2001). Transient down-regulation of L-type Ca^{2+} channel and dystrophin expression after ballon injury in rat aortic cells. *Cardiovasc Res* 49(1): 177-188.

Raizen DM and Avery L (1994). Electrical activity and behavior in the pharynx of *Caenorhabditis elegans*. *Neuron* 12: 483-495.

Raizen DM, Lee RY and Avery L (1995). Interacting genes required for pharyngeal excitation by motor neuron MC in *Caenorhabditis elegans*. Genetics *141*: 1365-1382.

Ratz PH and Flaim SF (1984). Mechanism of 5-HT Contraction in Isolated Bovine Ventricular Coronary Arteries Evidence for Transient Receptor-Operated Calcium Influx Channels. *Circ.Res.* 54 (2)

Ratz PH, Berg KM, Urban NH, Miner AS (2005). Regulation of smooth muscle calcium sensitivity: KCl as a calcium-sensitizing stimulus. *American Journal of Physiology - Cell Physiology*, 288(4): 769–783.

Ratz PH, Berg KM (2006). 2-Aminoethoxydiphenyl borate inhibits KCl-induced vascular smooth muscle contraction. *Eur. J. Pharmacol.* 541(3): 177-183.

Rawat S, Jugran AK, Bahukhandi A, Bahuguna A, Bhatt ID, Rawal RS, Dhar U (2016). Anti-oxidant and anti-microbial properties of some ethno-therapeutically important medicinal plants of Indian Himalayan Region. *3 Biotech*. 6(2):154.

Re M, Pampillo M, Savard M, Dubuc C, McArdle CA, Millar RP, Babwah AV (2010). The Human Gonadotropin Releasing Hormone Type I Receptor Is a Functional Intracellular GPCR Expressed on the Nuclear Membrane. *PLoS ONE,* 5(7)

Regmi SG, Rolland SG, Conradt B (2014). Age-dependent changes in mitochondrial morphology and volume are not predictors of lifespan. *Aging (Albany NY)* 6:118–130.

Riddle DL, Blumenthal T, Meyer BJ, *et al.,* editors (1997). *C. elegans* II. 2nd edition. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 1997. Section II, Sexual Dimorphism.

Robinson A, Colbran R (2013). Calcium/calmodulin-dependent Protein Kinases. In Lennarz W, Lane D (eds). Encyclopedia of Biological Chemistry (2nd ed). *Elsevier* 304-309.

Robishaw JD, Berlot CH (2004). Translating G protein subunit diversity into functional specificity. *Curr. Opin. Cell. Biol.* 16(2): 206-209

Rochette L, Lorin J, Zeller M, Guilland JC, Lorgis L, Cottin Y, Vergely C (2013). Nitric oxide synthase inhibition and oxidative stress in cardiovascular diseases: possible therapeutic targets? *Pharmacol Ther.* 140(3): 239-57.

Rodat-Despoix L, Aires V, Ducret T, Marthan R, Savineau JP, Rousseau E, Guibert C (2009). Signalling pathways involved in the contractile response to 5-HT in the human pulmonary artery. *Eur Respir J* 34: 1338-1347

Rodrigo E, Maeso R, Muñoz-García R, Navarro-Cid J, Ruilope LM, Cachofeiro V and Lahera V (1997). Endothelial dysfunction in spontaneously hypertensive rats. *J Hypertens* 15(6): 613–618.

Rodriguez R, Ventura-Martinez R, Santiago-Meija J, Avila-Costa M, Fortoul TI (2009). Altered responsiveness of the guinea-pig isolated ileum to smooth muscle stimulants and to electrical stimulation after in-situ ischaemia. *Br J Pharmacol.* 147(4): 371-378

Roffel AF, Elzinga CR, and Zaagsma J (1990). Muscarinic M3 receptors mediate contraction of human central and peripheral airway smooth muscle. *Pulm Pharmacol Ther* 3(1): 47–51

Rosenbaum DM, Rasmussen SG, Kobilka BK (2009). The structure and function of G-protein-coupled receptors. *Nature*. 459(7245): 356-63.

Rosker C, Graziani A, Lukas M, Eder P, Zhu MX, Romanin C, Groschner K (2004). Ca^{2+} signaling by TRPC3 involves Na^{+} entry and local coupling to the Na^{+}/Ca^{2+} exchanger. *J Biol Chem*. 279: 13696–704.

Ross M and Pawlina W (2011). *Histology: A Text and Atlas. Sixth edition*. Lippincott Williams & Wilkins.

Roush GC and Sica DA (2016). Diuretics for Hypertension: A Review and Update. *Am. J. Hypertens.* 29(10): 1130–1137.

Rubanyi GM (1991). Endothelium-derived relaxing and contracting factors. *J Cell Biochem.* 46(1): 27-36.

Rubio AR, Morales-Segura MA (2004). Nitric oxide, an iceberg in cardiovascular physiology: far beyond vessel tone control*. Arch. Med. Res*. 35: 1–11.

Ruffolo RR, Messick K (1985). Evaluation of the alpha-1 and alpha-2 adrenoceptor mediated effects of a series of dimethoxy substituted tolazoline derivatives in the cardiovascular system of the pithed rat. *JPET* 232(1): 94-99.

Ruschitzka F and Taddei S (2012). Angiotensin-converting enzyme inhibitors: first-line agents in cardiovascular protection? *Eur. Heart J.* 33(16): 1996–1998.

Rusch NJ, De Lucena RG, Wooldridge TA, England SK, Cowley AJ (1992). A Ca^{2+} dependent K^{+} current is enhanced in arterial membranes of hypertensive rats. *Hypertens* 19: 301–307.

Saini HK, Sharma SK, Zahradka P, Kumamoto H, Takeda N and Dhalla NS (2003). Attenuation of the serotonin-induced increase in intracellular calcium in rat aortic smooth muscle cells by sarpogrelate. *Can. J. Physiol. Pharmacol.* 81(11): 1056–1063.

Salgueiro WG, Xavier MC and Duarte LF (2014). Direct synthesis of 4-organylsulfenyl-7-chloro quinolines and their toxicological and pharmacological activities in Caenorhabditis elegans. *Eur J Med Chem*. 75: 448–459.

Sandu OA, Ito M, Begum N (2001). Selected contribution: Insulin utilises NO/cGMP pathway to activate myosin phosphatase via Rho inhibition in vascular smooth muscle. *J Appl. Physiol*. 91:1475-1482

Sarasija S, Laboy JT, Ashkavand Z, Bonner J, Tang Y, Norman KR (2018). Presenilin mutations deregulate mitochondrial Ca^{2+} homeostasis and metabolic activity causing neurodegeneration in *Caenorhabditis elegans*. *Elife*. 10(7): e33052.

Saur T, DeMarco SE, Ortiz A, Sliwoski GR, Hao L, Wang X, Buttner EA (2013). A genome-wide RNAi screen in Caenorhabditis elegans identifies the nicotinic acetylcholine receptor subunit ACR-7 as an antipsychotic drug target. *PLoS genetics* 9(2): 1003313.

Scriabine, A and Peklak, GJ (1970). Electrical stimulation of isolated guinea pig ileum. *J. Appl. Physiol.* 28(6): 860–862.

Schafer WR (2005). Deciphering the Neural and Molecular Mechanisms of *C. elegans* Behavior. *Curr. Biol.* 15: 723–729.

Sharif-Naeini R, Dedman A, Folgering JH, Duprat F, Patel A, Nilius B, Honore E (2008). TRP channels and mechanosensory transduction: insights into the arterial myogenic response. *Pflugers Arch* 456: 529-540

Shaye DD and Greenwald I (2011). OrthoList: a compendium of *C. elegans* genes with human orthologs. *PLoS ONE* 6.

Seca A and Pinto D (2018). Plant Secondary Metabolites as Anticancer Agents: Successes in Clinical Trials and Therapeutic Application. *Int J Mol Sci* 19(1): 263

Senadheera S, Kim Y, Grayson T.H, Toemoe S, Kochukov MY, Abramowitz J, Housley GD, Bertrand RL, Chadha PS, Bertrand PP (2012). Transient receptor potential canonical type 3 channels facilitate endothelium-derived hyperpolarization-mediated resistance artery vasodilator activity. *Cardiovasc. Res.* 95: 439–447

Sharma V, He C, Sacca-Schaeffer J, Brzozowski E, Martin-Herranz DE, Mendelowitz Z, Fitzpatrick DA, O'Halloran DM (2013). Insight into the family of Na^{+}/Ca^{2+} exchangers of Caenorhabditis elegans. *Genetics* 195(2): 611-9.

Shattock MJ, Ottolia M, Bers DM, Blaustein MP, Boguslavskyi A, Bossuyt J, Bridge JH, Chen-Izu Y, Clancy CE, Edwards A, Goldhaber J, Kaplan J, Lingrel JB, Pavlovic D, Philipson K, Sipido KR, Xie ZJ (2015). Na^{+}/Ca^{2+} exchange and Na^{+}/K^{+}-ATPase in the heart. *J Physiol*. 593(6): 1361-82.

Shen L, Xiao J, Ye H, Wang D (2009). Toxicity evaluation in nematode Caenorhabditis elegans after chronic metal exposure. *Environ Toxicol Pharmacol*. 28: 125–132.

Shen Q, Rigor RR, Pivetti CD, Wu MH, Yuan SY (2010). Myosin light chain kinase in microvascular endothelial barrier function. *Cardiovasc Res*. 87(2): 272-80.

Shen WW and Demaurex N (2012). Calcium Cycling in Synthetic and Contractile Phasic or Tonic Vascular Smooth Muscle Cells. *Biochem. Soc. Trans.* 40(1): 1128

Shimokowa H, Yasutake H, Fujii K, Owada MK, Nakaike R, Fukumoto Y, Takayanagi T, Nagao T, Egashira K, Fujishima M, Takeshita A (1996). The importance of the hyperpolarizing mechanism increases as the vessel size decreases in endothelium-dependent relaxations in rat mesenteric circulation. *J. Cardiovasc. Pharmacol.* 28: 703–711.

Shimozono S, Fukano T, Kimura KD, Mori I, Kirino Y, Miyawaki A (2004). Slow Ca^{2+} dynamics in pharyngeal muscles in Caenorhabditis elegans during fast pumping. *EMBO Rep.* 5(5): 521–526.

Shogakiuchi Y, Kanaide H, Nakamura M (1991). Cytosolic calcium transients differ between porcine coronary arterial and aortic smooth muscle cells in primary culture. *Circ. Res* 202(2): 143-9.

Shtonda BB and Avery L (2005). *CCA-1, EGL-19 and EXP-2 currents shape action potentials in the Caenorhabditis elegans pharynx*. *J Exp Biol* 208: 2177–2190.

Shukla V, Saxena S, Tiwari S (2016). *Caenorhabditis elegans* as a Toolkit for Studying Mammalian Aging Pathways. *J Nutr Food Sci* 6:5.

Shyn SI, Kerr R and Schafer WR (2003). Serotonin and Go modulate functional states of neurons and muscles controlling *C. elegans* egg-laying behavior. *Curr. Biol*. *13:* 1910–1915.

Sica DA, Prisant LM (2007). Pharmacologic and therapeutic considerations in hypertension therapy with calcium channel blockers: focus on verapamil. *J Clin Hypertens.* 9:1-22.

Silva BR, Laena Pernomian L, and Bendhack LM (2012). Contribution of oxidative stress to endothelial dysfunction in hypertension. *Front Physiol.* 3: 441.

Singh BN, Collett JT, Chew CYC (1978). Verapamil: A review of its pharmacological properties and therapeutic use. *Drugs* 15: 169-97.

Singh A, Hildebrand ME, Garcia E, and Snutch TP (2010). The transient receptor potential channel antagonist SKF96365 is a potent blocker of low-voltage-activated T-type calcium channels. *Br J Pharmacol.* 160(6): 1464–1475.

Smith JM, Jones SB, Bylund DB, Jones AW (1987). Characterization of the α_1-adrenergic receptors in the thoracic aorta of control and aldosterone hypertensive rats: Correlation of radioligand binding with potassium efflux and contraction. *Pharmacol Exp Ther* 241: 882-890

Sobey CG (2001). Potassium channel function in vascular disease. *Arterioscler. Thromb. Vasc. Biol.* 21: 28–38.

Sofola OA, Adegunloye BJ and Knill A (2003). The effects of nifedipine and thapsigargin on the responses of pressurized rat mesenteric artery to 5-hydroxytryptamine and norepinephrine. *Vascul Pharmacol.* 40(1): 29–33.

Son HG, Altintas O, Kim EJE, Kwon S, Lee SV (2019). Age-dependent changes and biomarkers of aging in *Caenorhabditis elegans. Aging Cell*. 18(2): e12853.

Sonkusare S, Palade PT, Marsh JD, Telemaque S, Pesic A, Rusch NJ (2006). Vascular calcium channels and high blood pressure: pathophysiology and therapeutic implications. *Vascul Pharmacol*. 44(3): 131-42.

Somlyo AV, Bond M, Somlyo AP and Scarpa A (1985). Inositol trisphosphate-induced calcium release and contraction in vascular smooth muscle. *Proc. Natl. Acad. Sci. USA* 82: 5231 -5235.

Sowers JR, Epstein M and Frohlich ED (2001). Diabetes, Hypertension, and Cardiovascular Disease. *Hypertens* 37(4): 1053 -1059.

Sowers JR (2004). Insulin resistance and hypertension. *Am. J. Physiol. Heart Circ. Physiol.* 286(5): 1597–1602.

Standen NB and Quayle JM (1998). K^+ channel modulation in arterial smooth muscle. *Acta Physiol. Scand.* 164: 549–557.

Stangherlin A and Zaccolo M (2012). Phosphodiesterases and subcellular compartmentalized cAMP signaling in the cardiovascular system. *Am J Physiol Heart Circ Physiol.* 302(2): 379-90.

Strehler EE, Caride AJ, Filoteo AG, Xiong Y, Penniston JT, Enyedi A (2007). Plasma membrane Ca^{2+} ATPases as dynamic regulators of cellular calcium handling. *Annals of the New York Academy of Sciences* 1099: 226-36.

Stiernagle T (2006). Maintenance of *C.elegans* Wormbook ed The *C.elegans* Research Community, Wormbook

Striessnig, J, Ortner, NJ, and Pinggera A (2015). Pharmacology of L-type Calcium Channels: Novel Drugs for Old Targets? *Curr. Mol. Pharmacol.* 8(2): 110–122.

Stutz K, Kaech A, Aebi M, Kunzler M, Hengartner MO (2015). Disruption of the *C. elegans* Intestinal Brush Border by the Fungal Lectin CCL2 Phenocopies Dietary Lectin Toxicity in Mammals. *PLoS One* 10: e0129381.

Sugiyama T, Yoshizumi M, Takaku F, and Yazaki Y (1990). Abnormal calcium handling in vascular smooth muscle cells of spontaneously hypertensive rats. *J Hypertens* 8(4): 369–375.

Sulston JE and Horvitz HR (1977). Post-embryonic cell lineages of the nematode, *Caenorhabditis elegans*. *Dev. Biol.* 56: 110-156.

Sulston J and Hodgkin J (1988) Methods. in: Wood, W.B. (ed.) The Nematode *Caenorhabditis elegans.* Cold Spring Harbor Laboratory, Cold Spring Harbor: 587-606.

Sun T, Liu R and Cao YX (2011). Vasorelaxant and antihypertensive effects of formononetin through endothelium-dependent and -independent mechanisms. *Acta pharmacologica Sinica*, 32(8): 1009–1018.

Surendran P, Drenos F, Young R, Warren H, Cook JP, Barnes DR (2016). Trans-ancestry meta-analyses identify rare and common variants associated with blood pressure and hypertension. *Nature Genetics* 48: 1151.

Swiatkowski P, Sesti F (2013). Delayed pharyngeal repolarization promotes abnormal calcium buildup in aging muscle. *Biochem Biophys Res Commun*. 433: 354–357.

Svobodova B and Groschner K (2016). Mechanisms of lipid regulation and lipid gating in TRPC channels. *Cell Calcium* 59(6): 271–279.

Szasz T, Webb RC (2017). Rho-Mancing to Sensitize Calcium Signaling for Contraction in the Vasculature: Role of Rho Kinase. *Vascul Pharmacol* 78: 303–322.

Sze JY, Victor M, Loer C, Shi Y and Ruvkun G (2000). Food and metabolic signalling defects in a *Caenorhabditis elegans* serotonin-synthesis mutant. *Nature* 403(6769): 560–564.

Takata Y, Kato H (1996). Adrenoceptors in SHR: alterations in binding characteristics and intracellular signal transduction pathways. *Life Sci* 58: 91-106

Takeuchi T, Tanaka K, Nakajima H, Matsui M, Azuma YT (2007). M_2 and M_3 muscarinic receptors are involved in enteric nerve-mediated contraction of the mouse ileum: Findings obtained with muscarinic-receptor knockout mouse. *Am J Physiol Gastrointest Liver Physiol.* 292(1): 154-164.

Taniguchi S, Furukawa K, Sasamura S, Ohizumi Y, Seya K, Motomura S (2004). Gene expression and functional activity of sodium/calcium exchanger enhanced in vascular smooth muscle cells of spontaneously hypertensive rats. *J Cardiovasc Pharmacol*. 3(5): 629-37.

Tanoue A, Nasa Y, Koshimizu T, Shinoura H, Oshikawa S, Kawai T, Sunada S,Takeo S and Tsujimoto G (2002). The α_{1D}-adrenergic receptor directly regulates arterial blood pressure via vasoconstriction. *J Clin Invest* 109(6): 765–775.

Tammaro P, Smith AL, Hutchings SR, Smirnov SV (2004). Pharmacological evidence for a key role of voltage-gated K^+ channels in the function of rat aortic smooth muscle cells. *Br J Pharmacol*. 143(2): 303-17.

Teramoto N (2006). Physiological roles of ATP-sensitive K+ channels in smooth muscle. *J. Physiol. (Lond.)* 572: 617–624.

Thilo F, Loddenkemper C, Berg E, Zidek W, Tepel M (2009). Increased TRPC3 expression in vascular endothelium of patients with malignant hypertension. *Mod Pathol*. 22: 426–430.

Thornloe K, Nelson M. (2005). Ion channels in smooth muscle: regulators of intracellular calcium and contractility. *Can. J. Physiol. Pharmacol.* 83 215–242.

Tice RR, Austin CP, Robert JK. Bucher JR (2013). Improving the human hazard characterization of chemical: A Tox21 update. *Environ Health Perspect* 121(7): 756-765.

Tissenbaum HA (2015). Using *C. elegans* for aging research. *Invertebr Reprod Dev* 59: 59-63.

Tocci G, Battistoni A, Passerini J, Musumeci MB, Francia P, Ferrucci A, Volpe M (2014). Calcium Channel Blockers and Hypertension. *J. Cardiovasc. Pharmacol. Ther* 20(2): 121–130.

Toke A, Meyer T (2001). Hemodynamic effects of angiotenin II in the kidney. In: Wolf G, editor. The renin-angiotensin system and progression of renal diseases. 1st ed. Basel: Karger: 34–46.

Touyz RM, Alves-Lopes R, Rios FJ, Camargo LL, Anagnostopoulou A, Arner A, Montezano AC (2018). Vascular smooth muscle contraction in hypertension. *Cardiovasc Res* 114(4): 529–539.

Townsend N, Nichols M, Scarborough P, Rayner M (2015). Cardiovascular disease in Europe--epidemiological update. *Eur Heart J.* 21; 36(40): 2696-705.

Trippodo NC and Frohlich ED (1981). Similarities of genetic (spontaneous) hypertension: Man and rat. *Circ. Res.* 48: 309 319.

Trojanowski NF, Padovan-Merhar O, Raizen DM and Fang-Yen C (2014). Neural and genetic degeneracy underlies *Caenorhabditis elegans*feeding behavior. *J. Neurophysiol.* 112: 951–961.

Tsalik EL, Niacaris T, Wenick AS, Pau K, Avery L, and Hobert O (2003). LIM homeobox gene-dependent expression of biogenic amine receptors in restricted regions of the *C. elegans* nervous system. *Dev. Biol. 263*, 81-102.

Tuteja N (2009). Signaling through G protein coupled receptors. *Plant Signal Behav* 4(10): 942–947.

Tykocki NR, Jackson WF, Watts SW (2012). Reverse-mode Na^+/Ca^{2+} exchange is an important mediator of venous contraction. *Pharmacological research : the official journal of the Italian Pharmacological Society* 66(6): 544–554.

Tykocki NR, Thompson JM, Jackson WF, Watts SW (2013). Ryanodine receptors are uncoupled from contraction in rat vena cava. *Cell Calcium* 53: 112-119

Unno T, Matsuyama H, Sakamoto T, Uchiyama M, Izumi Y, Okamoto H, Komori S (2005). M_2 and M_3 muscarinic receptor-mediated contractions in longitudinal smooth muscle of the ileum studied with receptor knockout mice. *Br J Pharmacol* 146(1): 98–108.

Utz J, Eckert R, Trautwein W (1999). Changes of intracellular calcium concentrations by phenylephrine in renal arterial smooth muscle cells. *Pflugers Arch.* 438(6): 725-31.

Valet B, Dupuis B, Chopin C (1991). Dobutamine: mechanisms of action and use in acute cardiovascular pathology. *Ann Cardio Angeiol* 40(6): 397-402.

van Breemen C (1969) Blockade of membrane calcium fluxes by lanthanum in relation to vascular smooth muscle contractility. *Arch Int Physiol Biochim* 77: 710-717.

van Brummelen P, Jie K, van Zwieten PA (1986). Alpha-adrenergic receptors in human blood vessels. *Br J Clin Pharmacol.* 21(1): 33-39.

Vanhoutte PM, Boulanger CM and Mombouli JV (1995). Endothelium-Derived Relaxing Factors and Converting Enzyme Inhibition. *Am. J. Cardiol.* 76(15): 3–12

Vanhoutte PM, Boulanger CM (1995). Endothelium-dependent responses in hypertension. *Hypertens Res.*18(2): 87-98.

Vanhoutte PM, Shimokawa H, Tang EHC, Félétou M (2009). Endothelial dysfunction and vascular disease. *Acta Physiol* 196:193–222.

van Zwieten PA, Jie K, van Brummelen P (1987). Postsynaptic α_1 and α_1-adrenoceptor changes in hypertension. *Cardiovasc Pharmacol* 10(4):68-75.

Vazquez-Prieto MA, Lanzi RC, Lembo C, Galmarini RC, Miatelio RM (2011). Garlic and onion attenuates vascular inflammation and oxidative stress in Fructose-fed rats. *J Nutr Metab* 475216.

Veber DF, Johnson SR, Cheng HY, Smith BR, Ward KW, Kopple KD (2002). Molecular properties that influence the oral bioavailability of drug candidates. *J Med Chem* 45(12): 2615-2623.

Venkatachalam K, Zheng F, and Gill DL (2003). Regulation of canonical transient receptor potential (TRPC) channel function by diacylglycerol and protein kinase C. *J. Biol. Chem.* 278: 29031–29040.

Verma S, Buchanan MR, Anderson TJ (2003). Endothelial function testing as a biomarker of vascular disease. *Circulation* 108: 2054–9.

Villazón M, Padín JF, Cadavid MI, Enguix MJ, Tristán H, Orallo F, Loza MI (2002). Functional characterization of serotonin receptors in rat isolated aorta. *Biol Pharm Bull.* 25(5): 584-90.

Visser AJ and van Mastrigt R (2000). The role of intracellular and extracellular calcium in mechanical and intracellular electrical activity of human urinary bladder smooth muscle. *Urol Res.* 28(4): 260-8.

Walsh MF, Barazi M , Pete G, Muniyappa R, Dunbar JC, and Sowers JR (1996). Insulin-Like Growth Factor I Diminishes in Viva and in Vitro Vascular Contractility: Role of Vascular Nitric Oxide. *Endrocrinology* 137(5)

Wang B, Xiong S, Lin S, Xia W, Li Q, Zhao Z, and Zhu, Z. (2017). Enhanced Mitochondrial Transient Receptor Potential Channel, Canonical Type 3–Mediated Calcium Handling in the Vasculature From Hypertensive Rats. *J. Am. Heart Assoc* 6(7).

Wang M, Xing N, Wu L, Huang WC, Xu Z and Liu G (2018). Regulation of spontaneous contractions in intact rat bladder strips and the effects of hydrogen peroxide. *Biomed Res Int* 2925985.

Wang Y, Tang S, Harvey KE, Salyer AE, August Li T, Rantz EK, Lill MA and Hockerman GH (2018). Molecular Determinants of the Differential Modulation of $Ca_{v1.2}$ and $Ca_{v1.3}$ by Nifedipine and FPL 64176. *Mol. Pharmacol.* 94(3): 973-983.

Waterston RH (1988). Muscle. In The Nematode *Caenorhabditis elegans*, W.B. Wood, ed. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), 281-335.

Watts SW, Cox DA, Johnson BG, Schoepp DD, Cohen ML (1994). Contractile serotonin-2A receptor signal transduction in guinea pig trachea: importance of protein kinase C and extracellular and intracellular calcium but not phosphoinositide hydrolysis. *J Pharmacol Exp Ther.* 1(2): 832-44.

Watts SW, Schenck KW, and Cohen ML (1994). Selectlve desensitization of 5-hydroxytryptamine 2A receptor mediated contraction in guinea pig trachea. *Can J Physiol Pharm* 72(5): 463–470.

Webb RC (2003). Smooth muscle contraction and relaxation. *Adv Physiol Educ* 27(4): 201–206.

Weber MA, Schiffrin EL, White WB, Mann S, Lindholm LH, Kenerson JG *et al.,*(2014). Clinical practice guidelines for the management of hypertension in the community a statement by the American society of hypertension and the international society of hypertension. *J. Hypertens*. 32: 3–15.

Wen Q, Po MD, Hulme E, Chen S, Liu X, Kwok SW, Gershow M, Leifer AM, Butler V, Fang-Yen C, Kawano T, Schafer WR, Whitesides G, Wyart M, Chklovskii DB, Zhen M, Samuel AD (2012). Proprioceptive coupling within motor neurons drives C. elegans forward locomotion. *Neuron*. 76(4): 750-61.

Wettschureck N and Offermanns S (2005). Mammalian G Proteins and Their Cell Type Specific Functions. *Physiol Rev* 85(4): 1159–1204.

White JG, Southgate E, Thomson JN, and Brenner S (1986). The structure of the nervous system of the nematode *Caenorhabditis elegans*. *Philosophical Transactions of the Royal Society London B. Biological Sciences* 314(1): 1–340.

Widlansky ME, Gokce N, Keaney JF, Jr, Vita JA (2003). The clinical implications of endothelial dysfunction. *J Am Coll Cardiol.* 42:1149–1160.

Wieland T, Lutz S, Chidiac P (2007). Regulators of G protein signalling: a spotlight on emerging functions in the cardiovascular system. *Curr. Opin. Pharmacol,* 7(2): 201-207

Wilkins JT, Ning H, Berry J, Zhao L, Dyer AR, Lloyd-Jones DM (2012). Lifetime risk and years lived free of total cardiovascular disease. *JAMA* 308(17): 1795-1801

Wilson L, Wickramasinghe K, Bhatnagar P, Leal J, Luengo-Fernandez R, Burns R, Rayner M, Townsend N (2017). *European Cardiovascular Disease Statistics 2017*. European Heart Network, Brussels.

Wind S, Beuerlein K, Armitage ME (2010). Oxidative stress and endothelial dysfunction in aortas of aged spontaneously hypertensive rats by NOX1/2 is reversed by NADPH oxidase inhibition. *Hypertens.* 56(3): 490–497.

Wirth A and Offermanns S (2012). G-Protein-Coupled Receptors in Smooth Muscle. Muscle: *Fundamental Biology and Mechanisms of Disease*. First Edition 1145–1153.

Wiysonge CS, Bradley HA, Volmink J, Mayosi BM, Opie LH (2017). Beta-blockers for hypertension. *The Cochrane database of systematic reviews* 1(1): 2003

Wood JG, Rogina B, Lavu S, Howitz K, Helfand SL, Tatar M, Sinclair D (2004). Sirtuin activators mimic caloric restriction and delay ageing in metazoans. *Nature*. 430:686–689.

Woodsome TP, Polzin A, Kitazawa K, Eto M, Kitazawa T (2006). Agonist and depolarisation-induced signals for myosin light chain phosphorylation and force generation of cultured vascular smooth muscle cells. *J Cell Sci* 119: 1769-1780

Wynne BM, Chiao CW and Webb RC (2009). Vascular Smooth Muscle Cell Signaling Mechanisms for Contraction to Angiotensin II and Endothelin-1. *J Am Soc Hypertens* 3(2): 84–95

Xiao R, Xu XZ (2011). *C. elegans* TRP channels. *Adv Exp Med Biol*. 704: 323–339.

Xing J, Yan X, Estevez A, Strange K (2008). Highly Ca^{2+}-selective TRPM channels regulate IP_3-dependent oscillatory Ca^{2+} signaling in the *C. elegans* intestine. *J Gen Physiol*. 131(3): 245–255.

Yagil Y and Yagil C (2001). Genetic models of hypertension in experimental animals. *Exp. nephrol.* 9(1): 1–9.

Yamaguchi O and Chapple C (2007). β_3-adrenoceptors in urinary bladder. *Neurol Urodyn* 26: 752-756

Yang X, Jiang C, Hsu-Kim H, et al. Silver nanoparticle behavior, uptake, and toxicity in Caenorhabditis elegans: effects of natural organic matter. *Environ Sci Technol*. 48: 3486–3495.

Yan ZY, Ban T, Fan Y, Chen WR, Sun HL, Qiao QF, Li BY (2015). Na^+-induced Ca^{2+} influx through reverse mode of Na^+-Ca^{2+} exchanger in mouse ventricular cardiomyocyte. *Oncotarget* 6(27): 23272-23180

Yim HE, Yoo KH (2008). Renin-Angiotensin system - considerations for hypertension and kidney. *Electrolyte Blood Press*. 6(1): 42-50.

Yorimitsu K, Moroi K, Inagaki N, Saito T, Masuda Y, Masaki T, Seino S, Kimura S (1995). Cloning and sequencing of a human endothelin converting enzyme in renal adenocarcinoma (ACHN) cells producing endothelin-2. Biochem. *Biophys. Res. Commun.* 208: 721–727.

Yuan JP, Lee KP, Hong JH, Muallem S (2012). The closing and opening of TRPC channels by Homer1 and STIM1. *Acta Physiol* 204(2): 238-47.

Yusuf S, Rangarajan S, Teo K, Islam S, Li W, Liu L, Dagenais G (2014). Cardiovascular Risk and Events in 17 Low-, Middle-, and High-Income Countries. *N. Engl. J. Med.* 371(9): 818–827.

Zalba G, Beaumont FJ, San José G (2000). Vascular NADH/NAD(P)H oxidase is involved in enhanced superoxide production in spontaneously hypertensive rats. *Hypertens.* 35(5): 1055–1061.

Zarkower, D (2006). Somatic sex determination (February 10, 2006), *WormBook*, ed. The *C. elegans* Research Community.

Zhang MQ, Wilkinson B (2007). Drug discovery beyond the 'rule of five'. *COBIOT* 18(6): 478-488

Zhang J, Ren C, Chen L, Navedo MF, Antos LK, Kinsey SP, Iwamoto T, Philipson KD, Kotlikoff MI, Santana LF, Wier WG, Matteson DR, Blaustein MP (2010). Knockout of Na^+/Ca^{2+} exchanger in smooth muscle attenuates vasoconstriction and L-type Ca^{2+} channel current and lowers blood pressure. *Am J Physiol Heart Circ Physiol.* 298: 1472–83.

Zhang J, Fallahzadeh MK, McCullough PA (2016). Aging Male Spontaneously Hypertensive Rat as an Animal Model for the Evaluation of the Interplay between Contrast-Induced Acute Kidney Injury and Cardiorenal Syndrome in Humans. *Cardiorenal Med*. 7(1): 1-10.

Zhao, Y, Vanhoutte PM, and Leung SWS (2012). Endothelial Nitric Oxide Synthase-Independent Release of Nitric Oxide in the Aorta of the Spontaneously Hypertensive Rat*. J. Pharmacol. Exp. Ther.* 344(1): 15–22*.*

Zheng H, Loh HH, Law PY (2010). Agonist-selective signaling of G-protein-coupled receptor: mechanisms and implications. *IUBMB Life,* 62(2): 112-119.

Zhong H, Minneman KP (1999). Alpha1-adrenoceptor subtypes. *Eur J Pharmacol.* 375(1-3): 261-76.

Zhou Y, Shao L, Li J, Han L, Cai W, Zhu C, Chen D (2011). An efficient and novel screening model for assessing the bioactivity of extracts against multidrug-resistant Pseudomonas aeruginosa using Caenorhabditis elegans. *Biosci. Biotechnol. Biochem.* 75(9): 1746–1751.

Zhu MX (2005). Multiple roles of calmodulin and other $Ca(^{2+})$-binding proteins in the functional regulation of TRP channels. *Pflugers Arch*. 451: 105–115.

Zhu X, Jiang M, Birnbaumer L (1998). Receptor-activated Ca^{2+} influx via human Trp3 stably expressed in human embryonic kidney (HEK)293 cells. Evidence for a non-capacitative Ca^{2+} entry. *J Biol Chem*. 273: 133–142

Zou Q, Zhang C, Guo Y (2015). TRPC3 and TRPC6 Contribute to the Pathogenesis of Hypertension. *Am. J. Mol. Biol.* 5: 124-133

Zulian A, Baryshnikov SG, Linde CI, Hamlyn JM, Ferrari P, Golovina VA (2010). Upregulation of Na^+/Ca^{2+} exchanger and TRPC6 contributes to abnormal Ca^{2+} homeostasis in arterial smooth muscle cells from Milan hypertensive rats. *Am J Physiol Heart Circ Physiol* 299: 624–633

Online sources:

Asian Plant: *Ficus schwarzii* Koord.
http://www.asianplant.net/Moraceae/Ficus_schwarzii.htm. Last accessed on 19th Feb 2019.

DrugBank database. http://www.drugbank.ca/. Various accessed time.

Eisenmann, DM, Wnt signaling (June 25, 2005), *WormBook*, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.7.1 http://www.wormbook.org.

Encyclopaedia Britannica: *Ficus* plant genus.
http://global.britannica.com/plant/Ficus. Last accessed on 29th January 2019.

European Pharmaceutical Industry 2015 Update: Putting animal welfare principles and 3Rs into action. http://www.efpia.eu/uploads/EFPIAAnimalWelfare_3Rs_2015__FINAL.pdf. Last accessed on 3rd July 2019.

IUPHAR database. http://www.guidetopharmacology.org/. Various accessed time

Kerr RA (2006). Imaging the activity of neurons and muscles. 2006 Jun 2. In: WormBook: The Online Review of *C. elegans* Biology [Internet]. Pasadena (CA): WormBook; 2005-2018.

PubChem database. http://www.ncbi.nlm.nih.gov/pccompound. Various accessed time.

Ficus Schwarzii Koord:
https://www.google.com/url?sa=i&source=images&cd=&cad=rja&uact=8&ved=2ahUKEwjHhsTDmfrkAhWoILcAHajyC3cQjhx6BAgBEAI&url=https%3A%2F%2Fwww.flickr.com%2Fphotos%2Fadaduitokla%2F13398693115&psig=AOvVaw0MR3lOoZI63VpBaPgSLzTT&ust=1569989696970566.Various accessed time

World Health Organisation (2018). Cardiovascular diseases 17th May

World Heart Federation Fact Sheet (2012). http://www.world-heartfederation.
org/heart-facts/fact-sheets/cardiovascular-disease- terms/. Last accessed on 15th Jan 2019.