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Synergistic Interaction of Lithium Ions and α_1-Adrenoceptor Agonists in Vascular and Non-Vascular Porcine Smooth Muscle

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Abstract

Phenylephrine, L-erythromethoxamine and metaraminol are selective α_1-adrenoceptor agonists having phenethylamine chemical structure and used to treat a variety of clinical conditions, including sepsis, anaesthesia associated hypotension, nasal congestion, haemorrhoids, and the evaluation of eye function. Their action involves activation of α_1-adrenoceptor on the smooth muscle of major organs to induce a contraction and the resultant therapeutic benefit is dependent on the maintenance of stable response. The signalling mechanism most closely linked to α_1-adrenoceptors in smooth muscle is stimulation of phosphatidylinositol metabolism, which induces subsequent elevation of intracellular calcium ions. Lithium ions are also used clinically in the treatment of bipolar disorders and are known to prevent degradation of a key mediator of phosphoinositide metabolism and there are a few reports suggest that this cation can enhance or maintain contractions mediated by α_1-adrenoceptors. In the present study, I have investigated the interaction between lithium ions and constrictor responses to a variety of agonists, including selective α_1-adrenoceptor agonists, with a particular focus on both the potency of the constrictor agent and the time course of responses. I have also used pharmacological approaches to better understand the basis of a novel interaction between lithium ions and selective α_1-adrenoceptor agonists.

Two methods have been employed. Isometric tension recording of isolated vascular and urethral smooth muscle determined using a standard approach of maintaining the tissue in Krebs-Henseliet solution gassed with 95%O_2/5%CO_2. Agonists were added either cumulatively or as a single concentration and the magnitude and time course of the contraction assessed with lithium and in the presence of selective inhibitors. In addition, the time of course of changes in human skin blood flow, using laser Doppler flowmetry, to the iontophoretic application of phenylephrine has also been evaluated.

Our *in vitro* results show that the contractions of the isolated vascular smooth muscle to KCl and the thromboxane mimetic (U46619), were sustained for up to three hours. In contrast contraction to high concentrations to noradrenaline, 5HT, histamine, vasopressin, angiotensin II, carbachol and the selective α_1-adrenoceptor agonist were not. Furthermore, in porcine isolated splenic artery segments, the use of therapeutic concentration of lithium (1mM) increased the maximum response to the selective α_1-adrenoceptor agonists (and carbachol) almost 2 fold it did not increase the sensitivity of the tissue to these drugs. In contrast, the time course of responses to noradrenaline, cirazoline, 5HT, histamine, vasopressin and angiotensin II was not affected by lithium. Interestingly, similar interaction was also reported in isolated porcine renal and mesenteric arteries, splenic vein and urethral smooth muscle.

Moreover, our results showed that this interaction is sensitive to Ca^{2+} and abolished in the absence of this cation. This interaction prevents the desensitisation of the receptors which develop following the repetitive exposure to a high concentration of α_1-adrenoceptor agonists. Furthermore, we confirm that this synergistic interaction is not related to internalisation, or inhibition of inositol monophosphatase, glycogen synthase kinase, Rho kinase or nitric oxide pathways. Although we reported similar

enhancement in the time course of phenylephrine using nM of Na^+-K^+ ATPase inhibitor (ouabain), the magnitude of the response was much smaller than that reported with lithium.

Our *in vivo* results demonstrate that the time course of contraction induced by iontophoresis of phenylephrine was relatively stable for more than 30 minutes which suggest that the concentrations reaching the superficial blood vessels were low and therefore there was no point of investigating the effect of lithium on the contraction induced by phenylephrine in this system.

In conclusion, I have demonstrated that the use of high concentration of phenethylamine derived α_1-adrenoceptor agonists synergistically interact with therapeutics concentration of LiCl in various smooth muscle preparations and that phosphoinositide metabolism is not involved. This synergistic effect appeared as sustain in the time course of the contraction induced by these agonists and could be accompanied by an enhancement in the magnitudes of the response. Further clinical studies using different approaches are needed to detect any potential clinical uses of this interaction.

Abstracts for Conferences

1. **S. Yahya**, A. Abdulrahman, S. Rayment, R. Mahajan, J. Scholefield, S. Chan, V. Wilson. Lithium ions selectively enhance contractions of the porcine isolated splenic artery to high concentrations of phenylephrine and L-erythromethoxamine. *Pharmacology* 2016 Queen Elizabeth II Conference Centre London. www.pA2 online.org, 152P

2. **S. Yahya**, R. Mahajan, V. Wilson. Synergistic interaction of lithium ions with L-erythromethoxamine and phenylephrine contractions in porcine blood vessels: no role for inhibition of inositol monophosphatase. *Pharmacology* 2016 Queen Elizabeth II Conference Centre London. www.pA2 online.org, 142P

3. **S. Yahya**, R. Mahajan, V. Wilson. Factors influencing the time course of noradrenaline-induced contractions of the porcine isolated splenic artery. *Pharmacology* 2017 Queen Elizabeth II Conference Centre London. www.pA2 online.org, 134P

Award

March 2018	Second best poster in the Whole School Away Day from the School of Life Sciences, Faculty of Medicine and Health, University of Nottingham Sciences.
July 2018	Third outstanding presentation in the Postgraduate Research Symposium, from the School of Life Sciences, Faculty of Medicine and Health, University of Nottingham Sciences.

Dedication

To my beloved late brother,

Mohammed Yahya,

Who support my family during the liberation

of Mosul and help me to achieve my goals, I miss

you so much my young brother

May your soul rest in peace.

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I prised *ALLAH Lord of the universe* for providing and supporting me to finish my thesis and to pass the hard time which I faced during this journey especially losing my young brother.

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Most importantly, I would like to thank my father and mother who raised me and offered me all I need to be a qualified person who can serve the community. Also, my sincere thanks and love to my wife Zinah and my beloved kids Mohammed, daughter Tiba and my little angle Jana.

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Abbreviation

^0C	Temperature degrees in Celsius
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanine monophosphate
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
EDTA	Ethylene diamine tetraacetic acid
GPCR	G protein coupled receptors
IP_3	Inositol-1, 4, 5-triphosphate
KCl	Potassium chloride
L-NAME	N^G-Nitro-L-arginine methyl ester hydrochloride
NO	Nitric oxide
NOS	Nitric oxide synthase
PLC	phospholipase C
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
PIP_2	Phosphatidylinositol 4,5-biphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
SEM	Standard error of mean

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Chapter 1: General Introduction

1.1 General outline

The work in this thesis will consist of two main parts, the first part is the ex-vivo study using the isometric tension recording to characterise the interaction between the contractile agents with a therapeutic dose of LiCl in porcine isolated vascular and non-vascular tissue. I will examine the time course, tissue involvement, concentration that will be used to induce this interaction in chapter 3. In chapter 4, I will highlight the tissue and non-tissue related factors responsible for the decline in the time course of contraction induced by noradrenaline in porcine splenic artery. In chapter 5 of this thesis, I will investigate the possible mechanisms responsible for the positive interaction between LiCl and α_1-adrenoceptors and excluding the suggested mechanisms responsible for the action of LiCl in bipolar disorder. The second part of my work will be described in chapter 6 which involve the *in vivo* mirroring of the main observation from my *in vitro* results using laser Doppler flowmetry to measure the changes in superficial blood flow following the iontophoresis of phenylephrine in a healthy volunteer.

The first chapter which is the general introduction will cover three main sections. Firstly, I will provide the essential knowledge to describe the vascular structure, function and reactivity. This will be followed by a review of the mechanisms for vasoconstrictor agents in general and will be a focus on the α-adrenoceptors mechanisms (role of inositol phosphoinositide and the release of intracellular calcium) as the main target. Finally, I will describe lithium uses, side effect and the suggested mechanisms of action before highlighting some studies that have raised a suggestion that lithium ions can modify contractile mechanisms and what we understand about how this ion is handled by vascular smooth muscle.

1.2 Vascular system

1.2.1 Blood vessels structure and function

Blood vessels structure varies according to their function, but in general, large blood vessels (both venous and artery) comprise three layers: the innermost layer (tunica intima) which consists of endothelial cells that form a single smooth flat layer. This layer is surrounded by a thick wall of smooth muscle cells embedded in a matrix of collagen which represent the middle layer (tunica media). The media is innervated by motor nerves and controlled by the sympathetic nervous system. The outer layer

(tunica externa or adventitia) contains collagen fibres and elastin fibres which add flexibility and strength to the arterial walls in addition to the nerve innervation by sensory neurons (Rhodin 1980), see Figure 1.1.

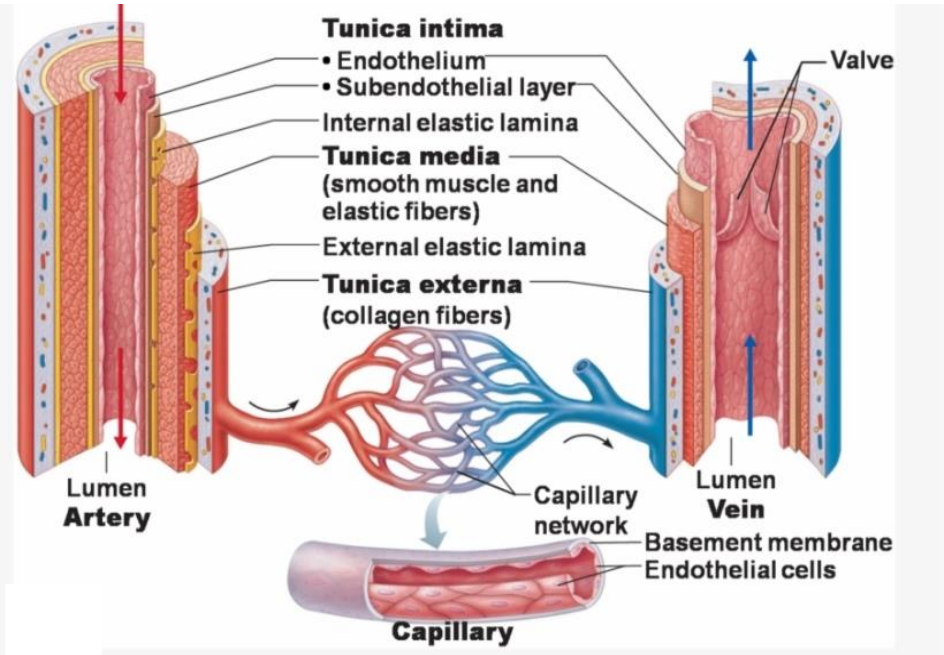


Figure 1-1 Structure and composition of the blood vessel (artery and vein and capillary). The tunica intima (endothelium) surrounded by tunica media while the outer layer is tunica externa. Figure adapted from (Teach me anatomy.info 2019, website)

These blood vessels maintain the absorption of essential nutrients, the removal of the cellular waste product in addition to controlling the haemostasis during different situations. The seminal observation made by the physiologist William Harvey who described for the first time the systemic circulation in 1628, since that time there was an increasing interest in research conducted in this failed to highlight the importance of the vascular function and their role in many pathophysiological conditions, these research helped in understanding the structure of cells and the development of many drugs that used in the treatment of vascular diseases (Pugsley and Tabrizchi 2000). The capillaries blood vessels contain no smooth muscle and consist of a single layer of endothelial cells surrounded by a basal membrane. The main functions of blood vessels are to maintain blood flow from the heart to the tissues and bring it back to the heart, in addition to oxygenation of the blood in the lung (Rutishauser, 1994; Klabunde, 2012). The endothelial cells and due to their nature of being in contact with the blood, they have a major role in maintaining the haemostasis either by secreting special

substances that can alter vessels tone or by interfering with the action of the peripheral nervous system. In addition to that endothelial cells also involved in a different cellular process such as coagulation and thrombolysis as well as taking a part in the inflammatory and immunological process inside the blood vessels (Vanhoutte 1989).

1.2.2 Vascular smooth muscle

The vascular smooth muscle cells are spindle-shaped cells with a diameter ranging from 5-10μm and a length ranging from 50-300μm. They are formed from actin and myosin as contractile proteins in addition to tropomyosin and innervated by autonomic nerve fibres. The actin protein filaments are joined together by the dense body to form a band called dense band these bands help to fix the actin filaments within the smooth muscle cell. Each single myosin filament is surrounded by several actin filaments, see Figure 1.2. The VSM cells connected with each other by gap junctions, these junctions provide cell-to-cell propagation of electrical current which results in depolarization and blood vessels contraction (Prosser, 1974; Klabunde, 2012; Walsh, 2014).

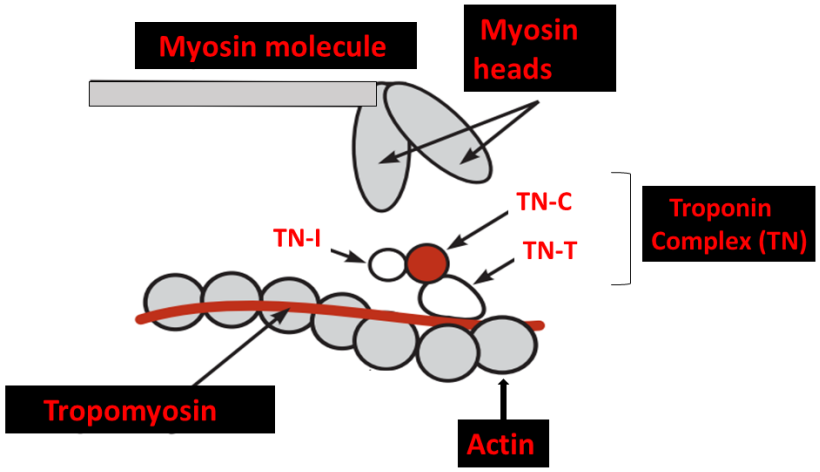


Figure 1-2 The composition of smooth muscle contractile proteins. The thick filaments consist of myosin molecules, each molecule characterised by having two myosin heads. The thin filaments consist of actin, tropomyosin in addition to a regulatory protein known as troponin complex (TN). There are three subunits of TN: TN-C is the site for binding with Ca^{2+}. TN-T, is the site of binding with tropomyosin proteins and TN-I, represent the inhibitory troponin that blocks the binding between actin and myosin. The contraction induced when Ca^{2+} binds to TN-C and initiate conformational changes in TN-T lead to hydrolysis of adenosine triphosphate (ATP) and exposes the myosin-binding site on the actin protein.

1.2.3 Contractile apparatus of VSMs

Vascular smooth muscle contractility can be affected and modified depending on the amount of free intracellular Ca^{2+}ion. The increase in Ca^{2+} ions concentration can result from either:

- Activation of inositol 1,4,5-trisphosphate (IP_3) after being activated with an agonist which leads to mobilization of Ca^{2+} from the sarcoplasmic reticulum.
- An influx of Ca^{2+} ions from the extracellular compartment via voltage-gated or receptor-operated Ca^{2+} channel.
- Or could be a combination of both mechanisms (Poulsen *et al.*, 2011, Eckert *et al.*, 2000).

For the contraction to occur, an agonist could couple with G protein-coupled receptors (GPCR) to activate the L-type Ca^{2+} channel which allows free Ca^{2+} to enter the cell. This free Ca^{2+} combines with a protein called calmodulin to form a Ca^{2+}-calmodulin complex. This complex activates MLCK resulting in phosphorylation of MLC in the presence of adenosine triphosphate (ATP). See Figure 1.3 and 1.4 (Somlyo and Somlyo, 2000; Clark and Pyne-Geithman, 2005).

1.2.4 Smooth muscle contraction:

The autonomic nervous system innervates the vascular smooth muscle. In addition to this, vascular smooth muscle receives various types of stimuli from neurotransmitters, hormones, endothelial-derived factors, and integrate them in order to keep the contractile apparatus active. As shown in Figure 1.3, contraction of individual vascular smooth muscle cells can usually be triggered by changes in Ca^{2+} ion concentration and/ or change in membrane potential due to the movement of ions across the cell membrane. The contraction is an active process in which phosphorylation of the myosin light chain occurs; the myosin light chain kinase (MLCK) enzyme is responsible for contraction (Webb 2003).

On the other hand, the myosin light chain phosphatase (MLCP) enzyme is the second enzyme which acts in the regulation of phosphorylation. It acts by removing the high energy phosphate from MLC to induce relaxation, see Figure 1.3. This enzyme consists of three subunits, the catalytic subunits, the variable subunits and most importantly myosin-binding subunit. Phosphorylation of myosin-binding subunit results in inhibition of the activity of MLCP and maintaining the contraction state in the cell (Somlyo and Somlyo 2003). The activity of the MLCP enzyme is highly affected by the

G protein Rho and its target Rho kinase. The Rho kinase or serine/threonine kinase maintains the phosphorylation state by phosphorylating myosin-binding subunit in MLCP enzyme. The use of Rho kinase inhibitors induces relaxation in the vascular smooth muscle (Chitaley *et al.,* 2001; Lee *et al.,* 2004).

Other factors might be involved in the regulation of phosphorylation processes and can affect the activity of MLCK and MLCP enzymes. The calmodulin-dependent protein kinase II can induce smooth muscle relaxation by lowering the sensitivity of Ca^{2+} to the MLCK enzyme (Surks *et al.* 1999; Clark and Pyne-Geithman 2005).

1.2.5 Smooth muscle relaxation:

Smooth muscle relaxation may result from either removing the stimuli (vasoconstrictor) that initiate the contraction or adding a direct agent that interferes with the mechanism of contraction (vasodilator). In both cases, lowering of the concentration of Ca^{2+} ions, in addition to increasing the activity of MLCP enzyme, is common (Somlyo *et al.,* 1999; Morgan 1990). The concentration of Ca^{2+} can be lowered by a specific enzyme known as Ca^{2+}-Mg^{2+} ATPase which acts to translocate the Ca^{2+} from the cytoplasm to its storage site in the sarcoplasmic reticulum. In addition to the previous enzyme, a specialised group of $Ca^{+\ 2}$ binding proteins are located in the sarcoplasmic reticulum, including calsequestrin and calreticulin which can bind with the free Ca^{2+} and lower its concentration (Brooks 2003). The plasma membrane also has its own Ca^{+}-Mg^{2+} ATPase and Na^{+}-Ca^{2+} exchange in addition to Ca^{2+} channels these enzymes can alter the concentration of Ca^{2+} by pumping it to the extracellular compartment. All these mechanisms can be work together to decrease the concentration of free Ca^{2+}ions and elicit relaxation (Webb 2003; Demaurex *et al.* 2009).

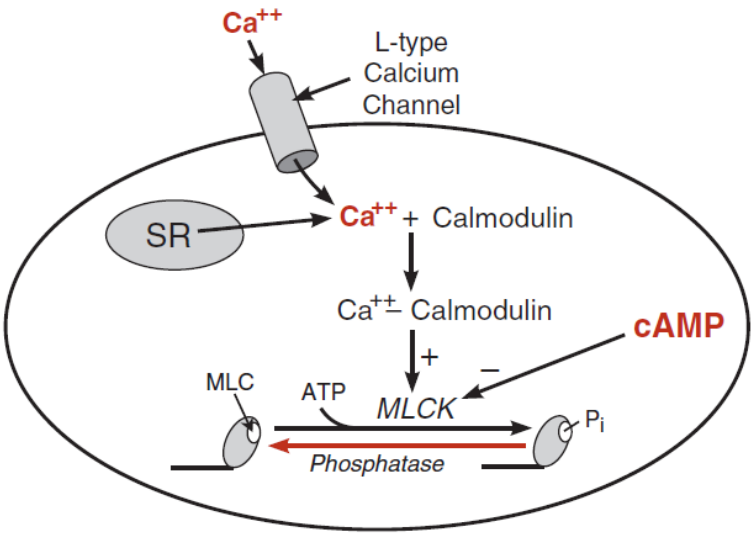


Figure 1-3 The mechanism for modulating vascular smooth muscle contraction and the role of MLCK enzyme in initiating contraction by increasing the concentration of Ca^{2+} and phosphorylation of Ca^{2+} -calmodulin complex; the role of MLCP enzyme-inducing relaxation by dephosphorylating the Ca^{2+} -calmodulin complex, (Klabunde, 2012).

1.2.6 Regulation of the contractile activity of VSM

1.2.6.1 Ca^{2+} dependent contraction and relaxation in vascular smooth muscles (VSMCs):

As described previously, any rise in Ca^{2+} concentration initiates the contraction in the smooth muscle cells. Stimulation in the VSMCs will trigger the entrance of Ca^{2+} ions into the cell via different types of Ca^{2+} channels. This is called trigger Ca^{2+} which stimulates the cell to release higher amounts of Ca^{2+} from the store (sarcoplasmic reticulum and mitochondria) and it represents the major source of Ca^{2+} induced contraction in the cell. After the concentration of Ca^{2+} increases, it binds to calmodulin proteins to form a complex called calcium-calmodulin complex. The latter activates MLCK enzyme to phosphorylate the light chain (Jackson 2000).

As shown in Figure 1.4, several agents such as catecholamines, angiotensin II, endothelin I, acetylcholine, vasopressin and α-adrenoceptors can bind to a specific receptor linked to a G_q protein on the cell membrane. This binding stimulates the phospholipase C enzyme (PLC). This enzyme catalysis the hydrolysis of phosphoinositol bisphosphate (PIP_2) to form two compounds: inositol triphosphate (IP_3) and diacylglycerol (DAG). These two compounds act as second messengers to perform specific roles inside the cell. The IP_3 binds to a specific receptor in the

sarcoplasmic reticulum and initiates an increase in the level of Ca^{2+} in the cytoplasm. The DAG activates the protein kinase C (PKC) enzyme; this enzyme is responsible for contraction by its role in the phosphorylation of L- type Ca^{2+} channels (Webb 2003). Each cell provided with Ca^{2+} homeostatic mechanism that regulates the concentration of free Ca^{2+} ion inside the cell through stimulation of Ca^{2+} extrusion to extracellular compartment via Ca^{2+} pump or Ca^{2+}-Na^{+} exchanger in addition to the enhancement of reuptake of Ca^{2+} by SR and mitochondria (Khalil 2010). The ions channels can play a pivotal role in the regulation of contraction and relaxation; several ion channels are involved and include the following.

1.2.6.2 The role of ion channels in maintaining vascular tone:

Potassium channels can contribute to and regulate vascular tone. The activity of these channels is an important factor in the determination of the action potential of the cell membrane. Under the normal resting condition, the concentration of K^{+} ions inside the cell is much higher than outside the cell. Opening K^{+} channels will result in the efflux of K^{+} ions from inside the cell to extracellular compartments; which will result in membrane hyperpolarization and produce vasodilation. By contrast, closing these channels will stimulate membrane depolarization and result in vasoconstriction (Nelson and Quayle 1995; Jackson 2005).

The voltage-gated Ca^{2+} channels also have a major role in controlling both the action potential and the blood vessels tone. Vasodilation will result from membrane hyperpolarization after the closing of these channels whereas opening these channels will result in vasoconstriction (Nelson *et al.,* 1990; Hughes 1995)

Chloride channels were proposed to participate in controlling blood vessels tone. Opening these channels will result in the efflux of negatively charged Cl^{-} ions from inside the cell, depolarization and vasoconstriction of blood vessels *(*Yamazaki *et al.,* 1998; Large and Wang 1996).

1.2.6.3 Role of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) in controlling vascular tone

After we described the role of membrane events and contractile process, we will outline the role of cyclic nucleotides in reversing or preventing the contractile process. Sutherland's group was the first group to describe the chemical structure of cAMP. After the second messenger hypothesis of Sutherland which states that cyclic

nucleotide such as cAMP and cGMP have the ability to mediate the action of several hormones and neurotransmitters, the number of studies examining the role of cAMP increased dramatically (Sutherland *et al.,* 1968). The cAMP formed from the action of AC acting on ATP molecules. This enzyme is present in the mitochondria, cytoplasm, and endoplasmic reticulum (Polson and Strada 1996). The level of cAMP is controlled by two main enzymes: the AC and the cyclic phosphodiesterase (PDE). After the agonist ligand binds to the GPCRs, it stimulates AC enzyme to produce cAMP from the ATP. This is followed by activation of cAMP-dependent protein kinase A (PKA) (Pierce *et al.,* 2002). On the other hand, if a ligand binds to the inhibitory Gi and stimulates it; or if the PDE enzyme was activated this resulted in inhibition of cAMP activity. The level of these enzymes is not stable inside the cell and many pathways can interfere with it such as the Ca^{2+} signalling pathway and the G protein subunits in addition to, receptors tyrosine kinase (Bruce *et al.,* 2003). Furthermore, the cAMP can modulate the action of nucleotide-gated ion channels. These are cationic channels such as Na^{+} or K^{+} and Ca^{2+} which control the transfer of Ca^{2+} across the cell membrane. Hence, they can affect the action potential across the cell membrane (Zaccolo and Pozzan 2003). In 1977 Murad and colleagues reported that nitric oxide (NO) which is released from nitrate compound and induce relaxation can stimulate the production of cGMP by activating the guanylyl cyclase enzyme (GC) (Hofmann *et al.,* 2000).

Cyclic GMP is a second messenger needs to interact with some cellular receptors to produce its effect. In smooth muscle cell, cGMP can stimulate the serine/threonine receptors protein (Rho) and activate cGMP-dependent protein kinase (PKG); the formation of former enzyme stimulates numerous phosphorylation mechanism that leads to relaxation (Francis and Corbin 1994). Cyclic GMP induces smooth muscle relaxation through two proposed mechanisms, the first mechanism includes its ability to reduce the concentration of Ca^{2+} ions in the cytoplasm, as it is well known that the intracellular level of Ca^{2+} should be increased in order to phosphorylate the MLC and produce contraction (Lincoln and Cornwell 1991).

The cGMP can reduce the concentration of free Ca^{2+} ions by acting on different sites as it stimulates the Ca^{2+} pump to extrude Ca^{2+}, inhibits the Ca^{2+} voltage-gated channels and inhibits the coupling of an agonist with GPCRs (Nelson *et al.,* 1995). The second mechanism by which the cGMP produces relaxation is by reducing Ca^{2+} sensitisation; it was suggested that cGMP can interfere with the activity of MLCK enzyme and inhibits its action (Van Riper *et al.,* 1997). Other studies suggested that PKG can stimulate MLCP enzyme and induce relaxation in contracted smooth muscle (Torrecillas *et al.,* 2000).

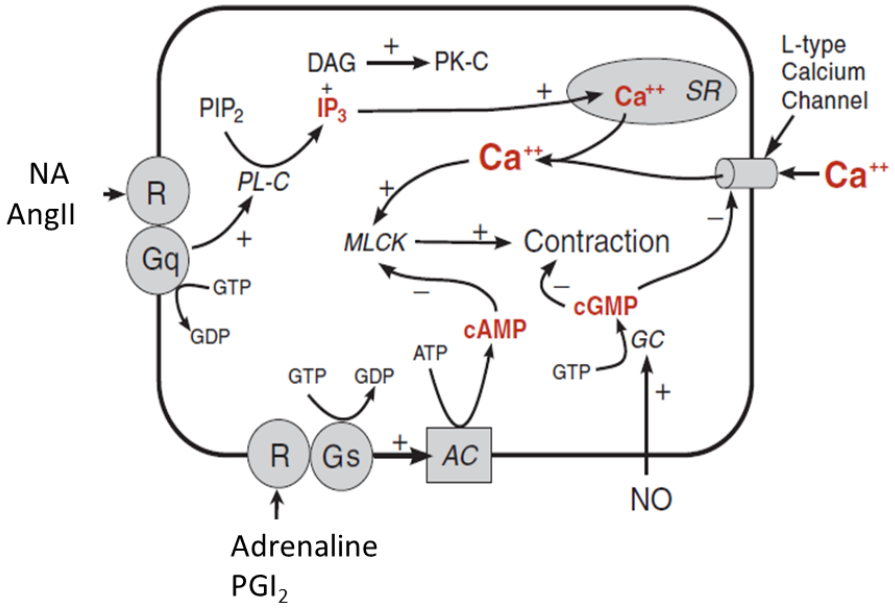


Figure 1-4 The signalling pathways and receptors which regulation smooth muscle contraction. stimulatory G protein (Gs); adenylyl cyclase (AC); phospholipase C (PLC); Gq, PLC coupled G protein; phosphatidylinositol bisphosphate (PIP_2); inositol triphosphate (IP_3); SR; phosphokinase C (PK-C); angiotensin II (AngII); noradrenaline (NA); adrenaline; prostacyclin (PGI_2); receptor (R) (Klabunde, 2012).

1.2.6.4 Endothelial role in regulating vascular tone

The discovery of prostacyclin in 1976 by Moncada and his group could be recognised as the earliest approach to identify the arterial wall as a paracrine gland *(Moncada et al.*, 1976). In 1977 Moncada showed that prostacyclin was highly secreted from the endothelial layer rather than from other tunica layers and was providing local control of vascular tone (Moncada *et al.,* 1977). This was augmented by the discovery of Furrgout Zawadzki in 1980 who reported that the endothelial cells can release a relaxing factor known as EDRF which was called latter NO (Furchgott and Zawadzki 1980). In addition to NO and prostacyclin, H_2S which is another gasotransmitter that has been identified significantly in the last two decades can also affect vascular tone through multiple pathways including the interaction with metalloproteins, reversible protein sulfahydration in addition to its antioxidant effect. H_2S can act on several target molecules such as potassium channels, PDE and NOS enzymes and alter the tone of smooth muscle (Dongó *et al.*, 2018). Furthermore, the smooth muscle tone was also regulated by the action of adipose tissue surrounding the adventitia (Löhn *et al.*, 2002). This fat tissue can induce relaxation in the blood vessels through the secretion

of transferable substances known as adipose-derived relaxing factors, which act through stimulating potassium channels and induced relaxation (Cherry *et al.*, 1982).

1.3 Receptor history

The British physiologist John Newport Langley and Nobel Prize winner Paul Ehrlich were the first scientists who introduced the term receptor to explain the action of the drug in the early 20th century. Langley used the term receptive substance to describe the effect of nicotine and curare on nerve impulses in dog skeletal muscle. In his work, he assumed that these receptive substances act as a relay point where they receive the impulse or stimuli and transmitting them to the target organ to induce the action (Langley 1905). Dale in 1906, was the first scientist who describes the possibility of the presence of multiple receptors. He examined the action of epinephrine in different physiological responses in cat, rabbit, dog, monkey and fowls using crude alkaloid preparation (ergotamine). His work emphasised that ergot not only inhibited the vasoconstriction action of epinephrine but also stimulated the vasodilation action in a phenomenon called "epinephrine reversal". (Dale 1906).

Contemporary to Langley, the German scientist Paul Ehrlich described the idea of the receptor during his work which ends up with the important discovery for the first clinical effective drug in the management of syphilis which he called it the magic bullet "Salvarsan" (Maehle 2005).

The concept of membrane-bound "receptive" substance was taken further by the work of Ahlquist. The term adrenotropic receptor" was first used in 1948 to describe the molecules or structures located near the glands or muscles in different places that can show response if exposed to adrenaline. He refused to simply classify the adrenotropic receptor to excitatory and inhibitory depending on the nature of their response to the stimulation only. Ahlquist reported that the response can vary depending on the location of the receptor in the sympathetic nervous system and the sympathomimetic agent used to initiate the response if both the amount and the methodology of addition were similar. He compares the response initiated by different amines (adrenaline, noradrenaline, isoprenaline, methyl-noradrenaline and methyl-adrenaline) to differentiate between adrenoceptors presented in effector organ in intact as well as isolated tissue from cats, rabbits and dogs. His main observation was the relative potency of these amines produced two different orders proposing the presence of two

different adrenoceptors. He suggested calling the first vasoconstriction response *α-adrenotropic* while the vasodilation response referred to as a *β-adrenotropic*. The potencies for α and β were as follow respectively:

Adrenaline>noradrenaline>methyl-noradrenaline>methyl-adrenaline >isoprenaline

Isoprenaline>adrenaline>methyl-adrenaline>methyl-noradrenaline >noradrenaline,(Ahlquist 1948).

In 1957, Brown & Gillespie reported that the use of two α_1-adrenoceptors antagonists (dibenamine and phenoxybenzamine) enhanced the release of sympathin (neurotransmitter) from spleen in the cats (Brown & Gillespie, 1957). These actions can now be explained to be potentiation of the release of noradrenaline from the neurons. In 1971 when Starke and his colleagues reported similar enhancement in noradrenaline release following the use of phentolamine with a minimal role of uptake mechanism in this action (Starke *et al.,* 1971). In 1972, Furchgott used the relative potency to rank the responses in different tissue to adrenoceptor agonists. He suggested that both adrenaline and noradrenaline and any other agent which can initiate a response in the tissue following the interaction with adrenergic receptors known as an "agonist". In addition to that, he used the phentolamine as a competitive inhibitor of the α- adrenergic receptors and referred to it as "antagonist" (Furchgott 1972). The pharmacologist starts using more complicated tool, probes and techniques to elucidate the subtypes of α and β-adrenoceptors. The radioligand binding method was one of this method in which a radioactive biochemical ligand used for diagnosis or for receptors study. The ligand binds to its specific receptors. The isotope which binds to the ligand can then be measured (Brunton *et al.,* 2011). The action of drugs on these receptors subtypes has been found to be useful in managing diseases in major organs across the body systems. Some of these diseases include glaucoma, asthma, benign prostatic hypertrophy, hypertension, hypotension, congestive heart failure, dysrhythmia and angina (Bylund *et al.,* 1994).

1.3.1 Heterogeneity of adrenoceptors

It is clearly established that both types of α-adrenoceptors can be located postjunctionally in a variety of mammalian vascular system including man (Elliott & Reid, 1980; Kobinger & Pichler, 1980; Hamilton & Reid, 1982; Tsujimoto *et al.*, 1987). These receptors have a crucial role in the control of blood flow and the degree of constriction in vascular blood vessels. Bylund and his group suggested dividing

adrenoceptors to three main groups: α_1, α_2, and β adrenoceptors. Each main classes is subdivided into three subclasses resulting in nine different pharmacological groups of adrenoceptors (α_{1A}- α_{1B}, α_{1D}, α_{2A}, α_{2B}, α_{2C}, β_1, β_2 and β_3) which identified by protein sequence method (Bylund *et al.,* 1994). An alternative classification suggested by Muramatsu and his group in (1990) to subdivide α_1-adrenoceptors depending on their affinity to prazosin into α_{1L} (low affinity) and α_{1H} (high affinity). The α_{1H} was matching the current classification of α_1- adrenoceptors yet, the α_{1L} did not fit this classification. In 2008, a study conducted on mouse prostate showed that knock –out the α_{1A}-adrenoceptor completely eliminate the pharmacological activity of α_{1L} emphasising that the latter is a phenotype of α_{1A}-adrenoceptor (Gray *et al.*, 2008)

Table 1-1 Shows, the location, the main targeted GPCR, principle effect and the main outcomes of stimulation of α_1, α_2, β_1 and β_2 -adrenoceptors across the body. PLC: phospholipase C; PLA: phospholipase A; AC: adenylyl cyclase; cAMP: cyclic adenosine monophosphate; PKA: protein kinase C.

	G protein coupling	**Principle effect**	**Location**	**Physiological outcomes**
α_1	$G\alpha_{q11, q14, q16}$	⬆ PLC, PLA ⬆ Ca^{2+} channels ⬆ Na^+/H^+ exchange	Heart, lung, liver, smooth muscle, anal sphincter, bladder/ urethral smooth muscle blood vessels, spleen, kidney, prostate, vas deferens	Vasoconstriction in blood vessels, The contraction in the anal sphincter and bladder/urethral smooth muscle Vasoconstriction in the aorta and large resistance artery in skeletal muscle
α_2	$G\alpha_I$	⬇ AC-cAMP-PKA pathway Opening K channels	Platelets, sympathetic neurons, autonomic ganglia, pancreas, Liver, kidney Coronary and Central nervous system (CNS) vessels	Inhibition of sympathetic neurons, Vasoconstriction of blood vessels, Inhibition of hormone release from the adrenal medulla
β_1	$G\alpha_s$	⬆ AC-cAMP-PKA pathway ⬆ L-type Ca^{2+} channels	Heart, kidney, adipocytes, brainstem, cortex, skeletal muscle	Positive inotropic and chronotropic in heart
β_2	$G\alpha_s$	⬆ AC-cAMP-PKA pathway ⬆ L-type Ca^{2+} channels	Heart, kidney, blood vessels, bronchial & GI smooth muscle, cortex	Smooth muscle relaxation, Skeletal muscle hypertrophy
B_3	$G\alpha_s$	⬆ AC-cAMP-PKA pathway ⬆ L-type Ca^{2+} channels	Adipose tissue	lipolysis

We now understand that adrenoceptors are expressed on the cell membrane and are part of seven transmembrane GPCR family. GPCR coupled with the G proteins. The latter is a specialised protein which has the ability to bind to guanosine diphosphate (GDP) or guanosine triphosphate (GTP), it is heterotrimeric having three subunits (α, β,γ)(Ruffolo and Hieble 1994). Once a ligand binds to adrenergic receptors in GPCR it produces a conformational change and exchange GDP for GTP followed by disassociation of α subunit.

This subunit can involve in one or more processors such as the production of AC, IP_3 and DAG second messengers or regulation of ion channels depending on the type of α subunit activated. It was reported that each adrenoceptors subtype has a favourable binding site to one of GPCR subunits: α_1-adrenoceptor selectively binds to $G_{q/11}$ while α_2-adrenoceptor binds to Gi and β- adrenoceptor linked to Gs (Bylund *et al.*, 1994; Foord *et al.*, 2005).

1.3.2 Characterisation and mode of action for β-adrenoceptors

The binding of an agonist to these receptors at Gs subunit resulted in increased concentration of AC enzyme, the latter is responsible for the conversion of ATP to cAMP that plays a major role as the second messenger. The β-adrenoceptors divided into three main groups, β_1, β_2 and β_3 adrenoceptors depending on the pharmacological response which they initiate. The β_1-adrenoceptors commonly found in the heart and intestinal muscles. Stimulation of these receptors resulted in an increase in contractility and heart rate and relaxation of intestinal smooth muscles (Hieble 2007).

The β_2-adrenoceptors are usually widespread in the respiratory system, uterine, heart, and vascular smooth muscle, liver and GIT tract. Activation of these adrenoceptors resulted in bronchodilation, relaxation of smooth muscle in the visceral, vasodilation, and glycogenolysis. The discovery of β_3 was in the adipose tissue where stimulation of these receptors enhances the lipolysis (Brunton *et al.,* 2011), see Table 1.1

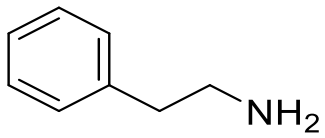
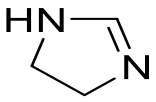
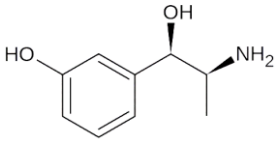
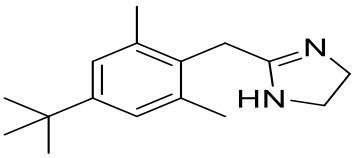
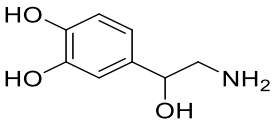
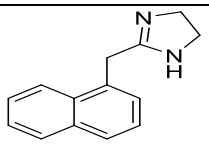
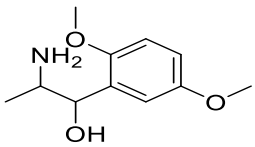
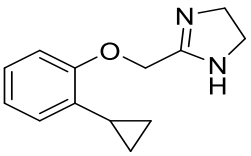
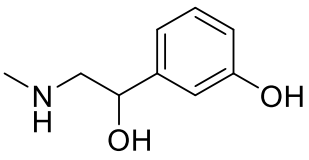
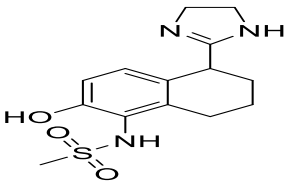
1.3.3 Classification of α-adrenoceptors

The α-adrenoceptors can be classified according to different criteria, however, for any future classification of adrenoceptors, it should ultimately involve the development of new agents that can be used clinically in the treatment of different conditions (Wilson *et al*., 1991).

1. According to their anatomical location, α-adrenoceptors can be classified into prejunctional and postjunctional. It was formal that stimulation of postjunctional adrenoceptors-mediated the sympathetic action at effector organ site this type of adrenergic receptor termed α_1-adrenoceptors. On the other hand, stimulation of prejunctional adrenoceptors is responsible for inhibiting the release of sympathetic neurotransmitter from this receptor which is termed α_2-AR (Starke *et al*., 1974).
2. According to their functional properties, because the anatomical classification failed to explain α-adrenoceptor inhibition of melanin release from melanocytes in the skin of frog and the inhibition of glycolysis by isoprenaline in hamster adipocytes and both this action are well documented to be controlled by α_2-adrenoceptors. Berthelsen and Pettinger suggested a functional classification in which the stimulation action attributed to α_1- adrenoceptor while the inhibitory actions were attributed to α_2- adrenoceptors (Berthelsen and Pettinger 1977). However, the functional classification was not applied in all instances. For example, both yohimbine and prazosin were able to inhibit the vasoconstriction produced in rat and cat blood vessels contracted by noradrenaline emphasising that postjunctional stimulation could be attributed to both α_1 and α_2-adrenoreceptors. So both anatomical and functional classification failed to provide a dependable method to classify adrenoceptors (Drew and Whiting 1979).
3. The pharmacological response to different agonist and antagonist were used to classify adrenoceptor depending on the potency of the agent used. α_1-adrenoceptors represents the responses to stimulation by cirazoline, phenylephrine, noradrenaline and methoxamine and completely blocked by prazosin while α_2-adrenoceptors responded to stimulation by clonidine, and guanfacine with a complete blocked by yohimbine and rauwolscine (Ruffolo and Hieble 1994).
4. According to their chemical structure, α-adrenoceptor ligands could be categorised into two main groups, imidazoline and phenethylamine. The imidazoline is a heterocyclic molecule which well bound to α-adrenoceptors the most common imidazoline are clonidine, lofexidine, naphazoline, A61603, brimonidine, cirazoline, xylometazoline, and oxymetazoline. On the other hand, the phenethylamine receptors are a natural alkaloid, they have a structure close to endogenous catecholamine, and they bound tightly to α_1-adrenoceptors and mainly act as a stimulant to hydrolysis of phosphoinositide. The main phenethylamine agonists are adrenaline, noradrenaline, isoprenaline,

methoxamine, phenylephrine and α-methyl-noradrenaline (Raulli and Crews 1991), see Table1.

Table 1-2 The chemical structure for some phenethylamine and imidazoline α_1-adrenoceptors agonists.

 phenethylamine	 Imidazoline
 **Metaraminol**	 **Xylometazoline**
 **Noradrenaline**	 **Naphazoline**
 **Methoxamine**	 **Cirazoline**
 **Phenylephrine**	 **A61603**

1.3.4 Characterisation and mode of action for α_1- adrenoceptors

These types of receptors are commonly distributed postjunctionally through the body in different tissue and organs including smooth muscle in blood vessels, bronchi, GIT, ureter, uterus, liver, bladder sphincter, salivary gland, pilomotor muscle and radial muscle in the iris. These receptors are located on the cell membrane. They are part of GPCR and specifically $G_{q/11}$ subunit. The binding of an agonist to theses subunit activates the second messenger system through stimulation of PLC enzyme. PLC enzymes hydrolysis the phosphoinositide on the cell membrane and produced IP_3 and DAG.

These are key second messenger responsible for the increase in the concentration of intracellular calcium that produces vasoconstriction in smooth muscle in blood vessels and increases glycogenolysis; as well as contraction pilomotor muscle, uterus, ureter, bladder sphincter, mydriasis. Increase glycogenolysis and saliva secretion and relaxation of GIT smooth muscle (Alexander *et al.*, 2008; Yu *et al.*, 1998). Since 1982 McGrath, suggested the presence of more than a_1 subtype of α-adrenoceptors (McGrath 1982).

The heterogeneity of α_1-adrenoceptor in rabbit isolated aortic rings was tested by Babich group. They compared the contraction induced by noradrenaline, adrenaline, phenylephrine and metaraminol using both functional and ligand binding methods. They reported that EC_{50} were 40±9nM, 45±7nM, 290±53nM and 1.2±0.2 for noradrenaline, adrenaline, phenylephrine and metaraminol respectively. All agents worked through stimulation of α_1-adrenoceptor (Babich *et al.,* 1987). With the use of receptors cloning, affinity labelling, gene expression, It was found that there are three main subtypes for α- adrenoceptors α_{1A}- α_{1B}, α_{1D} depending on their different affinity to the competitive blocker WB 4101 and alkylating with chloroethylclonidine (Johnson & Minneman, 1987; Wilson *et al*., 1991; Alexander *et al.*, 2011).

The α_{1A}-adrenoceptors are the main subtype responsible for the smooth muscle contraction in both artery and vein commonly located in blood vessels, heart, brain lung and prostate; α_{1B} adrenoceptors, mainly present in the heart, kidney, spleen, lung and cerebral cortex. They are responsible for maintaining the structure and cardiac growth. Finally, α_{1D} adrenoceptors located in coronary arteries, aorta, platelet, brain and prostate, they also maintain the vasoconstriction in the coronary artery and aorta (Brunton, *et al*., 2011).

1.3.5 Characterisation and mode of action for α_2- adrenoceptors

The α_2-adrenoceptors in the human body are located in nervous system compartments (brain stem, spinal cord, a neuron in addition to autonomic ganglia), platelet, and β-cells in the pancreas in addition to smooth muscle in the vasculature. They are part of GPCR and specifically Gi subunit. Coupling of an agonist to these receptors inhibits the release of AC enzyme. The latter is an essential enzyme in the synthesis of cAMP. The cAMP activates protein kinase which phosphorylates different protein and enzymes inside the cell. In addition to that, the activation of α_2-adrenoceptors will stimulate the opening of potassium channels and induced cell hyperpolarization with inhibition of Ca^{2+}entry.

The main physiological responses to the activation of these receptors are a reduction in the release of noradrenaline and acetylcholine in nerve terminals, inhibit the sympathetic outflow in the brain stem, induce platelet aggregation, decrease the release of insulin and glucagon from pancreatic cells and the inhibition of calcium channels (Gavras 2001). Similar to α_1- adrenoceptors, α_2-adrenoceptors have three subtypes (α_{2A}, α_{2B}, α_{2C}) (Bylund 1990). The α_{2A} mainly play an inhibitory role for the sympathetic release from sympathetic neurons and induce hypotension and hypothermia. While α_{2B} which usually present in liver and kidney blood vessels induce vasoconstriction. Finally, α_{2C} located in the cerebral cortex and adrenal gland upon stimulation they can modulate the release of dopamine and inhibit the release of neurotransmitter from the adrenal gland, see Table 1.1 (Brunton *et al.*, 2011).

1.3.6 Clinical roles of α-adrenoceptors

Both phenylephrine and metaraminol are selective α_1-adrenoceptors agonists while noradrenaline can act at both α_1 and α_2 adrenoceptors. they are effective pressor agents, commonly administered by infusion to treat hypotension associated with septic or cardiogenic shock (Morelli *et al.*, 2008), with spinal anaesthesia (Mon *et al.,* 2017). Phenylephrine can be administered topically and orally as a nasal decongestant by reducing the secretion of mucus and stimulating the vasoconstriction in the nasal mucosa (Eccles 2007).

Some other clinical uses of phenylephrine include induced mydriasis following the topical application on the eye (Ostrin and Glasser 2004). The suppositories dosage form of phenylephrine also provides a clinical value in the symptomatic relief of pain associated with a haemorrhoid *(Baek et al.,* 2011). Combination of phenylephrine with

some local anaesthetic reduces the pain during nasogastric intubation procedure (Craig *et al.,* 2015).

Phenylephrine maintains the contraction in smooth muscle mainly through stimulation of α_1-adrenoceptor. Many studies investigated the action of phenylephrine in different tissue and species and they have confirmed that it can stimulate different subtypes of α_1-adrenoceptor. Phenylephrine mediated the contraction in rabbit and human corpus cavernosum through stimulation of α_{1B} subtype of adrenergic receptors and the use of Rho kinase inhibitors relaxed the tissue with an EC_{50}: 2.2±0.25μM and 0.99±0.3μM for human and rabbit tissue respectively (Rees *et al.,* 2001). Another study examined the vasoconstriction action of α_1-adrenoceptor in rabbit penile artery using wire myography showed that the contraction induced by noradrenaline and phenylephrine resulted from stimulation of α_{1a} subunit mainly. Furthermore, phenylephrine-induced contraction is highly sensitive to prazosin (selective α_1-adrenoceptor antagonist) and RS100329 (subtype α_1-adrenoceptor antagonist). They also suggested the presence of α_2-adrenoceptors in the dorsal artery using UK 14,304 and rauwolscine (selective α_2-adrenoceptors antagonist) (Morton *et al.,* 2007). Another characterisation of α_1-adrenoceptor subtype involving in the contraction to phenylephrine in rat thoracic aorta, mesenteric and pulmonary arteries showed that α_{1D} is mainly responsible for the contraction in these vessels using antagonist method to test their theory (Hussain and Marshall 1997).

The hypotension which is a common side effect of spinal anaesthesia during caesarean surgery usually treated with α_1-adrenoceptor agents. Two of the most commonly prescribed drugs currently in use are metaraminol and phenylephrine. In a clinical study involving 90 women to compare the efficiency and the side effect associated with the use of these two agents with ephedrine, it was reported that although the three agents raised the blood pressure efficiently, nausea, vomiting and hypertension were more common with ephedrine. Indicating more safety profile related to phenylephrine and metaraminol during the treatment of hypotension induced by spinal cord anaesthesia (de Aragão *et al.,* 2014).

Another recent clinical study compared the prophylaxis use of phenylephrine and metaraminol to prevent the hypotension accompanied the spinal cord anaesthesia during a surgical procedure. The results of this double-blind trial showed regarding the incidence of hypotension, hypertension and neonatal acid-base balance both drugs have similar safety profile (McDonnell *et al*. 2017).

1.3.7 Porcine Splenic artery

We have used the porcine splenic artery in the majority of experiments in this thesis. Stimulation of α_1 and α_2 adrenoceptors suggested inducing contraction in VSMs however, the percentage of each receptor participation in this action was debated. The density of α-adrenoceptors in this porcine splenic artery was previously tested. According to Wright and his colleagues who used the saturation and ligand binding method using noradrenaline and phenylephrine as vasoconstriction agents. Their finding confirms that the contraction induced in the porcine splenic artery is mainly maintained by α_1-adrenoceptors with no role of α_2-adrenoceptors (Wright *et al.,* 1995). These results were confirmed by another study conducted by Barbieri and his group in 1998 who studied the α_1-adrenoceptors which mediated contraction and characterised receptors in the isolated porcine splenic artery using noradrenaline, phenylephrine as selective α_1-agonists. Their results showed that porcine splenic artery possesses only functional α_1-adrenoceptors, moreover, they have shown that α_{1A} subtype is responsible for the pharmacological activity in this tissue with no role of α_2-adrenoceptors and a minimal role for α_{1B} and α_{1D} subtypes (Barbieri *et al.,* 1998).

More studies examined the role of α_2-adrenoceptors in the vasoconstriction action in the vasculature. It was suggested that in the normal physiological condition it was difficult to detect a functional α_2-adrenoceptor, yet pre-contraction of the vascular smooth muscle can demonstrate the role of prejunctional α_2-adrenoceptors. It has been reported that the use of U46619 (thromboxane–mimetic agonist) enhanced the contraction induced by UK14303. The latter is a well-known α_2-agonist. This enhancement was abolished in the absence of Ca^{2+} (Bhattacharya and Roberts 2003). In light with these studies, we concluded that the contraction in this preparation is mainly due to the stimulation of α_1-adrenoceptors.

1.4 The discovery of lithium

In 1800 lithium was discovered in Sweden in a form of rocks thus its name was derived from the Greek word *lithos.* As summarised by Johnson (1980) since its discovery it had been used to alleviate symptoms in a variety of conditions including rheumatism, gout, infection associated with diabetes and renal stone without firm evidence for its therapeutic role in these conditions (F. Neil Johnson 1980). Lithium use in psychiatry was first introduced by Carl Lange in 1886, he described the value of lithium-containing mixture in the prevention of severe depression. For the time between 1929-1948, lithium was available for public use in a form of lithium citrate; it was used as one

component in the famous beverage drink ” Lithiated 7 Up” which was known as (Lithiated Lemon-lime Soda) launched by Dr Peppers company in the United State. The company claimed that this drink has a calming and mood-enhancing properties and advised the mothers to mix it with the milk to increase the milk tolerability in babies. While in adults the company claimed that it has slenderizing and antacid properties. The role of lithium was limited due to the severe side effects associated with its use.

Figure 1-5 Bottle cap, label and advertisement from 1930 showing the lithiated Lemon-Lemon Soda drink (Lithiated 7up web site source)

In 1949, Cade’s discovery initiated a series of research programmes across the world to confirm lithium roles in “calming” manic patients. In 1970 a group of Danish physicians conducted a big clinical research in which their findings were accepted by doctors across the world. They showed that lithium has a potential role in preventing the mania and depression at the same time the United States Food and Drug Administration (FDA) approved the use of lithium in the treatment of mania then extended this use in 1974 to involve the prevention of manic-depressive illness also. With the invention of new atypical antipsychotic and anticonvulsant, medication and due to the lack of commercial interest and the reported cases of chronic nephropathy and other renal problems related to the chronic usage of lithium, the therapeutic use of lithium across the world has slowed. (Hestbech *et al.*, 1977; Bauer and Gitlin, 2016)

1.4.1 Renaissance of lithium treatment

After more than 50 years of its approval by the FDA for the treatments and prevention of different psychological disorder, many new aspects of lithium effects in neuropsychiatry have been discovered. Its ability to prevent suicide was markedly acknowledged in the last 20 years. This gave lithium superiority over other mood stabilizer medication (Can *et al.,* 2014). In addition to that, some recent researches spotlighted the ability of lithium to protect the neurons that might be attributed to the treatment of neurodegenerative diseases and dementia (Lewitzka *et al*., 2015).

1.4.2 Recent position of lithium in modern psychiatry

The early use of lithium in psychiatry was mainly as a prophylactic agent for bipolar disorder and the treatment of acute manic attack. In 2014 two recent studies emphasise the effectiveness of lithium in preventing mood episodes (Severus *et al*., 2014). Nolen in 2015 recommended lithium as the preferred monotherapy first line for the long-term management of manic-depression disorder. This is in line with the recommendation of British NICE (National Institution for Health and Care Excellence 2006) in which lithium was considered as the solo first-line treatment of bipolar disorder (Nolen 2015).

The second most common and licensed use for lithium is an adjuvant of antidepressants especially in resistant cases of depression which do not respond to typical antidepressants. Many psychiatric organization approved the use of lithium in treating major depression episodes such as NICE, World Federation of Societies of Biological Psychiatry (WFSBP) and the British Association of Psychopharmacology (BAP) (Bauer *et al.*, 2013; Cleare *et al.*, 2015).

1.4.3 Optimal plasma concentration of lithium

According to the worldwide guidelines of lithium dosing, the target plasma concentration at 0.6-0.8 mmole/litre seems to be acceptable. According to the French HAS and the British NICE guidelines, for the immediate release preparation while higher plasma concentration at 0.8-1.2 mmole/litre was required for the sustained release preparation (Goodwin and Goldstein 2003).

In the USA, the American Psychological Association (APA) suggested a gradual increase in the plasma concentration starting from 0.5 mmole/litre until it reaches 1.2 mmole/litre (American Psychiatric Association 2002). The disparity in the reference's plasma concentrations was to provide an acceptable plasma level which controls the symptoms of bipolar disorder without causing a failure in the treatment associated with lower doses or mania associated with higher plasma concentration of lithium. Choosing the optimum dose of lithium depends on age, renal function, sex, and the responsiveness to treatment. It was found that 0.5mmole/litre rarely produced a pharmacological response while most male patients responded to maintenance dose at plasma concentration level at 1mmole/litre and higher level is predisposing to the risk of lithium toxicity because it has a narrow therapeutic window (Severus *et al.*, 2008; Malhi *et al.*, 2012; Nolen, 2015).

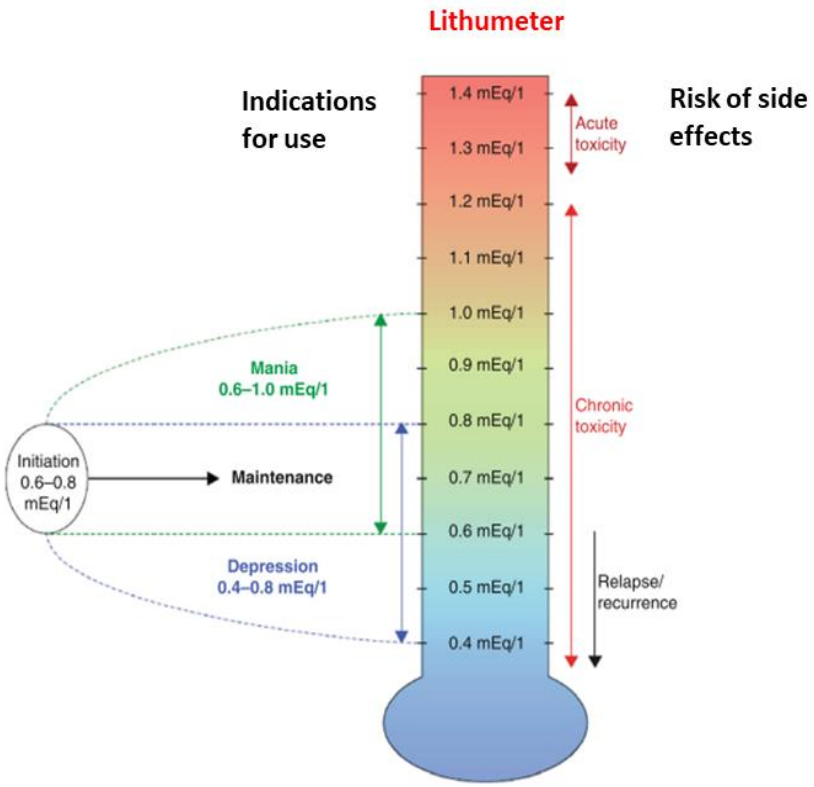


Figure 1-6 The lithiumeter, showing the initial and maintenance plasma concentration of lithium during the treatment of mania and depression (Malhi *et al.*, 2012).

1.4.4 Physiology of lithium

For more than 70 years, many studies focused on the use of lithium in the treatment and prophylaxis of bipolar disorder. Lithium usually influx into the cells by substitution of either sodium or potassium on the same transport pathways which normally transport these cations across the cell membrane. On the other hand, the efflux of lithium is limited to some transporting system that resulted in accumulation of lithium inside the cell. Lithium needs to be actively pumped out of the majority of cells. The ratio of the cell to plasma concentration of lithium is 2-4 in rat vascular tissues. Sodium –proton exchanger and epithelial sodium channels are the main proteins transporting lithium inside the cell. The permeability of epithelial sodium channels to both sodium and lithium is equal, thus this pathway considered as primary pathways through which lithium influx inside the cell. Furthermore, sodium-proton exchanger (Na^+/H^+ exchanger) is available in most cells and it is responsible for the reabsorption of a major portion of sodium in the proximal tubule. Due to the similarity in charge and size between lithium and sodium, lithium can replace sodium and transport through these pathways at a slower rate. Both of these transporting proteins are inhibited by amiloride. The Na^+/K^+ -ATPase could be suggested as a pathway to transport lithium across the cell membrane. However recent studies suggested that this pump under normal physiological conditions has less affinity for lithium than that for either sodium or potassium which reduced the possibility of its involvement in lithium movement across the basolateral membrane. (Finley *et al*., 1995; Lenox *et al.*, 1998).

Lithium efflux the cell mainly through sodium-sodium exchanger proteins which also known as sodium-lithium counter transport, this exchanger has 15-18 fold more affinity for lithium than sodium. Patient treated with lithium, the plasma concentrations for sodium and lithium is 140 mmol/l and 0.6-1.2 mmol/l respectively. The concentration of sodium intracellular is less than 10 mmol/l. The sodium will transport down its electrochemical gradient into the cell that resulted in pumping out of lithium outside the cell (Timmer, R.T. and Sands, J.M. 1999).

1.4.5 How does lithium work?

Bipolar disorder (BD) is a common and recurrent mood disorder that has a remarkable effect on both the patients and the society as a whole. The presences of manic attack characterised bipolar disorder from acute depression or monopolar disorder. After more than 70 years of using lithium in the treatment of bipolar disorder. There is a dramatic increase in the research and studies exploring the mechanism of action for

lithium. Following research and studies conducted to understand how lithium works, an only partial understanding of what mechanisms were reported with lots of questions which remain obscure. In 2015, Alda reported that lithium is a small molecule and we can predict that it has a simple mechanism. However, it is the opposite due to its simple structure, lacks the need for specific receptors mechanism and the similarity in its electronegativity with Mg^{2+}. lithium interfere with multiple biological processes inside the body such as maturation of embryo, differentiation of cells and tissues, and inhibition of cell ageing and apoptosis and can affect many signalling pathways inside the cell (Vestergaard and Licht, 2001; Alda, 2015; Bauer and Gitlin, 2016; Roux and Dosseto, 2017).

1.4.5.1 Lithium effect on electrolytes level, action potential and membrane transport

One of the early suggested mechanism of action for lithium is through the ability to balance electrolytes level in a patient with BD. It was found that the plasma level of sodium is increased with episodes of mania and depression and the use of lithium can normalize this level again (Coppen, 1967; El-Mallakh, 2004). The use of lithium also reported being responsible for the reduction in the action potential of the overexcited neurones (Schou 1957). In 1970, Mendels and Frazer suggested the use of a ratio between the intracellular and extracellular level of lithium, they proposed a direct relation between this ratio and the response to the treatment in a patient with BD. However the subsequent research failed to prove this relation especially after the discovery that lithium is not distributed evenly in all tissues (Mendels and Frazer, 1973; Spirtes, 1976; Rihmer *et al.*, 1982).

The transportation of lithium across the cell membrane and the net balance between the intracellular and extracellular level of lithium has an important role in its mechanism of action. Usually, lithium effluxes from the cell through lithium-sodium counter transporter proteins while the influx is usually by either passive diffusion or by epithelial sodium channels in the excitable tissues. The Na^+/K^+ ATPase has an only minimal role in the transportation of lithium across the cell membrane, however, its role is much more important in maintaining the level of Na^+ and to provide the cell with energy to maintain the action potential (Ehrlich and Diamond 1979).

This pump consumes energy to pump Na^+ outside the cell while pumping K^+ to the inside against their concentration gradients. The main source for energy for this pump is by glycolysis and from ATP located in the mitochondria (Fernández-Moncada and

Barros 2014). In BD patients, a considerable impairment in mitochondria function was reported and due to the importance of Na^+/K^+ ATPase in maintaining several cellular functions such as signal transduction and maintenance of calcium haemostasis, apoptosis process (Aperia 2007). The impairment in the activity of this pump was proposed as one of the pathophysiological mechanism of BD (Kirshenbaum *et al.,* 2012). Lithium was found to enhance the activity of this pump in overactive neurones by reducing the concentration of the intracellular level of Na^+ (Reddy *et al.,* 1992).

1.4.5.2 Lithium effect on a second messenger system

Lithium is well established to affect various signalling pathways inside the cell. Early studies investigated the effect of the lithium on the AC and protein kinase A (PKA) enzymes. AC enzyme is under control of G proteins which is either stimulatory (G_s) or inhibitory (G_i). Patients with BD have alteration in the function of these enzymes and the administration of lithium can further interfere with the signalling pathways of this enzyme. According to Young and his colleagues, the level of Gs α subunit was found to be high in the brain of postpartum BD patients with a significant increase in the concentration of cyclic adenosine monophosphate (cAMP) after exposure to forskolin (Young *et al.,* 1993). It was reported that the use of lithium can reduce the signalling amplitudes by inhibiting both G_s and G_i subunits (Manji, 1991; Jope, 1999).

The enzyme PKA which regulate transcription factors, generated following the catalytic effect of AC that converts ATP to cAMP. Lithium has minimal effect on the activity of PKA and AC at basal resting state, however, it inhibits both enzymes by competing with Mg^{+2} following acute but not chronic administration of the drug (Mørk and Geisler, 1987; Mørk and Geisler, 1989a; Mørk and Geisler, 1989b). Both G proteins and tyrosine kinase can activate phospholipase B and phospholipase C respectively- this resulted in hydrolysis of phospholipid at the cell membrane especially phosphatidylinositol 4, 5-bisphosphate (PIP_2). The hydrolysis of this compound resulted in the formation of two important second messenger: 1, 4, 5 triphosphate (IP_3) and diacylglycerol (DAG), the latter initiates the activation of kinase C enzymes while the IP_3 broken down to inositol bisphosphates (IP_2) and then to inositol monophosphates (IP). The enzyme IMPtase broke down the IP to myoinositol (Brown and Tracy 2013). Lithium at a therapeutics level inhibits IMPtase, which result in a depletion of myoinositol and subsequent reduction in the synthesis of DAG and IP_3 because myoinositol is a substrate for resynthesis of PIP_2 (Berridge *et al.*, 1989).

Many researchers examined the role of lithium on the phosphoinositide metabolism both *in vitro* and *in vivo.* It was found that the intracerebral injection of LiCl resulted in a time and dose-dependent increases in the labelled IP in rat brain. This was accompanied by a significant decrease in the labelled myoinositol level (Sun *et al*., 1992).

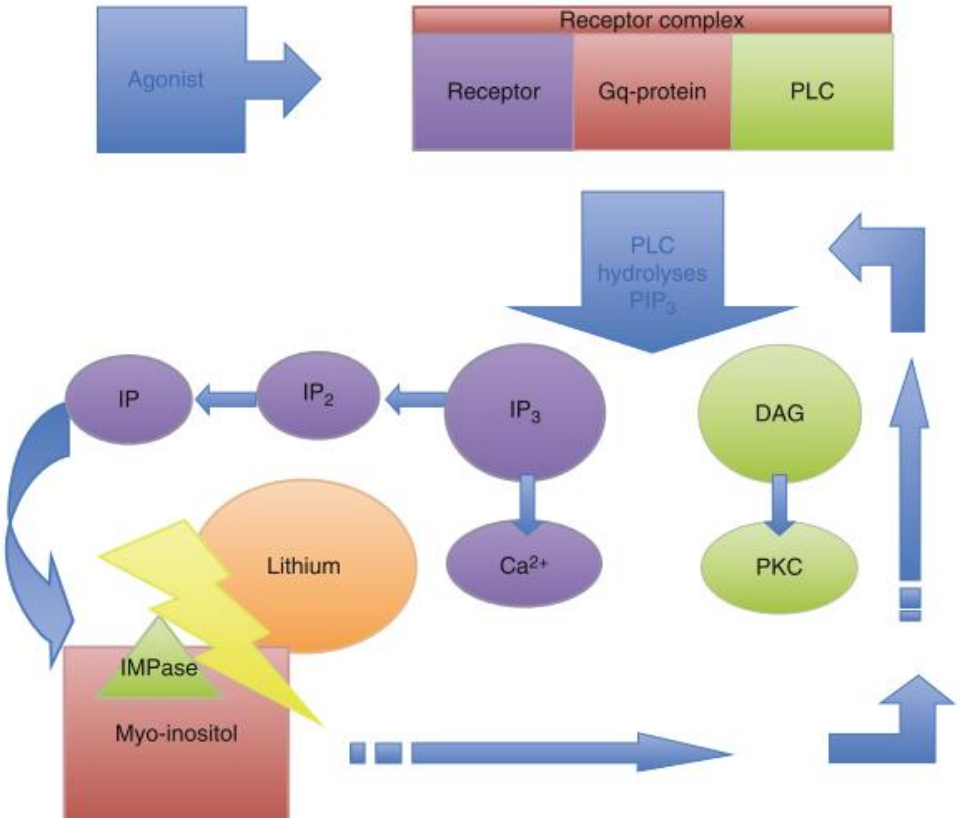


Figure 1-7 Mechanism of action of Lithium affecting cell signalling pathway; lithium inhibits inositol monophosphatase enzyme; leading to myoinositol depletion and hence it prevents the resynthesis of phosphatidylinositol 4,5-bisphosphate (PIP_2) and subsequent regeneration of IP_3 and DAG. Figure adapted from (Brown and Tracy 2013)

1.4.5.3 Effect of lithium on the glycogen synthase kinase 3 (GSK3)

GSK3 is a constitutively active enzyme, the first discovered action for these enzymes was a synthesis of glycogen. This enzyme can phosphorylate more than 50 substrates or proteins inside the cell and thus act as a hub to regulates several cellular processes such as gene expression, inflammation, apoptosis, development of the embryo, transmission of nerve impulses across neurones and regulate the function of the heart. The disturbance in the action of this enzyme can resulted in severe pathological

condition such as diabetes, psychiatric disorder, cardiac pathology, neurodegenerative diseases and cancer (Gao *et al.*, 2012; Cole, 2013; Llorens-MarÃ-tin *et al.*, 2014; Lal *et al.*, 2015; McCubrey *et al.*, 2017). Lithium can inhibit this enzyme at a therapeutic level ranging from 1-2mM by two pathways, the direct pathway by competing with Mg^{+2}. The indirect action of lithium is through the activation of protein kinase B (Akt), the latter act as an inhibitory regulator for the GSK3. Stimulation of dopamine receptor$_2$ (D_2R) or GPCR can trigger the formation of signalling complex which consists of three subunits β arrestin2 (βarr2), protein phosphatase $_2$A (PPA) and protein kinase B (Akt). This signalling complex inactivates Akt and maintains the action of GSK3 enzymes. The other indirect action of lithium is by enhancing the activity P13K which activates Akt directly (Klein and Melton, 1996; Stambolic *et al.*, 1996).

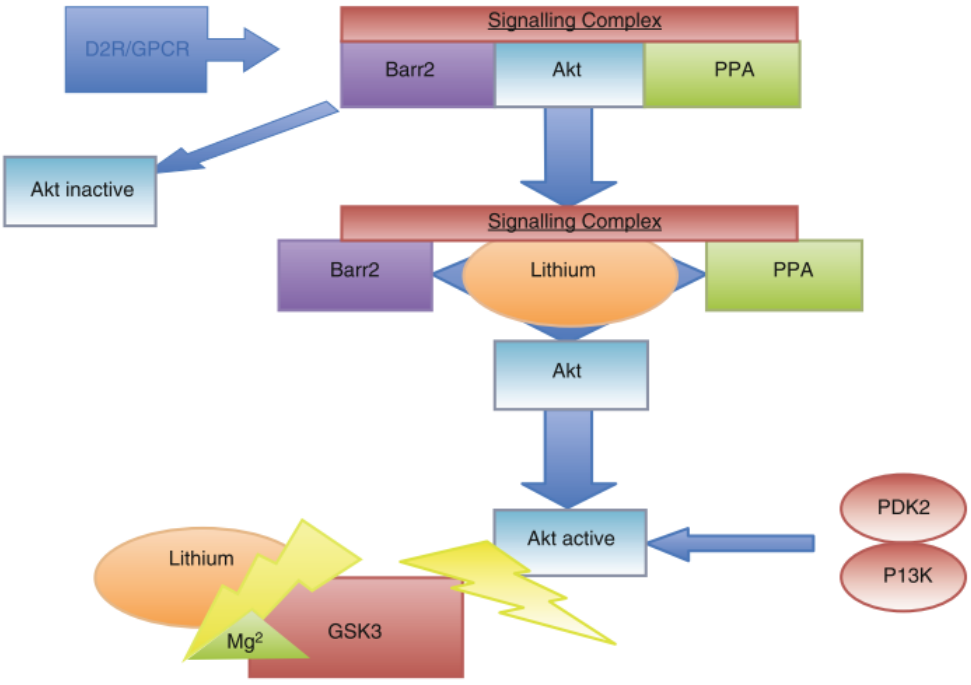


Figure 1-8 Inhibition of glycogen synthase kinase3 (GSK3) by lithium by direct and indirect pathways. Figure adapted from (Brown and Tracy 2013).

1.4.5.4 Effect of lithium on AC

Adenylyl cyclase enzymes (AC) is located in cell membrane it acts by converting ATP to 3, 5 cyclic adenosine monophosphate (cAMP). The latter is responsible for several cellular functions such as signal transduction, opening Ca^{2+} channels in addition to the activation of phosphokinase A (PKA) (Reches *et al.,* 1978). Lithium affects AC by two

mechanisms: first by competing with Mg^{+2} at the receptors binding sites. Second, by inhibiting $G_{\alpha s}$ subunit. This subunit is known to stimulate the synthesis of cAMP. The action of lithium was reported in human, rats and guinea pig where the use of therapeutic concentrations of lithium reduced the level of cAMP enzymes (Forn and Valdecasas, 1971; Belmaker *et al.*, 1980; Ellis and Lenox, 1990; Mann *et al.*, 2009).

1.4.5.5 Effect of lithium on neurotransmitter

The main neurotransmitters affected by lithium are dopamine, noradrenaline and serotonin they also are known as monoamines. Some drugs modulate the action of monoamines at their targets and are used to treat some psychiatric disorder such as BD. After ingestion of lithium, it is distributed in CNS and affects these monoamines neurotransmitters (Mota de Freitas *et al*., 2016). On serotonin neurotransmitter, animal research showed that lithium enhances serotonin by either increase its synthesis or release or by enhancing the uptake of tryptophan possibly through the inhibition of $5HT_{1A}$ presynaptically and activation of $5HT_{1A}$ postsynaptically with a reduction in $5HT_2$ receptors. The serotonergic effect of lithium could be attributed to its anti-suicidal, anti-aggressive effects as well as some of the antidepressant action. However, a two week follow up study in healthy volunteer failed to confirm such effect on the serotonergic neurotransmitter and only showed a small increase in noradrenaline release (Manji 1991). With respect to the dopaminergic system, the administration of lithium did not reduce the basal dopamine level. On the other hand, when dopaminergic activity is increased, lithium inhibits it via its action on β arrestin. The dopaminergic action of lithium possibly has a part in its anti-manic and antipsychotic effects (Grof and Grof 1990). Some clinical studies in human and animals showed that lithium can block the behavioural changes accompanied the administration of amphetamine which is maintained by dopaminergic neurotransmitter (Huey *et al.*, 1981).

Regarding the glutamatergic system, lithium shows several actions. After acute administration, lithium blocks the reuptake and enhances the release of glutamate by competing with Mg^{2+} ion. After several days of treatment, lithium reduced the concentration of glutamate at synaptic and increase the uptake of neurotransmitter (Dixon and Hokins, 1998; Akiska*l et al*, 1985). Opposite to the inhibitory role of lithium on the excitatory neurotransmitters (dopamine and glutamate) lithium enhances the action of γ–aminobutyric acid (GABA) neurotransmitter which is thought to have a crucial role in the release of both dopamine and glutamate in addition to its ability to enhance the release of neuroprotective protein and inhibits the pro-apoptotic proteins

inside the cells (Malhi *et al.*, 2013). In conclusion, lithium stimulates the inhibitory neurotransmitters and inhibits the excitatory neurotransmitters.

1.4.6 Interaction with intracellular calcium

In neurons, calcium is responsible for multiple functions. In addition to its role as a second messenger in the cells, it triggers the release of neurotransmitters in the presynaptic terminal. Furthermore, calcium enhances cell death, plasticity and synaptogenesis (Warsh *et al.*, 2004). Following the depolarization of the membrane, calcium enters the cells from extracellular and intracellular compartment via calcium channels. These channels are regulated by lithium in addition to the other second messenger pathways. In BD, it has been reported that the haemostasis of calcium is disturbed and the use of mood stabilizer help to regulate and normalize it (Emamghoreishi *et al.,* 1997; Petrova *et al.*, 2010).

1.4.7 Side effect and sign of toxicity

Most of the lithium side effects are dose-dependent. Reducing the dose will result in a reduction in the reported side effect associated with the use of lithium in both the prophylaxis as well as the maintenance treatment of psychiatric disorders (Albert *et al.,* 2013). Lithium can cause some minor side effect such as fine tremor in the hand which could be acute symptoms disappear after a period or could continue along with the treatment. Headache and nausea can result due to lithium-induced electrolyte imbalance. These are easily avoided by increasing water intake to normalize the electrolyte imbalance. It is well documented that lithium treatment can precipitate downbeat nystagmus which can last from days to months and is ceased by abstinence (Chan *et al.,* 2012; Bohlega and Al-Foghom, 2013).

Many clinical studies have connected lithium treatment with severe complication such as hypothyroidism (Tasevski *et al.,* 2000; Bschor and Bauer 2013; Sierra *et al.,* 2014; Özerdem *et al.*, 2014), weight gain (Grandjean and Aubry 2009), nephrogenic diabetes insipidus also connected with the treatment with lithium and suggested a complication with anti-diuretic hormones as possible explanation (Ibbeken *et al*., 2012; Gahr *et al.,* 2014). Permanent renal failure is also reported in some studies (De Groot *et al.,* 2014). The ability of lithium to inhibit GSK3 enzymes is thought to be responsible for the wide spectrum range of side effect associated with its use (Brown and Tracy 2013). Lithium is teratogenic (Hosseini *et al.,* 2010) and should not use during breastfeeding

1.4.8 Interaction of lithium with vasoconstriction and vasodilation agents

Due to the similarity in the suggested mechanism of action of lithium with many second messenger pathways inside the cell, a large number of studies focused on the interaction between lithium and different receptors systems such as adrenergic receptors, muscarinic receptors, histamine and angiotensin receptors, vasopressin receptors as well as α-adrenoceptors. These receptors act mainly through the mobilization of Ca^{2+} inside the cell through stimulation of phosphatidylinositol metabolism, and generation of IP_3. We have addressed some example of studies which highlighted the interaction between a therapeutic concentration of lithium with some of these receptors in different vascular and non-vascular tissue in different species, see Table 1.1.

A study conducted by Meknes and his group examined the effect of 2mM lithium solution on the phosphoinositide cycle. This cycle is known to be affected by the action of many hormones and neurotransmitters. The isolated tracheal smooth muscle segment from guinea pig was precontracted with carbachol, histamine and KCl in the presence and absence of 3mM LiCl and washed. The time of relaxation was measured. It was found that in the absence of lithium the time of relaxation to 50% of the tension produced by carbachol was 5 minutes. In contrast, incubation the tissue with LiCl prolonged that time to 12 minutes. They reported a similar sort of effect on the histamine but not with KCl induced contraction. The main outcome of this study was LiCl enhanced carbachol-induced contraction in this preparation (Menkes *et al.,* 1986).

In a different study, the effect of a range of LiCl concentrations was used to detect their action on the positive inotropic action of phenylephrine and isoprenaline on the rat heart using isometric tension technique. It was found that phenylephrine elicited positive inotropic increased in the presence of 0.25, 0.5, 1 and 3mM LiCl. This action was evident at low concentration and become statistically significant at 1 and 3 mM. This enhancement was accompanied by an increase in the potency and shift the response curve to the left. No enhancement in the inotropic effect of isoprenaline was reported (Skomedal *et al.,* 1991).

This interaction between lithium and α_1-adrenoceptors agonist not limited to vascular tissue. It also reported in non-vascular tissue. In their study, Dehpour and his colleagues reported that lithium enhanced the isolated rabbit anococcygeus muscle contraction in response to stimulation with methoxamine. The latter is a selective α_1-

adrenoceptors agonist. It was reported that methoxamine induces stable spontaneous contraction in this preparation at a concentration of 0.5μM (control). Incubation of the tissue with different concentrations of lithium reduced the minimal effective concentration of methoxamine required to initiate the contraction.

Another study reported the interaction between lithium and another contractile agent (vasopressin) in the smooth muscle of the rat urinary bladder. It was reported that vasopressin produced a dose-dependent contraction in this smooth muscle and the incubation of the isolated smooth muscle with lithium can enhance the sensitivity to stimulation by vasopressin. Vasopressin initiated contraction which reached its maximum response at a concentration of 500nM with a potency of 7.4±0.4. The preincubation the tissue with lithium at 0.5, 1 and 10mM increased the maximum response to become 115%, 130%, 150% respectively. This action was ascribed to the ability of lithium to inhibit inositol monophosphatase enzyme in this preparation (Dehpour *et al*., 1997). Bosch and his group highlighted the effect of incubation the tissue with lithium on the relaxation induced by an endothelium-dependent mechanism using Ach in two types of tissue (murine aorta and porcine cerebral artery) tested in isometric myography. It was reported that lithium at low concentration <0.4mM enhanced the Ach- induced relaxation. The use of higher concentrations (0.8-100mM) of lithium was associated with opposite action by inhibition of Ach induced relaxation. No effect for lithium incubation was reported on the r relaxation induced by sodium nitroprusside (Bosche *et al*., 2016a).

In line with those results obtained from Dehpour observation regarding lithium enhancement of methoxamine induced contraction in rabbit anococcygeus smooth muscle (Dehpour *et al.*, 1993) and In light with an early observation made by Rayment and colleagues, who reported that in internal sheep isolated anal sphincter, a preparation which endowed with α_1-adrenoceptors the stimulation with phenylephrine or methoxamine produced a contraction which declines after less than 60 minutes, and the incubation of the preparation with 0.5-3 mM LiCl prevented that decline in the maximally-effective concentration of both agonists (Rayment *et al.*, 2007).

Table 1-3 The main studies conducted in different species to detect the effect of adding different concentrations of lithium on vascular smooth muscle contraction/relaxation mechanism

Lithium concentration	**Species, site of interaction**	**Action of lithium**
0.1-10 mM	Guinea pig myenteric	Enhances myenteric plexus muscle to electrical stimulation [a]
0.5-1 mM	Human	Inhibits adrenaline induced rise in cGMP [b]
2mM	Guinea pig tracheal smooth muscle	Inhibits relaxation induced by carbachol and histamine in tracheal smooth muscle [c]
7 mM	Guinea pig atrium	Enhanced phenylephrine-induced positive inotropic in heart [d]
0.6-1 mM	Rat cortex	Inhibits the adrenergic and cholinergic increase in cGMP [e]
1-10 mM	Rat ventricles	Inhibits phenylephrine inotropic effect in the heart [f]
0.25-3 mM	Rat heart	Enhances the phenylephrine inotropic effect in the heart [g]
1,3 5 mM	Rabbit anococcygeus muscle	Enhances methoxamine induced contraction [h]
0.5, 1, 10 mM	Rat urinary bladder	Enhances vasoconstriction induced by vasopressin [i]
0.3 mM	Rat thoracic aorta	Enhances endothelium-dependent relaxation to Ach [j]
0.33 mM	Rat corpus cavernosum	Inhibits Ach induced relaxation [k]
1-5 mM	Rat anococcygeus muscle	Inhibits nitric oxide-mediated relaxation [l]
0.4 mM	Murine aorta & porcine cerebral artery	Enhanced Ach induced relaxation [m]
0.8 mM		Inhibits Ach induced relaxation [m]

a- (Hirsch *et al.,* 1978)
b- (Belmaker *et al.,* 1980)
c- (Menkes *et al.,* 1986)
d- (Molderings and Schiimann 1987)
e- (Abraham 1988)
f- (Mantelli *et al*., 1988)
g- (Skomedal *et al.,* 1991)
h- (Dehpour *et al.,* 1993)
i- (Dehpour *et al.*, 1997)
j- (Dehpour *et al.,* 2000)
k- (Sadeghipour *et al.*, 2007)
l- (Karimollah *et al.,* 2009)
m- (Bosche *et al*., 2016a)

1.5 Hypothesis and aims of the study

The hypothesis of the work in this thesis derived from the ability of lithium salts (used in a therapeutic concentration) to enhance either the magnitude and/or the time course of the response to phenylephrine in different vascular and non-vascular smooth muscle in the pig. As we mentioned previously, phenylephrine is commonly prescribed drug use in the management of many clinical conditions and the presence of lithium could reduce the frequency of the doses that result in an increase in patient compliance to the treatment.

This study has two main aims: First, to examine the time course of the contraction induced by different contractile agents in vascular and non-vascular isolated porcine tissue in the presence and absence of a therapeutic concentration of LiCl, with an attempt to characterise the mechanism of the interaction between LiCl and α_1-adrenoceptors agonists. Second, to test the ability to replicating ours *in vitro* results on the skin blood flow in man using the laser Doppler flowmetry to detect any potential clinical benefits of this interaction in a healthy volunteer.

To our knowledge, no one has compared the difference in the time course of the contractile agent and use a non-cumulative protocol to add the agonist to the tissue. In addition to that, in the recent years, researchers have shown an increased interest in the clinical uses of lithium and its interaction with other medication, however, little information regarding the interaction between lithium and α_1-adrenoceptors agonist-induced vasoconstriction in vascular and non-vascular porcine smooth muscle were provided.

Chapter 2: Methodology for Contractility Study

2.1 Methodology

Because we have used the isometric tissue recoding technique in the first three chapters in this thesis, it was reasonable to summarise the common points of the methodology section to avoid repetition. However, each chapter will contain the essential information including the tissue, chemicals used and their salts in addition to the protocol followed.

2.1.1 Materials buffer and chemicals

The Krebs Henseleit solution was prepared in the lab freshly and constituted the following chemicals (mM): KCl 4.7, NaCl 118, $CaCl_2 2H_20$ 1.3, $NaHCO_3$ 25, $MgSO_4 7H_2O$, 1.2, KH_2PO_4 1.2, to which 2.1 $gm.L^{-1}$ of D-glucose anhydrous needs to be added. All should be dissolved in distilled water, followed by gassing for 15 minutes with 95% O_2; 5% CO_2 (to maintain the pH at 7.4). Finally, the buffer is ready for use and could be stored at 4°C. The following chemicals were used in studying the contractility in this thesis, the concentrations and other details of each chemical, their salts, and their roles will be mentioned in the method section in each chapter.

Table 2-1 The list of chemicals and materials used in contractility study in this thesis

Reagents	**Solvents**	**Company name and production country**
Sodium Chloride	D.W	Fisher Scientific, Loughborough, UK
Potassium Chloride	D.W	
Sodium hydrogen carbonate	D.W	
Calcium Chloride	D.W	
Magnesium sulphate	D.W	
Potassium dihydrogen orthophosphate	D.W	
Glucose	D.W	
Cocaine	D.W	Nottingham, hospital pharmacy
Dobutamine	DMSO	Tocris Bio-Techne, Abingdon, UK
Endothelin1	DMSO	
Isoprenaline	D.W	
A61603	D.W	
Angiotensin II	DMSO	
Pargyline	ethanol	
Tolcapone	D.W	
Vasopressin	ethanol	
U46619	ethanol	
SB216367	DMSO	
L 690, 330 and L 690,448	D.W	
Noradrenaline	D.W	Sigma Aldrich, Germany
Phenylephrine	D.W	
Metaraminol	D.W	
L-erythromethoxamine	D.W	
DMSO (dimethylsulphoxide)		
Concanavalin A	DMSO	
Ouabain	DMSO	
Rubidium	D.W	
Concanavalin A	D.W	
Cirazoline	D.W	
α, β mATP	DMSO	
Amiloride	D.W	
Lithium (sulphate, chloride, carbonate)	D.W	
Ascorbate	D.W	
Corticosterone	DMSO	
Propranolol	DMSO	
Histamine	D.W	
5HT	DMSO	
Carbachol	D.W	BDH laboratory, Pool, UK
ethylenediamine tetraacetic acid (EDTA)	D.W	

2.1.2 Tissue collection and preparation

Unless otherwise stated, all experiments described in this report followed a standard protocol to record the isometric tension in the porcine splenic artery. Porcine tissues were obtained from the local abattoir in Nottingham and transported to the lab in ice boxes. Several centimetres of the dorsal branch of a splenic artery from both gender pig < than 12 months old was dissected, see Figure 2.1. And placed in Krebs Henseleit. Then the buffer stored overnight in a refrigerator at 4°C.

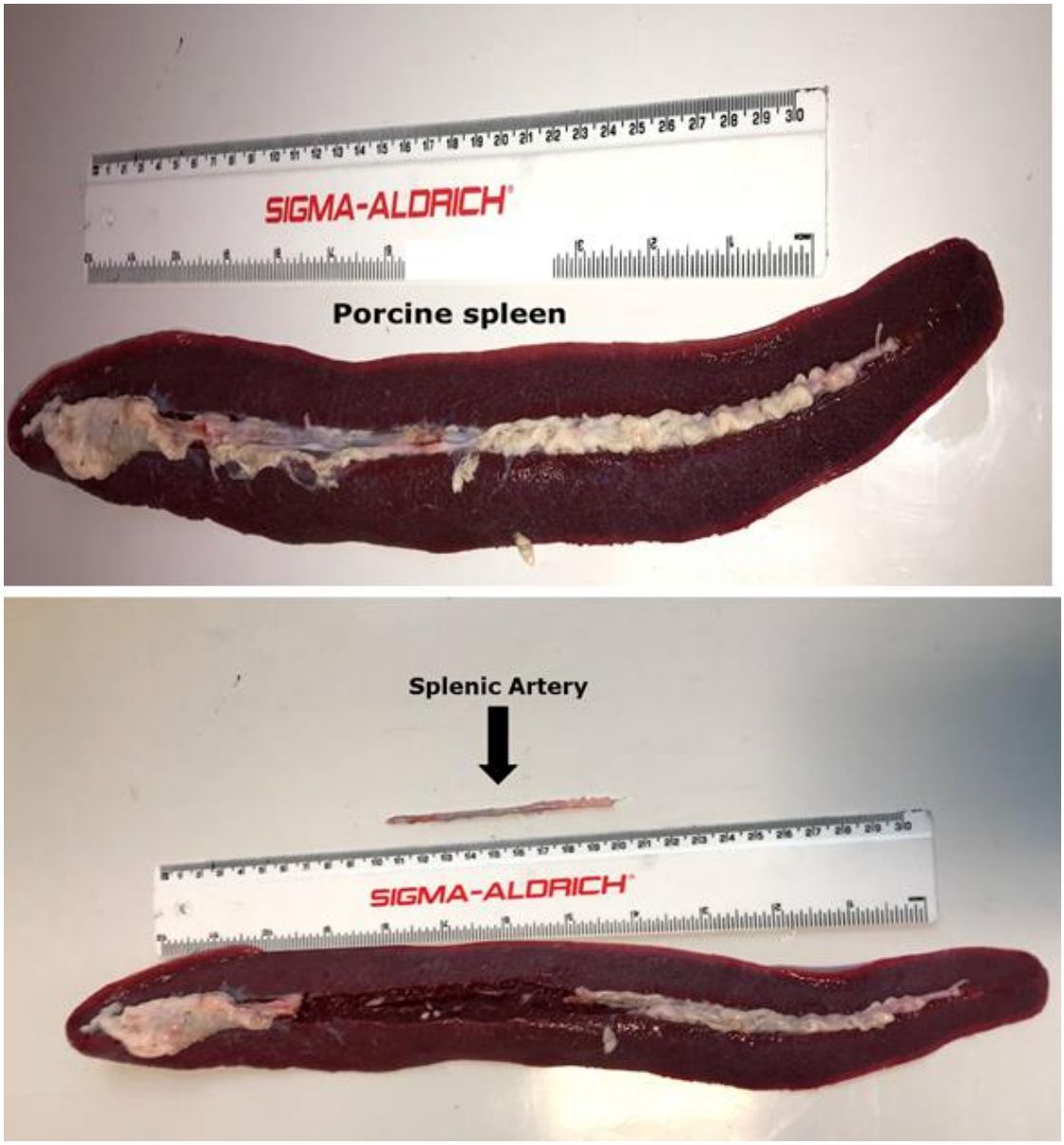


Figure 2-1 Porcine spleen (upper) and 10 cm of the porcine splenic artery (lower)

On the following day, a fine dissection was performed to clear the artery from the surrounding fat and connective tissues; it was then divided into four segments. Each 4 mm segment of porcine splenic artery was fixed in a 20ml organ bath filled with Krebs Henseleit and continuously gassed with 95%O_2 and 5%CO_2 using a stainless-steel hook to penetrate the lumen. Another hook was connected by a cotton string (0.4mm in diameter) and used to transfer tension to a Force displacement transducer, which was connected to an AD Instruments Quad Bridge pre-amplifier unit. A daily calibration for the transducers was done using 20-gram weight. The tension was recorded using either Mac lab chart v3.5 program run on a Macintosh LC IV computers or Mac lab chart v7 program run on Window XP computers. The temperature of the organ bath was maintained at 37° C using a Thermo circulator. Figure 2.1 shows the diagram for the organ bath.

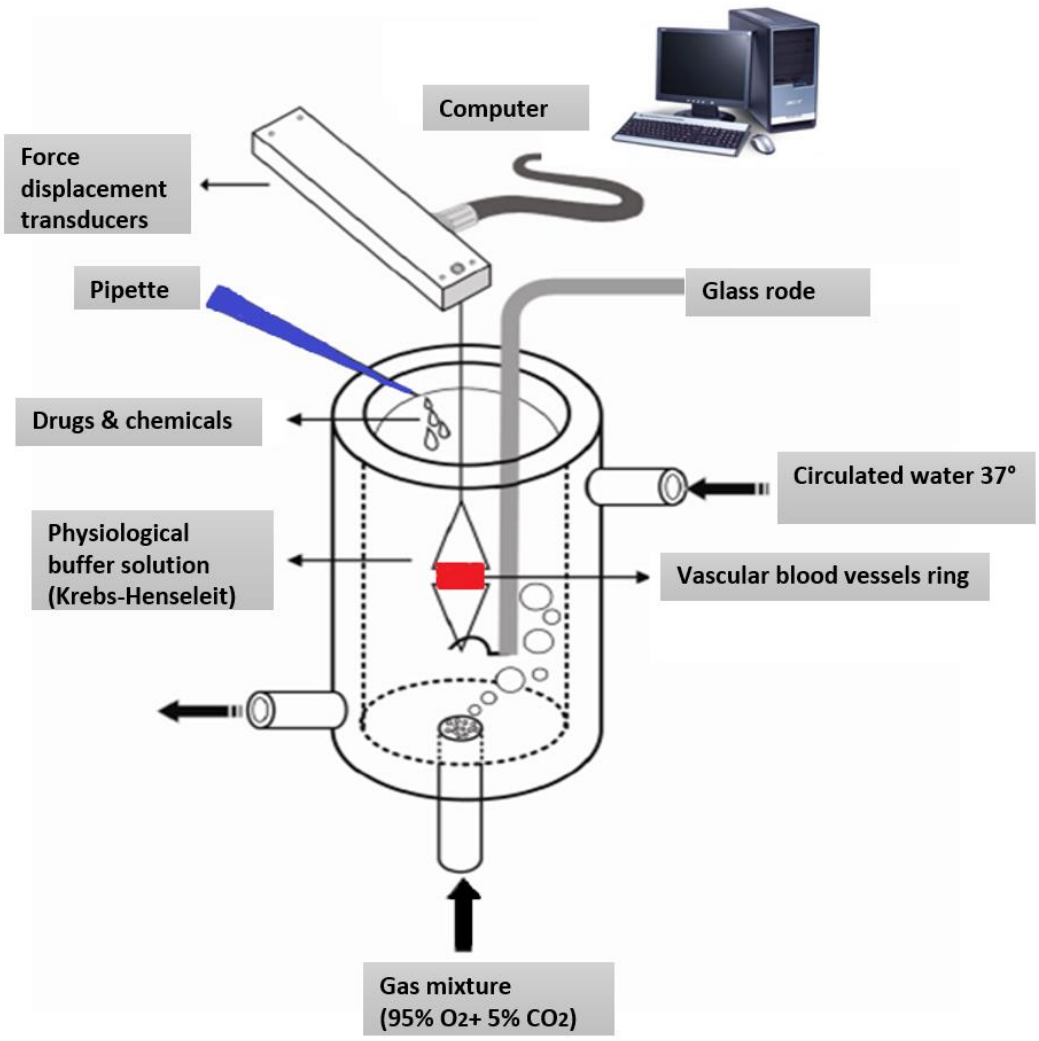


Figure 2-2 Schematic diagram of the organ bath set-up used to study contractility of porcine blood vessels in 20 ml warmed Krebs Henseleit buffer

2.1.3 Experimental protocol

After 40 minutes of acclimatisation, the tissues were placed in an organ bath without applying any tension to allow them to recover from the dissection trauma and to enable them to adapt to the new environment. Thereafter, an initial tension of 10 g wt (for porcine splenic, renal, mesenteric and coronary arteries). While the tension for porcine splenic vein was 7 g wt. The tension applied slowly applied to all segments. Then segments were allowed to relax gradually (usually for approximately 40 minutes) until it reached 2-4 g wt (baseline). To check the viability of tissues, three reproducible responses to 60 mM KCl were applied, for 15 minutes to each segment followed by

another 15 minutes for a washout with Krebs Henseleit buffer. Re-tension to 50% of the initial tension applied to all channels after the first KCl washout to exclude any possible movement of the string that might occur during the contraction of tissues by KCl, and to keep the baseline contraction above 1 g wt. The tissues were allowed to equilibrate for 30 minutes until they reached baseline after the third KCl exposure. This was followed by exposing the segments to (3μM) noradrenaline for 15 minutes to sensitise the adrenoceptors and to check the integrity of the endothelium (by adding 10 μM substance P, only in limited experiments), before being washed out by Krebs Henseleit buffer.

After that, different drugs were added in order to measure their effects on porcine splenic artery isometric tension. If a blocker or lithium ions need to be used in an experiment, they should be allowed to be in contact with the tissue for at least 40 minutes before other drugs are added. The addition of most agonists was either by a cumulative method (1, 3 and 10) per log cycle in order to generate a cumulative response curve or by adding a single high concentration of an agonist which allows us to record the concentration changes with time to generate time course of a drug. See the list of drugs, which were used in this study in Table 2.1

2.1.4 Data analysis

The contractions elicited by drugs were expressed as a gram weight (g wt.). All the tissue responses after a cumulative addition of agonist were recorded either as a percentage of the third 60mM KCl contraction or as a percentage of the maximum response produced using Microsoft Excel software. Logistic equation (Kaleidagraph version 4.5.2 Synergy software) was used to draw the best fit curve from which the following parameters were obtained: R_{max} (maximum response to cumulative addition of an agonist), EC_{50} (concentration causing 50% of maximum responses), pD_2 (–log EC_{50}). All the results were expressed as mean ± standard error (SEM). The number of observations in different animals for each experiment was expressed as (n) and this usually ranged from 4-14 for each experiment. In all experiments, 2-tailed paired Student's *t*-test was used to compare differences between the two groups and considered significant if the P-value was <0.05%.

Chapter 3: Characterisation of Interaction of LiCl and Contractile Agonists in Different Porcine Tissues

3.1 Introduction:

It is reported that vasoconstriction in vascular smooth muscle can result from the activation of GPCR subunit $G_{q/11}$ by different stimuli such as histamine, vasopressin, angiotensin II, muscarinic, 5HT and α_1-adrenoceptor agonists. This binding stimulates PLC enzyme located in the plasma membrane. This stimulation results in the activation of two important second messenger system IP_3 and DAG. The former is responsible for the increase in the concentration of free Ca^{2+} in the cytoplasm by releasing it from storage sites and stimulates the influx of Ca^{2+} by opening L-type Ca^{2+} channels. On the other hand, DAG is responsible for the activation of phosphokinase C (PKC) enzymes that control several cellular functions (Michell and Kirk, 1981; Berridge and Irvine, 1984; Costa, 1990; Janiak *et al.*, 2001; Ureña *et al.*, 2004).

The activation of postjunctional α_1 and α_{2-} adrenoceptors was reported to induce contraction in vascular smooth muscle (Rayment *et al.*, 2010). This contraction varies with different vessels and species. In a small mammal, the stimulation of α_{1-} adrenoceptors is mainly responsible for the vasoconstriction while in human and porcine blood vessels the α_{2-} adrenoceptors role also confirmed in addition to the action of α_1 (Barbieri *et al.,* 1998). Wright and colleagues suggested that in porcine vascular blood vessels from different organ including spleen, the stimulation of postjunctional α_1 and α_{2-} adrenoceptors with noradrenaline resulted in vasoconstriction. This contraction was attributed to α_{1-}receptors while the role of α_2 was considered elusive (Wright *et al*., 1995). Another study also looked at the contraction in isolated porcine splenic artery reported that porcine splenic artery contract after stimulation with phenylephrine. They demonstrated that this action is mainly due to stimulation of α_{1A} subtype with no role for α_2, α_{1B} and α_{1D} adrenoceptor (Barbieri *et al.,* 1998). Although both phenylephrine and porcine splenic artery have been used in the contractile study for decades, only limited research highlighted the fact that time course of contraction induced by this agonist in different porcine vascular tissue is not sustained and declines with time.

It has been reported that the use of 1μM of phenylephrine in porcine renal interlobar artery segments produced a rapid contraction that reached its maximum response after 3 minutes, however, this contraction start to decline and lost more than 50% of its peak response after less than 10 minutes of application (Ihara *et al.*, 2001; Derkach *et al.*, 2000).

In our lab, a similar observation was reported using phenylephrine and L-erythromethoxamine (phenethylamine α_1-adrenoceptor agonists) in sheep internal anal sphincter using isolated tissue bath recording (Rayment *et al.,* 2010). It also reported that incubating the tissue with a low concentration of lithium-ion (0.5-3mM) a drug commonly used in the prophylaxis and treatment of bipolar disorder better-maintained the contraction induced by these agonists in this preparation (Rayment *et al.,* 2007). As previously mentioned in the introduction of this thesis, It was described that lithium could interact and amplify the contraction induced by α-adrenoceptors and other receptors agonist that acts through the modulation of phosphoinositide metabolism (Berridge *et al.*, 1982; Skomedal *et al.*, 1991). To our knowledge, no other study has reported the interaction in multiple vascular tissues using a therapeutic concentration of Li-ions. Therefore, in this chapter, we aimed to achieve these points:

I. Compare the effect of lithium on the response of the porcine isolated splenic artery to noradrenaline and phenylephrine.
II. Compare the effect of lithium on the response of the porcine isolated splenic artery to phenethylamine-based and –imidazoline-based α_1-adrenoceptor agonists.
III. Examine the effect of lithium on the response of the porcine isolated splenic artery to non-adrenoceptor vasoactive receptors.
IV. Characterisation of lithium salts, concentrations in the interaction with α_1-adrenoceptors agonist in the porcine splenic artery.
V. Examine the effect of lithium on responses in different vascular and non-vascular porcine tissue.

3.2 Materials and methods

3.2.1 Methods

Porcine spleen, kidney, mesenteric, lung, urethra and heart from both genders were obtained from a local abattoir in iced boxes filled with Krebs Henseleit solution. Upon arrival to the lab coarse dissection was performed by dissecting out 5-8 cm length of the porcine artery, with the surrounding fat and connective tissue and then stored overnight in Krebs Henseleit solution at 4°C. On the next day, fine dissection was performed by removing the connective tissue and set-up the artery segments with a 4-5mm length in 20 ml organ bath. The segment was suspended between two 0.4 mm steel wires; the upper attached to Force transducer connected to AD Instruments Quad Bridge pre-amplifier unit while the lower was attached to a glass rode. The organ bath was filled with warm Krebs Henseleit solution at 37°C and gassed with 95%O_2 and 5%CO_2.

3.2.2 Protocols

Most of the observations in this chapter have been established in the conventional method, by examining this interaction following the cumulative increments in agonist concentration to reveal the enhancement in the contractions. We have confidence that this conventional method that used to generate the concentration-response curve might have some limitation. It was designed on the assumption that the tissue has reached equilibrium state after 3-5 minutes following the addition of the agonist without taking into consideration the stability and the time course of the response. Therefore, we have also employed single exposure to the high concentration of the agonists to demonstrate that LiCl can affect the duration of action to these agonists. While the non- cumulative method was used in limited experiments to compare the time course for the contraction induced by phenylephrine in the presence and absences of LiCl to better understanding the effect of lithium whether it's on peak or the sustained responses initiated by phenylephrine.

After 40-50 minutes equilibration in Krebs Henseleit solution, segments were placed under 10 g wt for all vascular tissue and urethral smooth muscle (except porcine splenic vein in which the tension was 6-7 g wt). tension and allowed to relax over 40 minutes. Unless it is stated before, all responses were expressed as a percentage to contraction induced by 60mM KCl. After the viability of tissue being tested with three additions of 60mM KCl (each addition last for 15 minutes before reaching a stable contraction where the system is washed with fresh buffer and allowed to relaxed for about 15 minutes), cumulative additions of an agonist to induce a contraction were added to isolate porcine splenic artery segments. In this chapter, and following the incubation of the tissue with different concentrations of chemicals for at least 30-40 minutes, four protocols were used to record the changes in tissue responses after the addition of the agonist.

I. The first protocol was conducted by the cumulative addition of the agonist to the tissue started from 0.01μM until the maximum concentration of the agonist was added at 30μM. The increase in the concentration after each addition was three folds of the previously added concentration. The next concentration was added after equilibrium was established between the organ bath concentration and the tissue.
II. In another set of experiments, a non- cumulative addition of phenylephrine was performed in which each response was allowed to attain equilibrium with the agonist and then replacing the bathing solution with a fresh Krebs Henseleit solution and allowing the contraction to return back to baseline before the addition of next agonist concentration. A minimum of 10 minutes was allowed between contractions.
III. The third protocol used in this chapter was keeping the tissue after it reached its maximum response to the cumulative addition of the agonist and recorded the response at different time points for the following 60-120 minutes. This protocol helped us to better estimates the time course of the response before adopting the single high concentration addition.
IV. The fourth protocol involved was exposing the tissue to a single high concentration of the agonist and record the changes in tissue response at different time points to obtain the time course curve for the agonist responses.

3.2.3 Chemicals and solution

The following concentrations of chemicals were used in this chapter: lithium chloride, rubidium chloride, 5HT, histamine dihydrochloride, α, β methylene ATP, lithium salts. In addition, either single concentration or cumulative concentration of the following drugs was used, noradrenaline bitartrate, phenylephrine hydrochloride, angiotensin II, vasopressin, Isoprenaline hydrochloride, dobutamine hydrochloride, cirazoline hydrochloride, L-erythromethoxamine hydrochloride, metaraminol bitartrate, carbachol and thromboxane A_2 receptor agonist U46619. The solubility and the source of these chemicals were previously reported in Table 2.1 in chapter two.

3.3 Results

3.3.1 Interaction between LiCl and α_1-adrenoceptors in a porcine splenic artery

3.3.1.1 The effect of adding LiCl on the concentration-response curve and time course of contraction induced by noradrenaline and phenylephrine in the porcine splenic artery

In porcine isolated splenic artery, exposing the tissue to 60mM KCl caused a sustained contraction equivalent to 15.5±2.1g wt. (n=36). This contraction indicates a stable response following the third addition of KCl in the tissue (all the next obtained values will be represented as a percentage to this value). Each tissue used in this study challenged three times with 60mM KCl and we only start the experiment when the difference in the magnitude of the response between second and third KCl is less than 10%.

Figure 3.1 shows that the cumulative addition of noradrenaline produces rapid, concentration-dependent contractions that took less than 5 minutes to attain peak equilibrium response. In contrast, comparable responses to phenylephrine were sustained but developed much slower in onset and at lower magnitudes. At the highest concentration examined for both agonists (30μM) the contractions were not sustained but declined slowly over time.

The addition of 1mM LiCl to the bathing solution did not affect either resting tone or responses to noradrenaline and phenylephrine. However, contractions to high concentrations of phenylephrine were enhanced by the presence of 1mM LiCl and the maximal responses were generally stable.

Figure 3.2 and Table 1 show that 1mM LiCl did not affect either the maximal response or the potency of noradrenaline in the porcine isolated splenic artery. In contrast, 1mM LiCl significantly enhanced the maximal response of phenylephrine and this was associated with a reduction in potency.

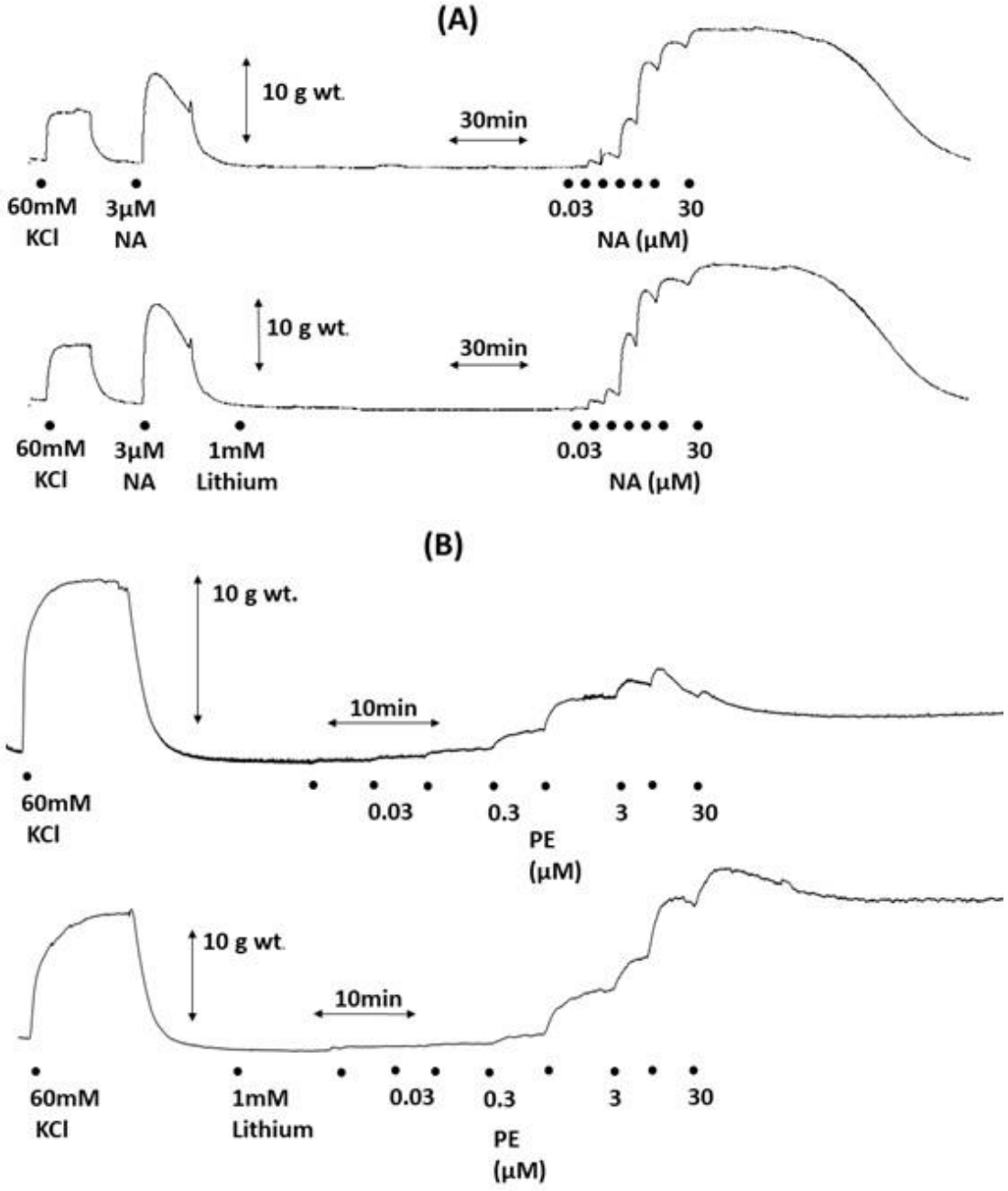


Figure 3-1 Representative trace recordings in paired segments of the porcine isolated splenic artery to the cumulative addition of (A) noradrenaline and (B) phenylephrine in the absence and presence of 1mM LiCl.

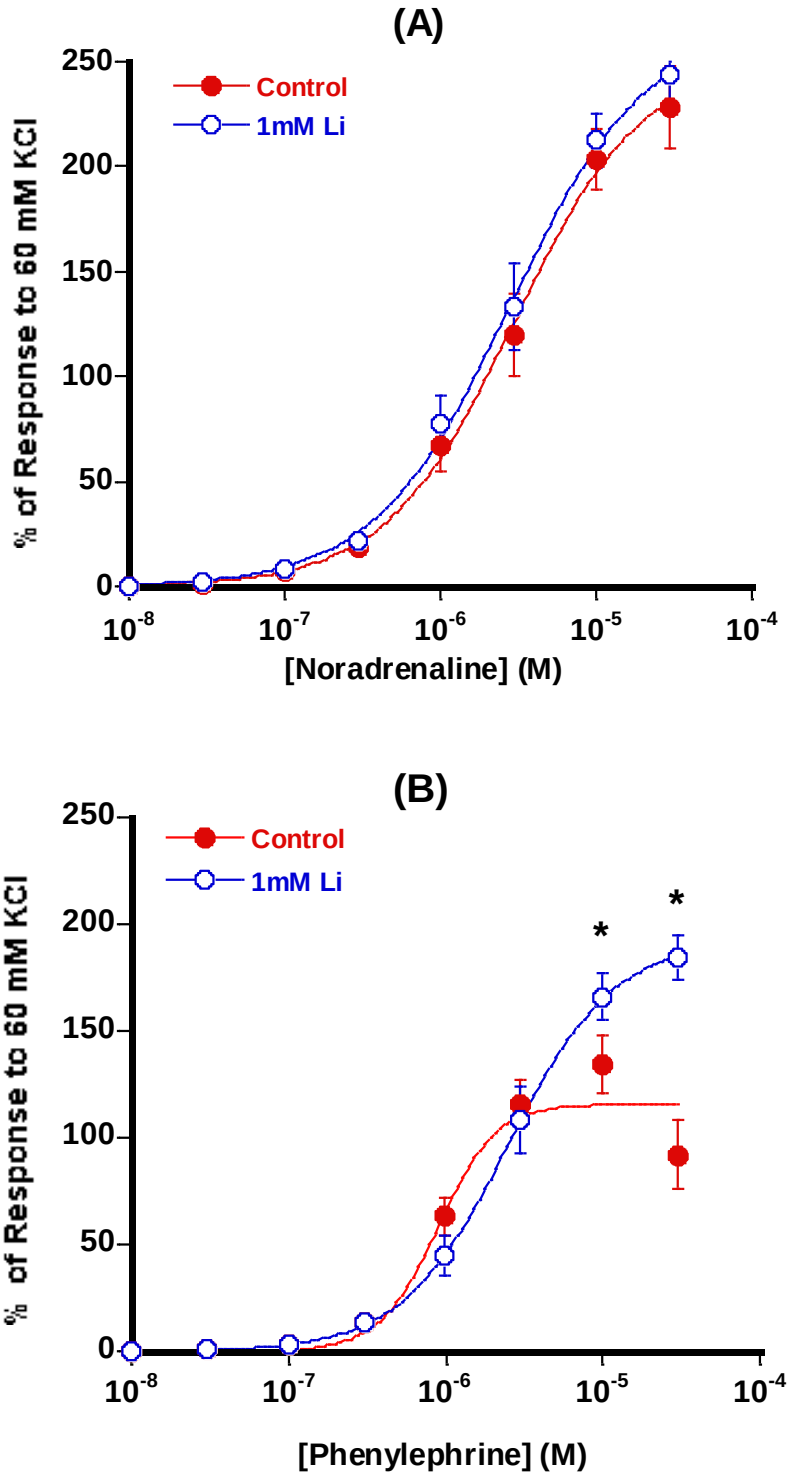


Figure 3-2 The effect of (A) noradrenaline (n=8) and (B) phenylephrine (n=6) on the porcine isolated splenic artery in the absence and presence of 1mM LiCl. Responses have been expressed as a percentage of the response to 60mM KCl and shown as the mean ± SEM. * - Denotes a significant difference between mean responses ($p < 0.05$; paired Student t-test).

Table 3-1 Summarises the responses to contractions induced by different α_1-adrenoceptor agonists in the absence and presence of LiCl in an isolated porcine splenic artery (PSA): where * represents significant changes using paired Student's *t*-test. Responses are expressed as a percentage of the contraction to 60mM KCl and are mean± SEM and n=represents the number of observations.

Agonist	Tissue	R_{max}	pD_2	n
Noradrenaline	PSA	241.4± 24.2	5.6± 0.09	8
Noradrenaline+ Li		256.2± 21.2	5.6± 0.11	
Phenylephrine	PSA	117.0±11.6	6.06±0.04	6
Phenylephrine+ Li		190.1±10.0*	5.6±0.07*	
LEM	PSA	93.8±16.0	6.29±0.07	8
LEM+ Li		268.8±36.0*	5.97±0.08*	
Metaraminol	PSA	51.3±8.5	5.9±0.04	7
Metaraminol+ Li		176.2±5.7*	5.4±0.03*	
Cirazoline	PSA	152.6± 8.1	7.1±0.05	10
Cirazoline+ Li		175.2± 5.2	7.15± 0.06	

Figures 3.3, 3.4 and 3.5 show an examination of the effect of 1mM LiCl on the non-cumulative exposure of the porcine isolated splenic artery to submaximal and maximal concentrations to phenylephrine. Figure 3.3 demonstrates that while low concentration < 0.3μM was associated with a slow-developing contraction that reached a peak after 7 min that was sustained. Higher concentrations > 3μM were characterised by a rapid contraction (peak response around 3 min) that declined by 30-50% over 30 min. In the presence of 1mM LiCl contractions to low concentrations < 0.3μM of phenylephrine were not affected, while contractions to the higher, non-cumulative concentrations > 3μM of phenylephrine were enhanced and better sustained. The consequence of what is measured for the concentration-response curve to phenylephrine, and the subsequent impact on the perceived effect of LiCl, is illustrated in Figure 3.4 and 3.5 The perceived effect of lithium is greater against the sustained response to phenylephrine (non-cumulative addition) compared to assessment based on the peak response to the agonist.

Figures 3.6 and 3.7 show that following the addition of either noradrenaline 30μM or phenylephrine 30μM the porcine isolated splenic artery contracted quickly, reaching a peak response within 10 min. However, while responses to noradrenaline 30μM were generally well maintained over 45 minutes, before beginning to decline, responses to

30μM phenylephrine decline rapidly within 15 minutes of attaining the peak response. For example, the peak response to 30μM phenylephrine was 133.0±9.1% of 60mM KCl (n=36) and declined to 33.5±3.4% of 60mM KCl (n=36) after 60 min. In contrast, in the presence of 1mM LiCl, the peak to 30μM phenylephrine was significantly increased to 190.1±10.0% of 60mM KCl (n=36) after 60 minutes declined to only 139.0±7.1% of 60mM KCl (n=36) after the same time. Thus after 5 minutes of exposure to 30μM phenylephrine responses in the presence and absence of LiCl were statistically different. In contrast, the presence of LiCl did not change the tissue response to contraction induced by noradrenaline 30μM in porcine splenic artery segments.

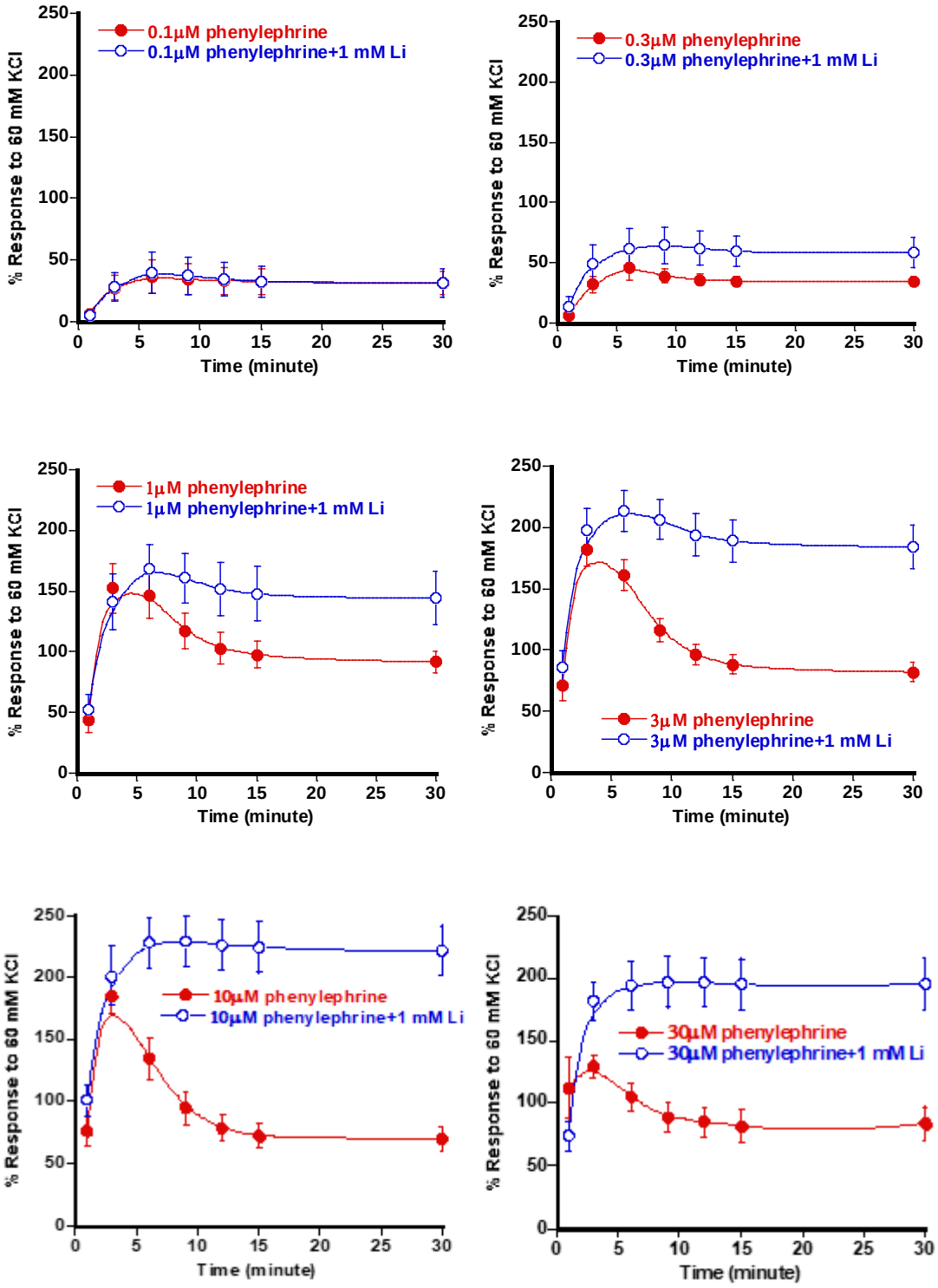


Figure 3-3 The effect of 1mM LiCl on the time course of contractions of the porcine isolated splenic artery following the non-cumulative addition of (100nM-30µM) phenylephrine (n=12). Responses have been expressed as a percentage of the contraction to 60mM KCl and represented as mean ± SEM.

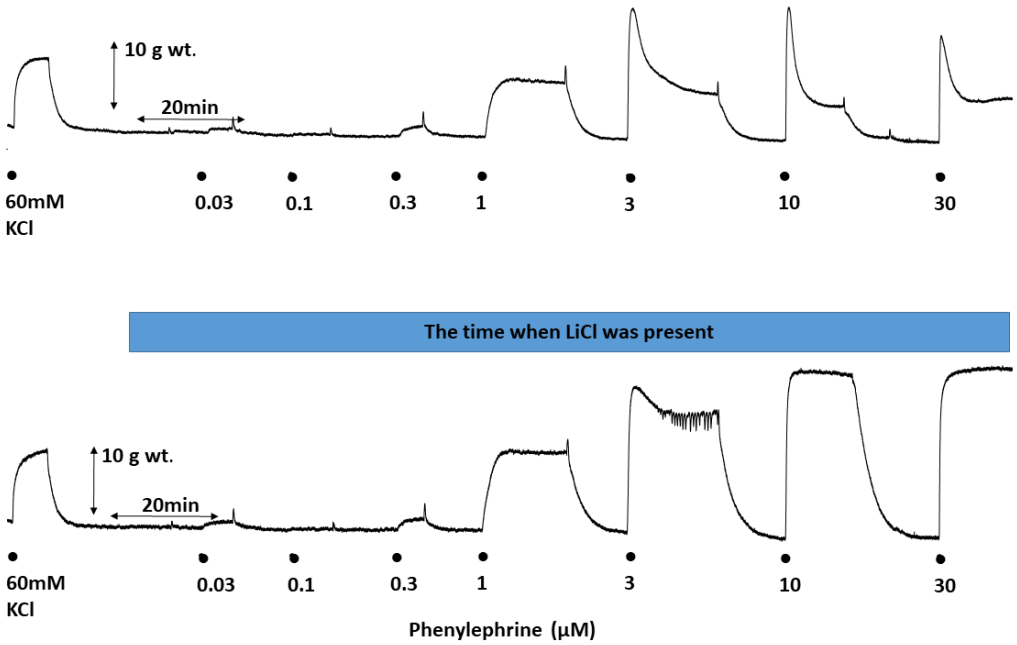
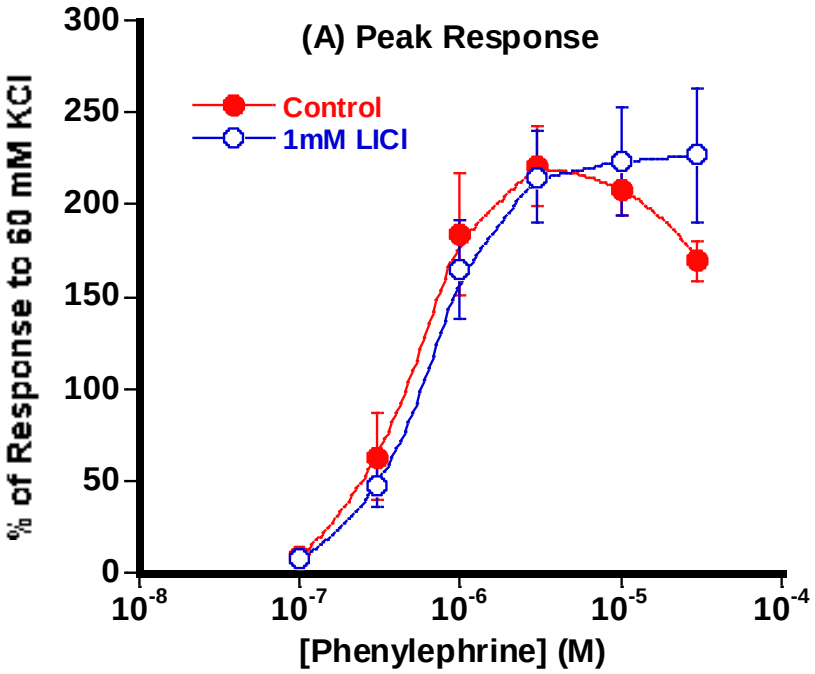


Figure 3-4 Representative trace recordings showing contractile responses of the porcine isolated splenic artery to the non-cumulative additions of phenylephrine (30nM-30µM) in the presence and absence of 1mM LiCl. Noted that a fresh buffer solution was added after the sustained response was reached in both conditions.



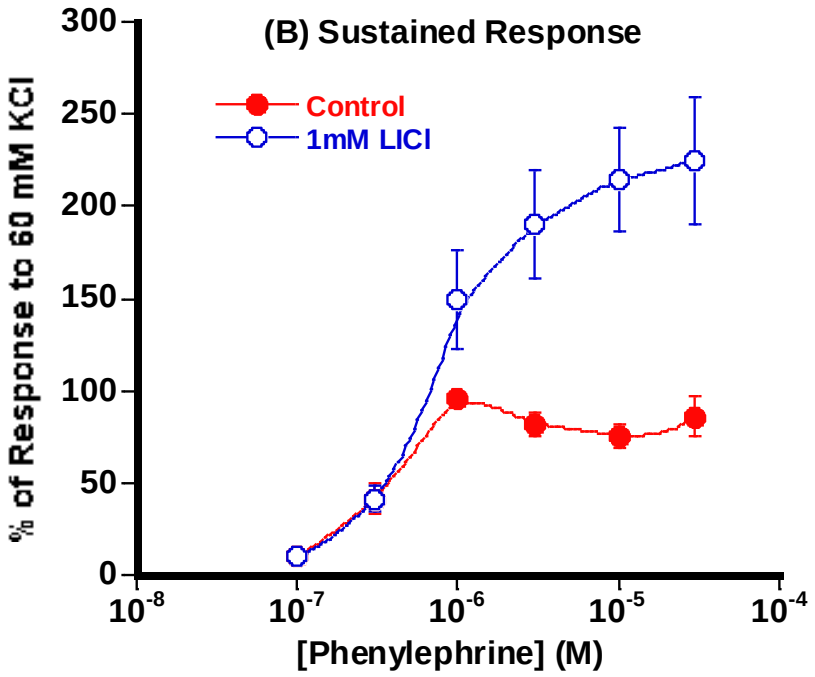


Figure 3-5 Compares the effect of 1mM LiCl on the peak response (A) and sustained response (B) to phenylephrine following non-cumulative addition of the agonist to isolated splenic artery segments (n=6). Responses have been expressed as a percentage of the contraction to 60mM KCl and represented as mean ± SEM.

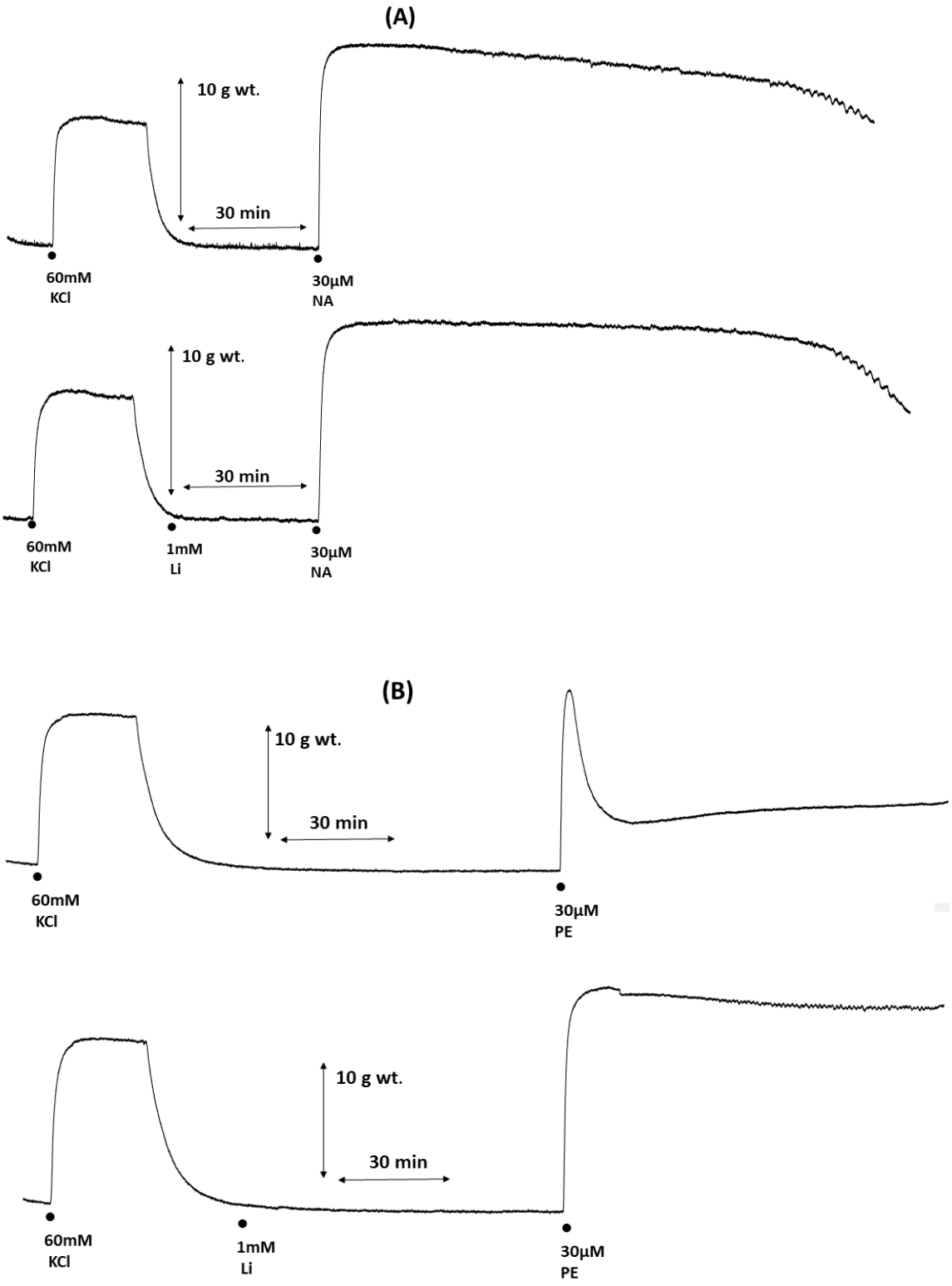


Figure 3-6 Representative trace recordings showing contractile responses of the porcine isolated splenic artery to the addition of 60mM KCl and single addition of either (A) noradrenaline 30µM or (B) phenylephrine 30µM in the presence and absence of 1mM LiCl.

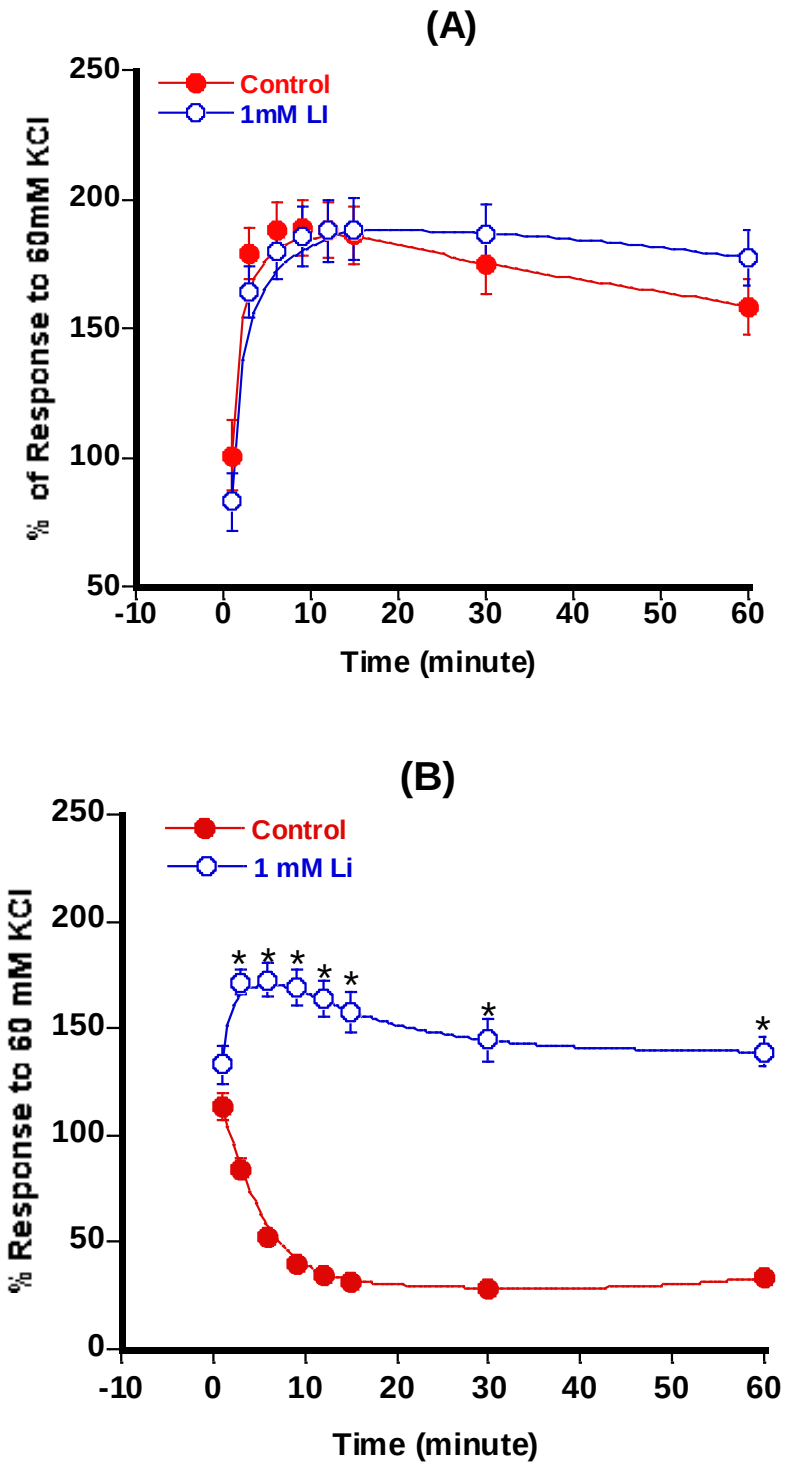


Figure 3-7 The effect of 1mM LiCl on the time course of contractions of the porcine isolated splenic artery following the single addition of either (A) 30μM noradrenaline (n=11) and (B) 30μM phenylephrine (n=36). Responses have been expressed as a percentage of the contraction to 60mM KCl and represented as mean ± SEM. * - Denotes a statistically significant difference ($p < 0.05$) between responses using a paired Student's t-test.

3.3.1.2 The effect of adding LiCl on the concentration-response curve and time course of contraction induced by L-erythromethoxamine (LEM) in the porcine splenic artery

Figure 3.8 (A) shows that the cumulative addition of L-erythromethoxamine produces rapid, concentration-dependent contractions. The presence of 1mM LiCl before the cumulative addition produced a significant enhancement in the induced vasoconstrictor effect as a percentage of contraction to 60mM KCl. Contractions to high concentrations of L-erythromethoxamine were significantly enhanced by the presence of LiCl and the maximal responses were generally stable. This was associated with a significant reduction in the potency of the agonist although this reduction in the estimated potency does not mean a reduction in the effectiveness of the agonist. See Table 3.1.

On another set of experiment and following the single addition of L-erythromethoxamine 30μM, the magnitude of the contraction was measured at different time points and expressed as a percentage to the response of 60mM KCl See Figure 3.8 (B). It was found that the presence of 1mM LiCl produced a significant enhancement in the time course curve of L-erythromethoxamine induced vasoconstriction. The peak of response after 3 minutes changed from 135.2±14.0% to 150.3±17.8% (n=4). However, the interesting observation was the ability of LiCl to better-maintained the response following 60 minutes of reaching its maximum response at 178.4±26.8% (n=4) in compare to the control in which the response declined to 23.5±5.6% (n=4) after the same period using paired Student's *t*-test.

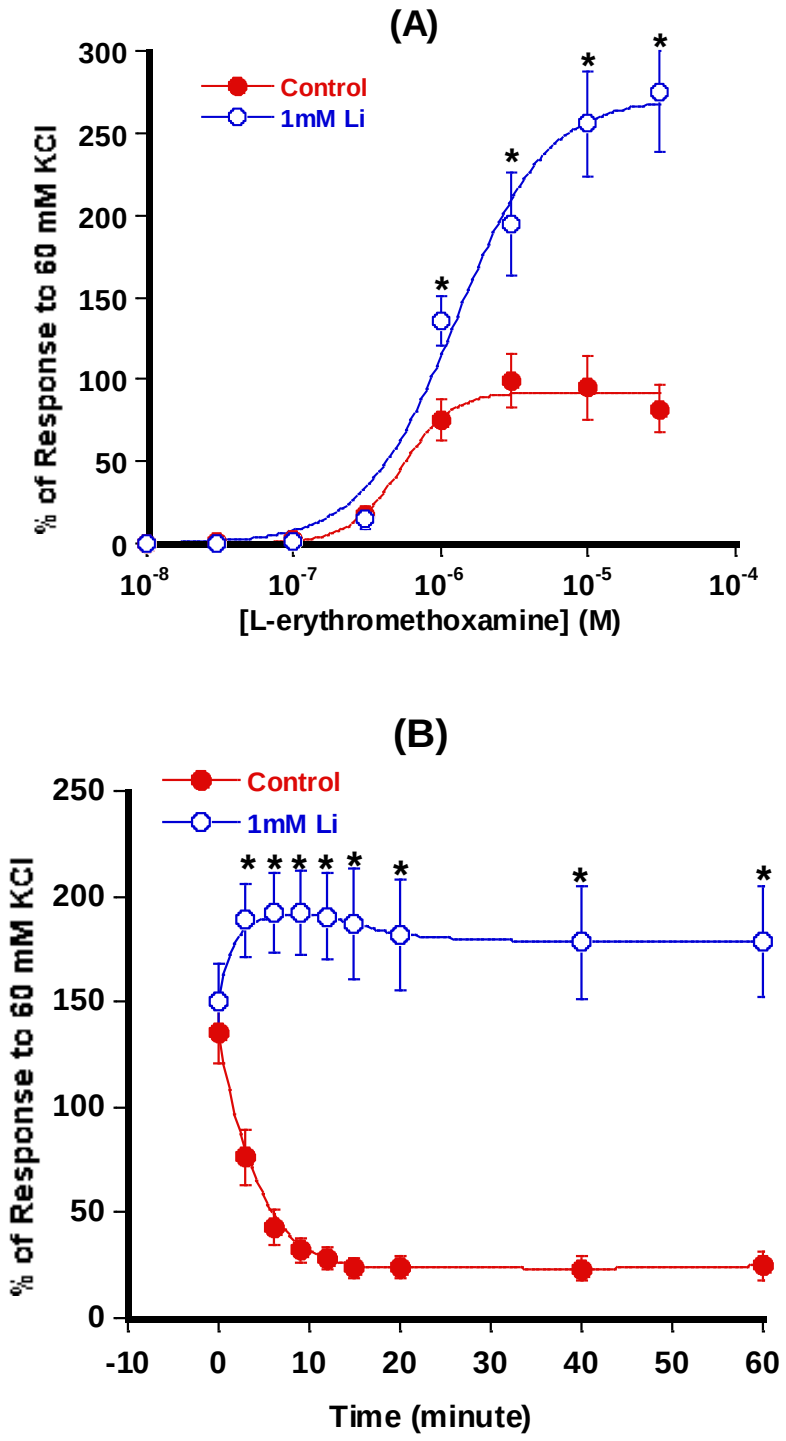


Figure 3-8 (A) The contractile response curve following cumulative addition of L-erythromethoxamine, (n=8) and (B) The time course curve following a single addition of 30μM L-erythromethoxamine (n=4). In the presence and absence of 1mM LiCl expressed as a percentage to contraction to 60mM KCl. Values are represented as mean ± SEM. * - Denotes a statistically significant difference ($p < 0.05$) between responses using a paired Student's *t*-test.

3.3.1.3 The effect of adding LiCl on the concentration-response curve and time course of contraction induced by metaraminol in the porcine splenic artery

In porcine splenic artery, the presence of 1 mM LiCl before the cumulative response curve of metaraminol produced a significant enhancement with the high concentration of metaraminol induced vasoconstriction as a percentage of contraction to 60mM KCl. The maximum response was significantly enhanced with an associated with a reduction in potency. See Figure 3.9 (A) and Table 3.1

On another set of experiment and following the single addition of metaraminol (30μM), the magnitude of the contraction was measured at different time points and expressed as a percentage to contraction induced by 60mM KCl. It was found that the addition of LiCl 1mM produced significant changes in the time course curve of metaraminol induced vasoconstriction. LiCl 1mM enhanced and better-maintained the response of metaraminol at 145.6±9.6% following 60 minutes of reaching its maximum response which was 158.7±11.3% (n=7) in compare with the control in which the percentage of response decline from 57.2±9.8% (n=7) to become 29.5±93.5% (n=7) after the same period using paired Student's *t*-test. See Figure 3.9 (B)

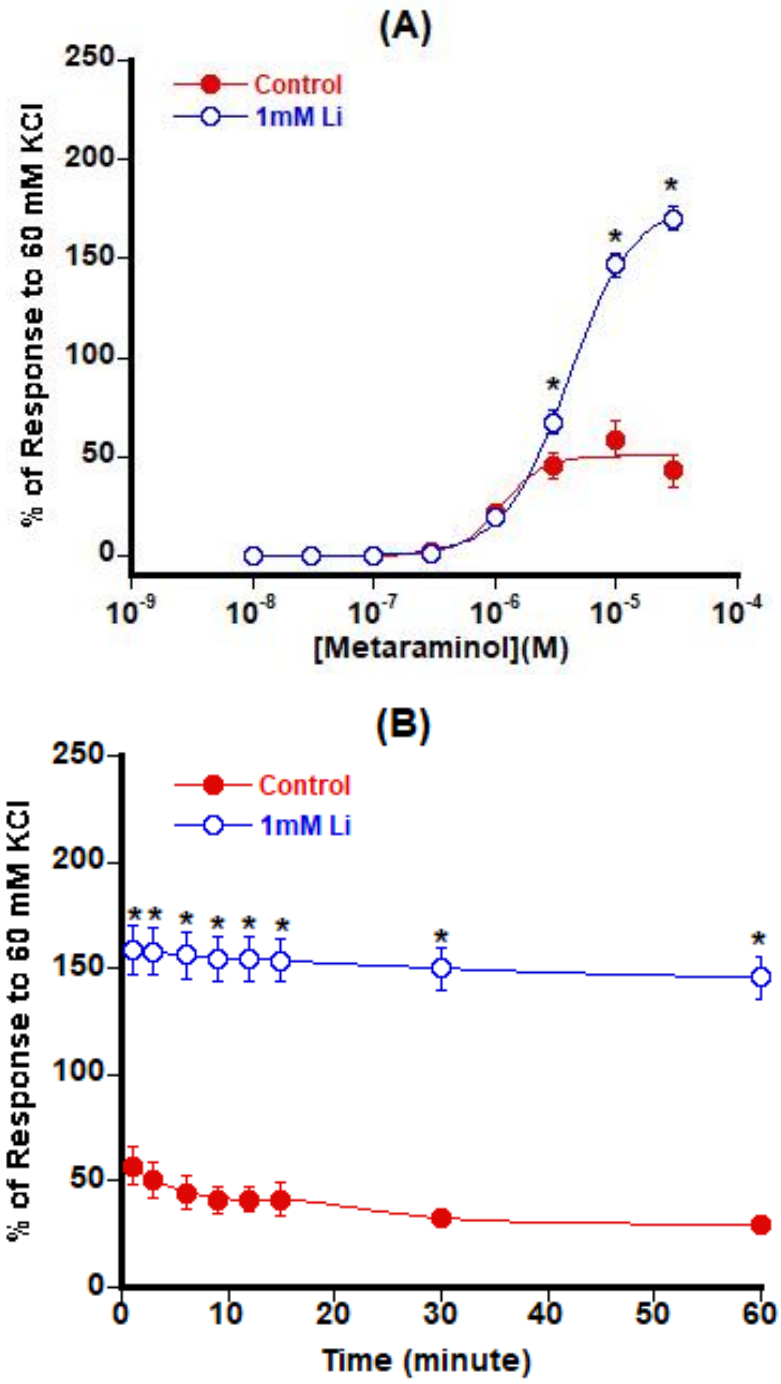


Figure 3-9 (A) The contractile response curve following cumulative addition of metaraminol, (n=7) and (B) The time course curve following a single addition of 30μM metaraminol (n=7). In the presence and absence of 1mM LiCl expressed as a percentage to contraction to 60mM KCl. Values are represented as mean ± SEM. * - Denotes a statistically significant difference ($p < 0.05$) between responses using a paired Student's *t*-test.

3.3.1.4 The effect of adding LiCl on the concentration-response curve and time course of contraction induced by cirazoline in the porcine splenic artery

Figure 3.10 (A) shows that in the porcine splenic artery the cumulative addition of cirazoline produced a rapid contraction, which reached its peak in less than 10 minutes, this contraction characterised by stability in compare with other α_{1-} adrenoceptor agonists previously tested in this chapter. Incubation the tissue with LiCl 1mM only produced a slight enhancement in the maximum response of cirazoline with a negligible effect on the potency Table 3.1

In a different set of experiments and following the single addition of cirazoline 30μM and recording the response at different time points showed that the contraction induced by cirazoline is stable and the presence of LiCl did not change tissue response to contraction induced by this agonist. Concentration after the max response was 188.02±18.6% in compare with control in which the response was 187.54±23.0%. While after 60 minutes of exposure, the concentration of LiCl incubated segments was 143.3±21.5% in compare to 135.8±20.4% for the control. Using a paired Student's *t*-test (n=8). See Figure 3.10 (B).

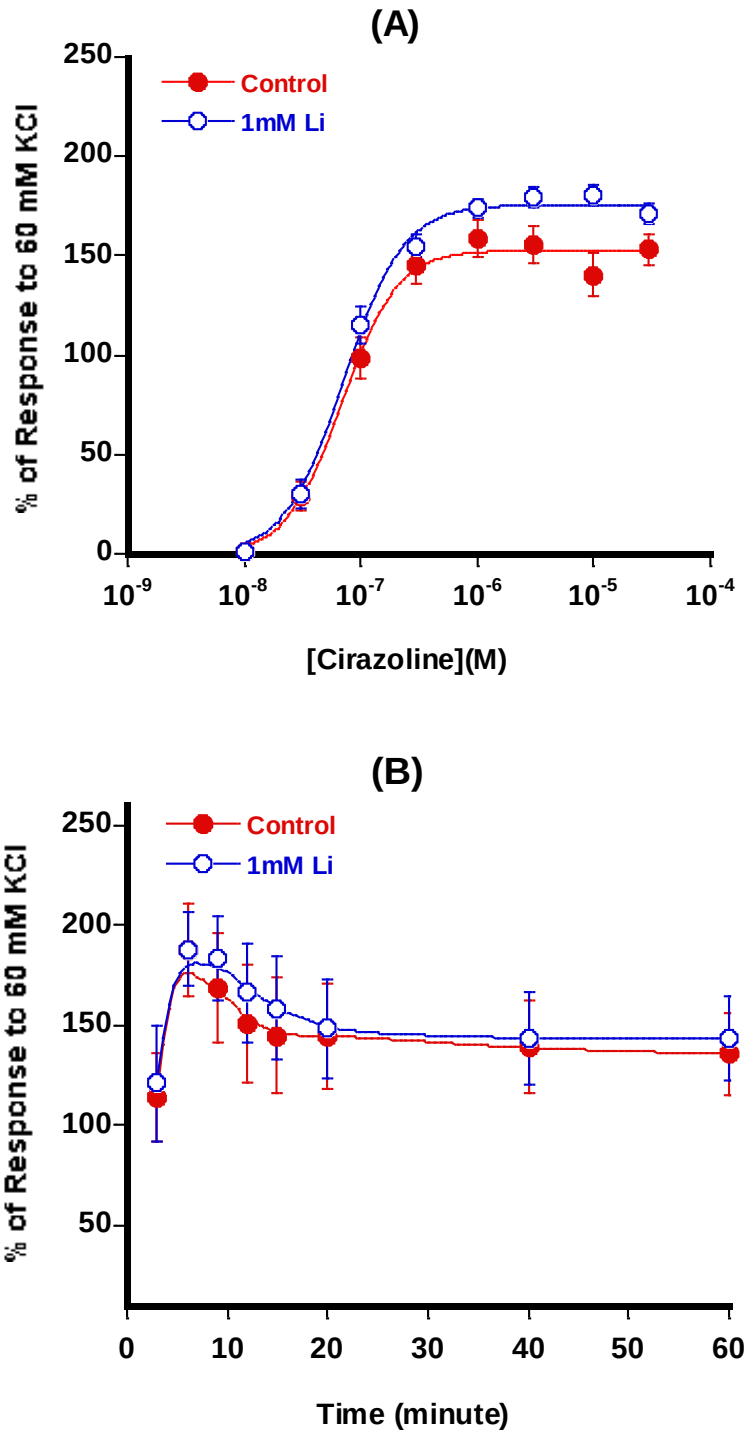


Figure 3-10 (A) The contractile response curve following cumulative addition of cirazoline, (n=10) and (B) The time course curve following a single addition of cirazoline 30μM (n=8). In the presence and absence of 1mM LiCl expressed as a percentage to contraction to 60mM KCl. Values are represented as mean ± SEM. * - Denotes a statistically significant difference ($p < 0.05$) between responses using a paired Student's t-test.3.3.2 Interaction between LiCl and phenylephrine in other porcine vascular tissues.

3.3.2 Interaction between LiCl and phenylephrine in other porcine isolated vascular and non-vascular tissues

3.3.2.1 The effect of adding LiCl on the concentration-response curve and time course of contraction induced by phenylephrine in a porcine mesenteric artery

In isolated porcine mesenteric artery segments exposing the tissue to 60mM KCl produced a sustained contraction equivalent to 10.8±2.1 (n=7). The preincubation of segments with 1mM LiCl showed a greater response to a high concentration of phenylephrine. Significant changes in the R_{max} and the potency was seen with a P-value< 0.05 using paired Student's *t*-test after the cumulative addition of the agonist. See Figure 3.11, 3.12 (A) and Table 3.2

On different set of experiments, following the single addition of phenylephrine 30μM to isolated porcine mesenteric arteries segments in the presence and absences of 1mM LiCl, The presence of LiCl produced significant changes with P-value<0.05 in the time course curve of phenylephrine-induced vasoconstriction in response to 60mM KCl using paired Student's t-test (n=7). After 3 minutes LiCl increased the response from 45.4± 11.2% to become 150.2± 13.0% and maintained it after 60 minutes of exposure at 129.1± 15.2% in compare with the control in which the % of response dropped to 12.6± 3.6% (n=7). See Figure 3.12 (B)

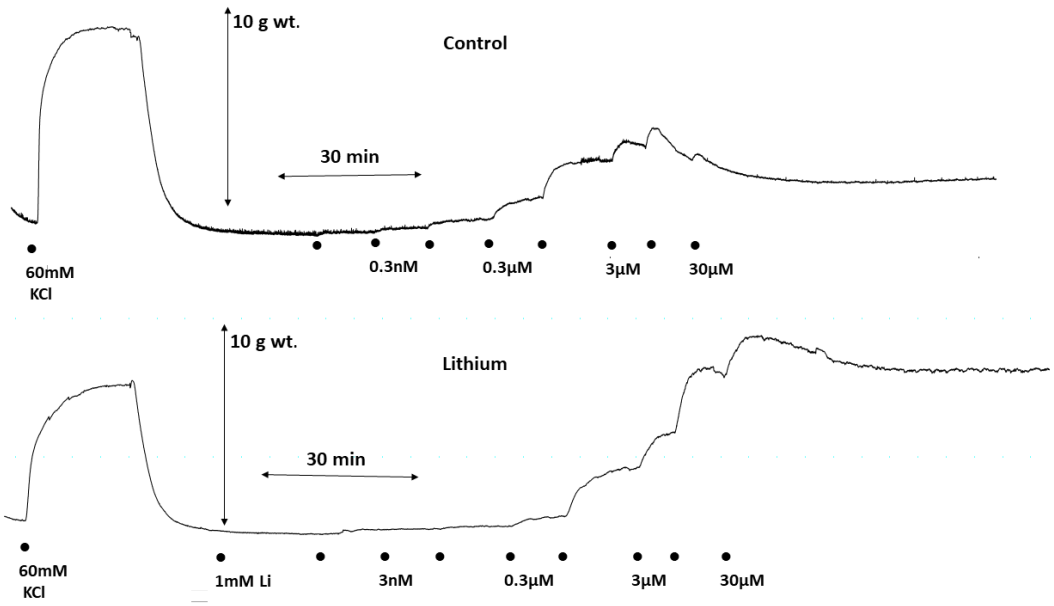


Figure 3-11 Representative trace recordings showing contractile responses of the porcine isolated mesenteric artery to the cumulative addition of (30nM-30µM) phenylephrine in the absence (upper) and presence (lower) of 1mM LiCl.

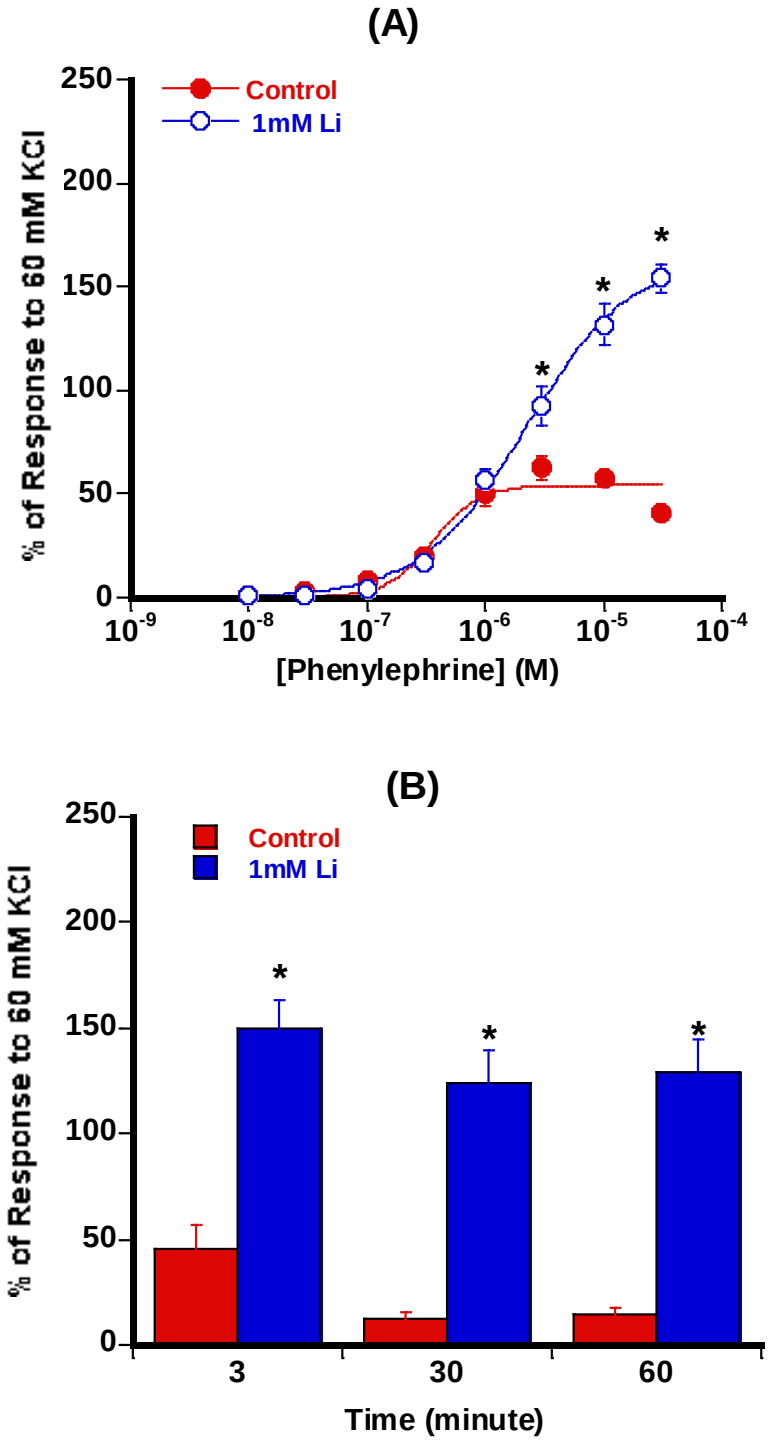


Figure 3-12 (A) The concentration-response curves for vasoconstrictor effect of cumulative addition of phenylephrine in the presence and absence of 1mM LiCl and (B) the bar chart for contractile response in the porcine mesenteric artery at a different time point after it reaches the maximum response. Data are expressed as a percentage of contraction to 60mM KCl and are mean ± SEM (n=7). * - Denotes a statistically significant difference ($p < 0.05$) between responses using a paired Student's *t*-test.

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3.3.2.2 The effect of adding LiCl on the concentration-response curve and time course of contraction induced by phenylephrine in a porcine renal artery

In porcine renal artery, exposing the tissue to 60mM KCl produced a sustained contraction equivalent to 7.5±1.2 (n=8). The preincubation with 1mM LiCl produced a greater response to a high concentration of phenylephrine. Significant changes in the R_{max} and the potency were seen with a P-value< 0.01 using paired Student's *t*-test. R_{max} changed from 130.7±9.2% to become 167.2±8.6%, (n=8) and the pD2 changed from 6.16± 0.05 to become 5.80± 0.14, (n=8). See Figure 3.13 (A) and Table 3.1.

Recording the responses at different time points following reaching the R_{max} showed that the presence of LiCl significantly enhanced and preserved the contraction after 60-minute; R_{max} for the control was 134.0±13.0% and in the LiCl treated segments the R_{max} was 164.3±9.0% (n=8). After 60 minutes, responses were 43.1±3.7% and 144.2±8.1% (n=8) for the control and LiCl incubated segments respectively. See Figure 3.13 (B)

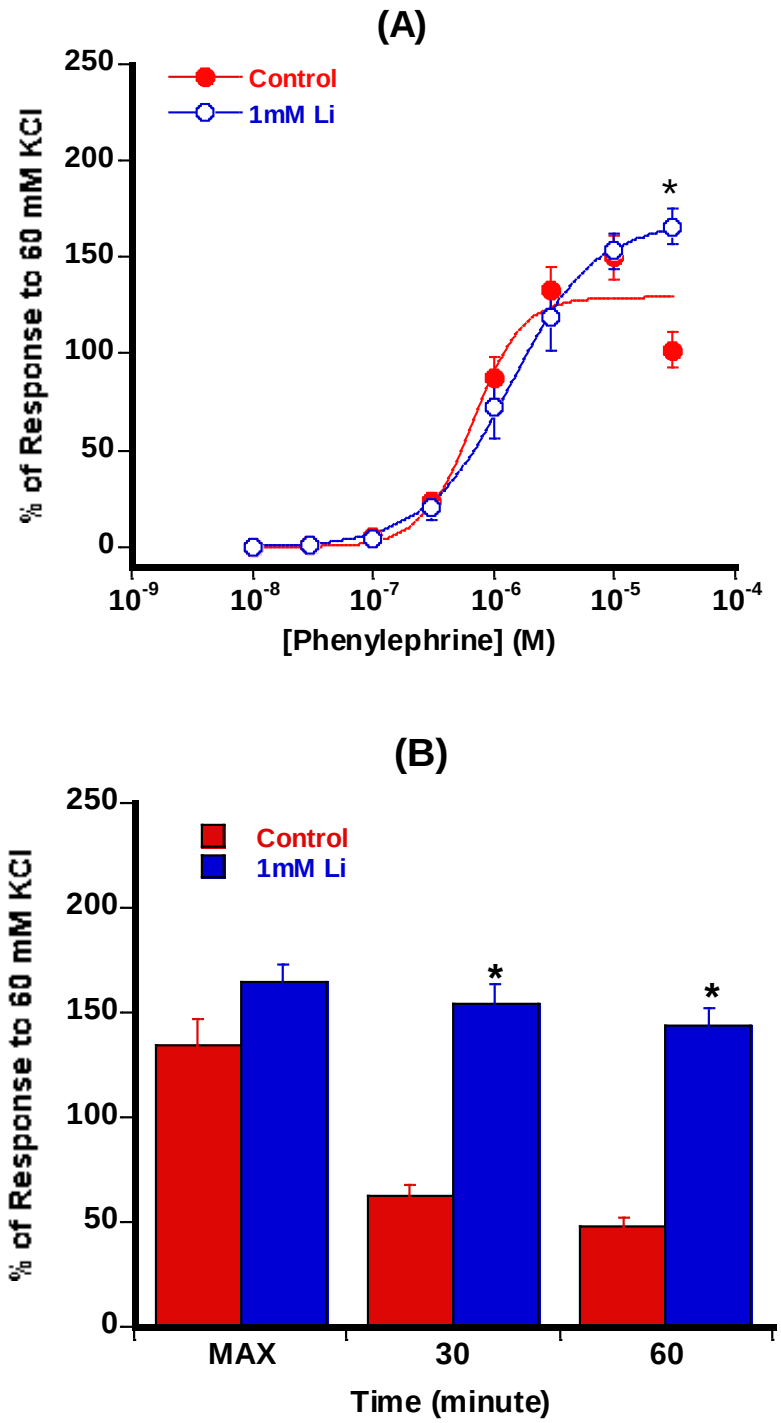

Figure 3-13 (A) The concentration-response curves for vasoconstrictor effect of cumulative addition of phenylephrine in the presence and absence of 1mM LiCl and (B) the bar chart for contractile response in the porcine renal artery at a different time point after it reaches the maximum response. Data are expressed as a percentage of contraction to 60mM KCl and are mean ± SEM (n=8). * - Denotes a statistically significant difference ($p < 0.05$) between responses using a paired Student's *t*-test.

3.3.2.3 The effect of adding LiCl on the concentration-response curve and time course of contraction induced by phenylephrine in a porcine pulmonary artery

It was difficult to get a recordable response following the cumulative addition of phenylephrine to segments of a porcine pulmonary artery due to limited availability of α_1- adrenoceptors at this site and because the contraction induced by α_1-agonist mainly results from stimulation of α_2-adrenoceptors (Jantschak and Pertz 2012). In porcine pulmonary artery adding phenylephrine 30µM failed to initiate a contraction and hence the effect of adding 1mM LiCl could not be recognized (n=6). See Figure 3.14

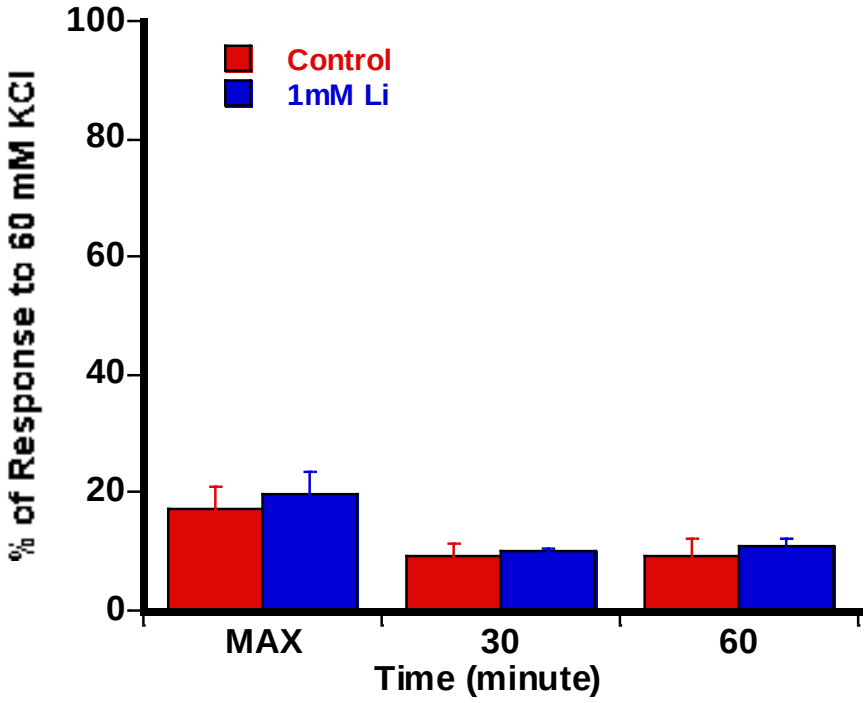

Figure 3-14 Time course for contractile response in the porcine pulmonary artery at a different time point. Following single addition of (30mM) phenylephrine in presence and absence of 1 mM LiCl. Data are expressed as a percentage of 60mM KCl and are mean ± SEM (n=6).

3.3.2.4 The effect of adding LiCl on the concentration-response curve and time course of contraction induced by phenylephrine in a porcine splenic vein

In the porcine splenic vein, exposing the tissue to 60mM KCl produced a sustained contraction equivalent to 5.6±1.1gm (n=8). The preincubation of 1mM LiCl enhanced the response to a high concentration of phenylephrine. A significant enhancement in the R_{max} was seen with a P-value< 0.05 using paired Student's *t*-test. R_{max} changed from 99.4± 10.40% to become 148.9±8.4%, (n=8). The pD2 slightly changed from 5.67± 0.13 to become 5.37±0.17, (n=8). See Figure 3.15 (A), Table 3.1.

Recording the responses at different time points after reaching the R_{max} showed that the presence of LiCl significantly enhanced and preserved the contraction after 60minute R_{max} for the control was 76.9±7.8% and for the LiCl treated segment the R_{max} was 116.6±7.7% (n=8). After 60 minutes, responses were 56.1±10 % and 103.1±8.1% (n=8) for the control and lithium incubated segments respectively. See Figure 3.15 (B).

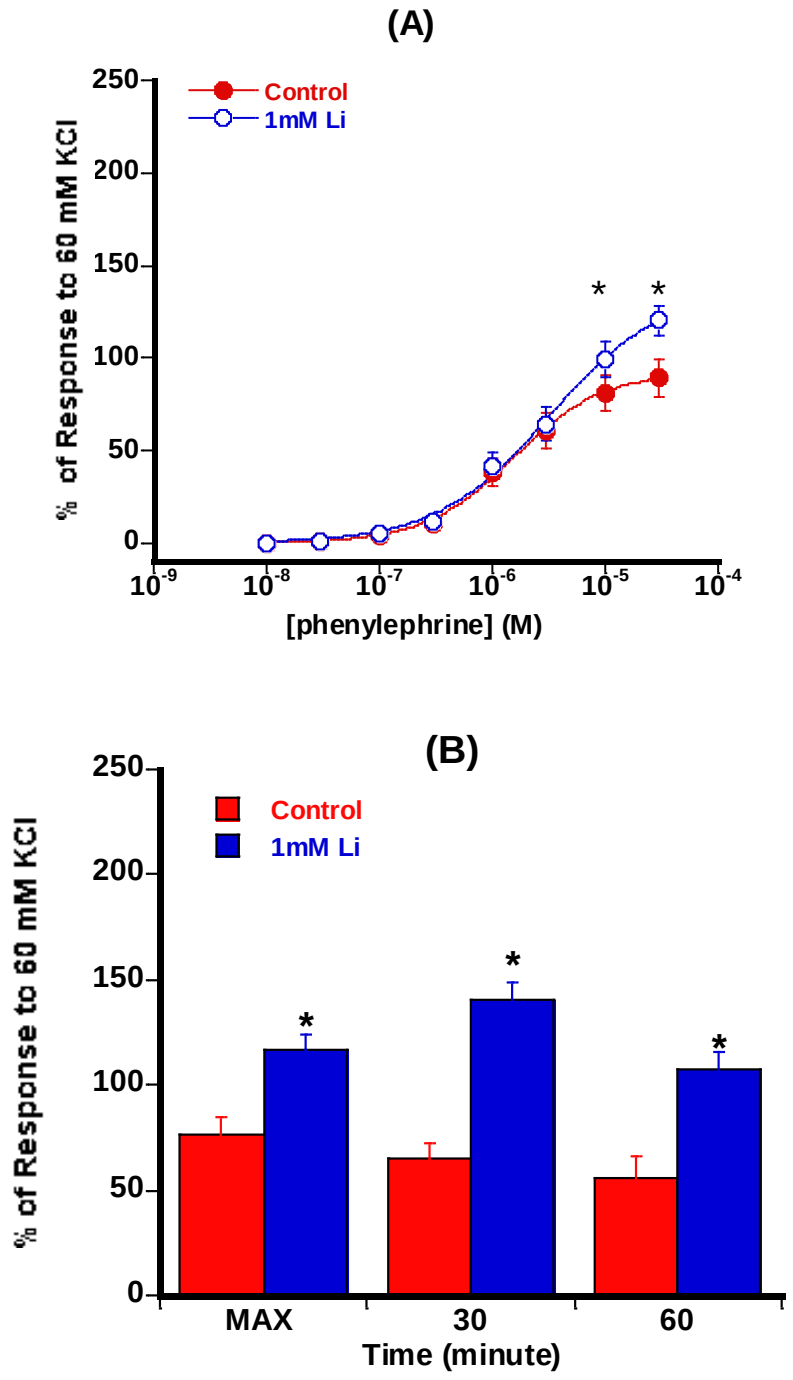

Figure 3-15 (A) The concentration-response curves for vasoconstrictor effect of cumulative addition of phenylephrine in the presence and absence of 1mM LiCl and (B) the bar chart for contractile response in the porcine splenic vein at a different time point after it reaches the maximum response. Data are expressed as a percentage of contraction to 60mM KCl and are mean ± SEM (n=8). * - Denotes a statistically significant difference ($p < 0.05$) between responses using a paired Student's *t*-test.

Table 3-2 Summarises the responses to contractions induced by phenylephrine in the presence and absence of LiCl in different porcine vascular tissues: where PMA, PRA, PSV and PUsm are porcine mesenteric, renal artery, splenic vein and porcine urethral smooth muscle respectively; where * represents significant changes using paired Student's t-test. Responses are expressed as a percentage of the contraction to 60mM KCl and are mean± SEM and n=represents the number of observations.

Agonist	**Tissue**	**KCl Response**	**R_{max}**	**pD_2**	**n**
Phenylephrine	PMA	10.8±2.1	54.7±2.8	6.4±0.07	7
Phenylephrine+ Li		9.2±1.4	171.5±5.9*	5.5±0.1*	
Phenylephrine	PRA	7.5±1.2	130.7±9.2	6.1±0.05	8
Phenylephrine+ Li		10.0±0.8	167.2±8.6*	5.8±0.1*	
Phenylephrine	PSV	5.6±1.1	99.4±10.4	5.67±0.13	8
Phenylephrine+ Li		6.3±1.3	148.9±8.4*	5.3±0.17*	
Phenylephrine	PUsm	1.±0.2	127.5±16.1	6.1±0.1	7
Phenylephrine+ Li		1.2±0.2	219.5±18.8*	5.5±0.2*	

3.3.2.5 The effect of adding LiCl on the concentration-response curve and time course of contraction induced by phenylephrine in the porcine urethra smooth muscle

In isolated porcine urethral smooth muscle segments exposing the tissue to 60mM KCl produced a slightly stable contraction equivalent to 1.4±0.17 g wt. (n=14). This contraction weakened with the successive exposure to KCl, so we used the 1st response as a reference in this set of experiments rather than the 3rd. The preincubation of segments with 1mM LiCl showed a greater response to a high concentration of phenylephrine. Significant changes in the R_{max} and the potency was seen with a P-value< 0.05 using paired Student's *t*-test after the cumulative addition of the phenylephrine in this preparation. See Figure 3.16, and Table 3.2

In a different set of experiments, using porcine urethra smooth muscle, the single addition of phenylephrine 30μM, initiated contraction in presence and absences of 1mM LiCl. The contraction reached its peak response within 3-5 minutes and declined slowly to lose about 50% of its peak response after 60minutes in control segments but not the LiCl incubated segments. The response was expressed as a percentage to contraction induced by first 60mM KCl. After 15 min the peak response in the absence and presence of LiCl was not significantly changed at 242.3±35.8% (n=11) to become 238.8±34.0 % (n=11). Interestingly, after 60 minutes, the changes were significant at 188.7±36.5% (n=11) in comparison with the control in which the percentage of response was 23.1± 13.8% (n=11), with a P-value <0.05, using paired Student's *t*-test. See Figure 3.17

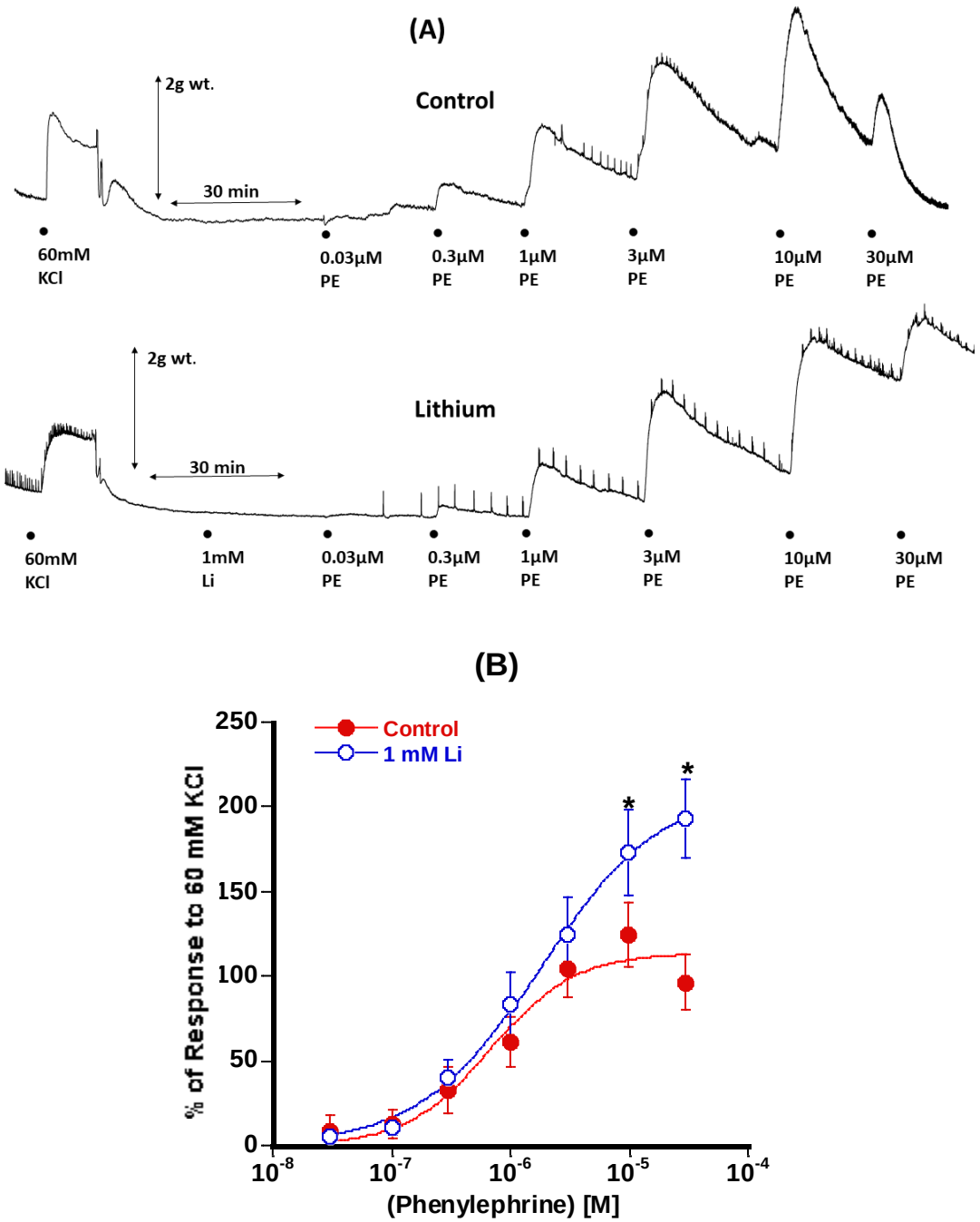

Figure 3-16 (A) Representative traces for the response of porcine urethral smooth muscle to contraction induced by addition of KCl and cumulative addition of phenylephrine in the absence and presence of LiCl 1mM. (B) The concentration-response curve for cumulative addition of phenylephrine in the presence and absence of LiCl 1mM. Data are expressed as a percentage of 60mM KCl and are mean ± SEM (n=7). * - Denotes a statistically significant difference ($p < 0.05$) between responses using a paired Student's *t*-test.

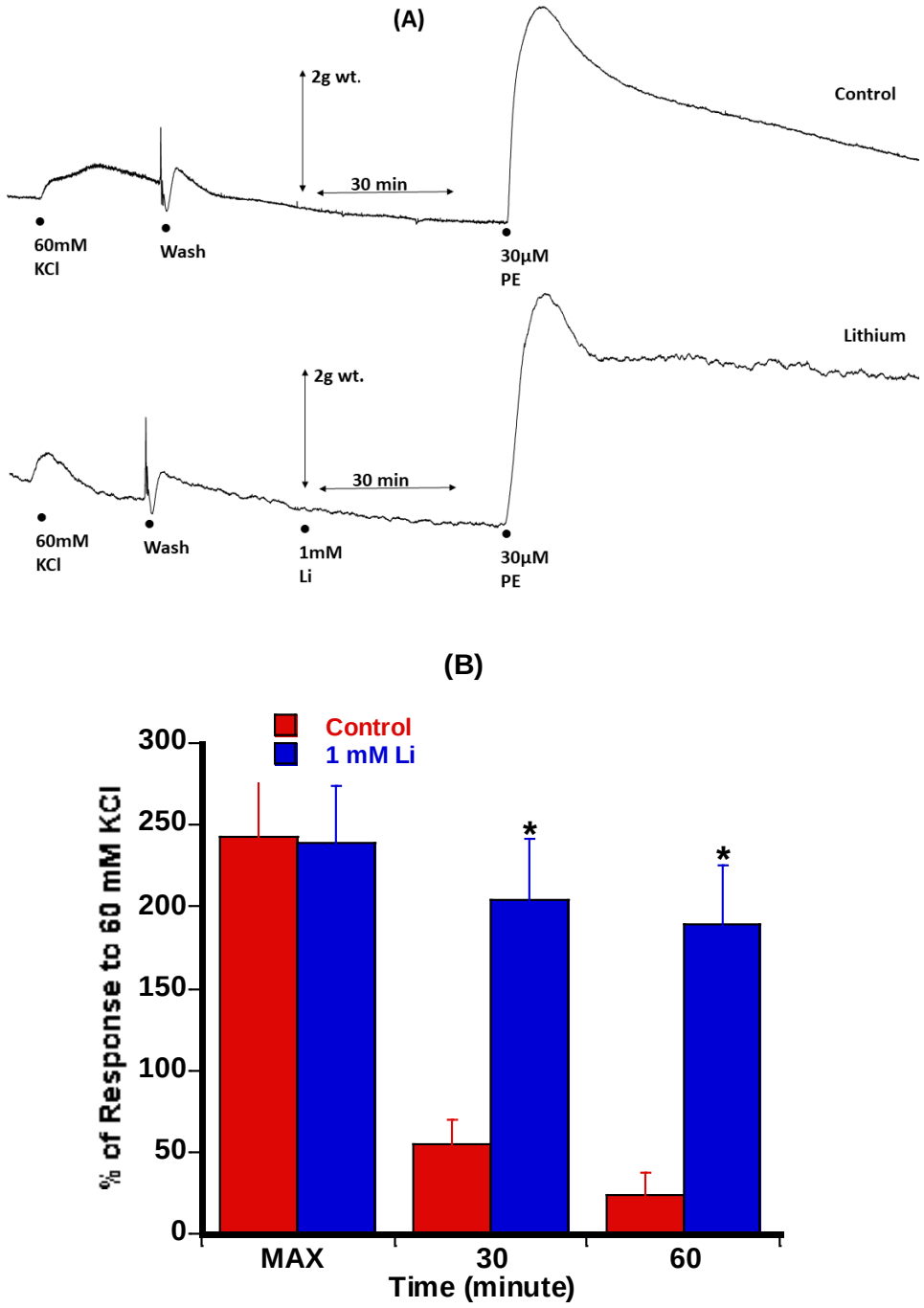

Figure 3-17 (A) Representative traces for the response of porcine urethral smooth muscle to contraction induced by addition of KCl and phenylephrine 30µM in the absence and presence of LiCl 1mM. (B) Time course for contractile response at a different time point. Following single addition of phenylephrine 30µM in presence and absence of LiCl 1mM. Data are expressed as a percentage of 60mM KCl and are mean ± SEM (n=11). * - Denotes a statistically significant difference ($p < 0.05$) between responses using a paired Student's *t*-test.

3.3.3 Interaction between LiCl and other vasoconstrictors receptor systems

3.3.3.1 The effect of adding LiCl on the time course of contraction induced by 5HT, histamine and α, β methylene ATP in the porcine splenic artery

In the porcine splenic artery, incubation the tissue with 1mM LiCl did not produce an effect on the time course for the contraction initiated by a purinergic agonist α, β, methylene ATP 3μM induced vasoconstriction in response to 60mM KCl (n=6). After 30 minutes both control and treated segments went back to baseline, See Figure 3.18 (A) and Figure 3.19

Moreover, the addition of 1mM LiCl to 5HT 10μM did not produce a statistically significant change in the time course of the 5HT. After 60 minutes of 5HT addition, the concentration decreases to less than 16.4±5.6% (n=8) of its peak response, in compare to 27.3±7.9% (n=8) of the 5HT in the presence of LiCl, using paired Student *t*-test. See Figure 3.18 (B)

On the other hand, the addition of 1mM LiCl to histamine 20μM produced a significant change in the time course of the histamine and the P-value <0.05, using paired Student's *t*-test. After 60 minutes of histamine addition, lithium raises the contraction to be 59.0±17.0% (n=8) of its peak response in comparison to control, where the contraction was 47.7±13.5% (n=8). Using a paired Student's *t*-test. See Figure 3.18 (C).

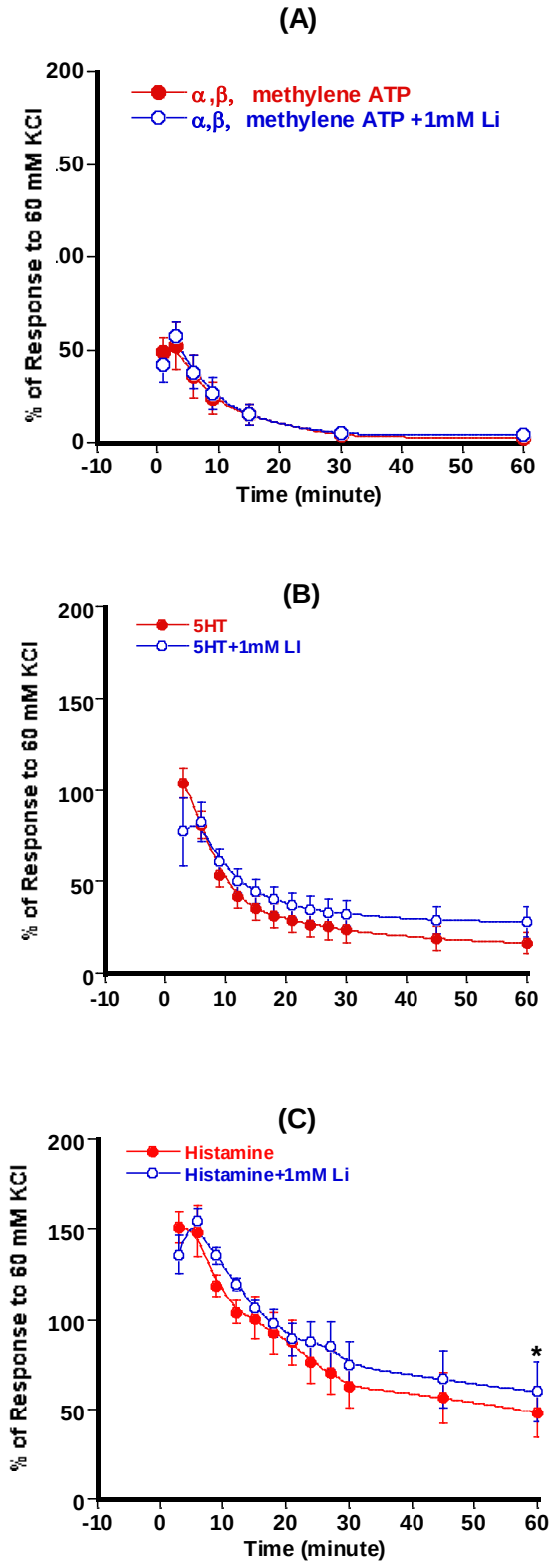

Figure 3-18 The effect of 1mM LiCl on the time course of responses to (A) α, β methylene ATP 3μM (n=6) (B) 5HT 10μM (n=8) and (C) histamine 20μM induced contraction in the porcine splenic artery (n=8), expressed as % to 60mM KCl. Data represented as mean± SEM. P* value <0.05, Using paired Student's *t*-test.

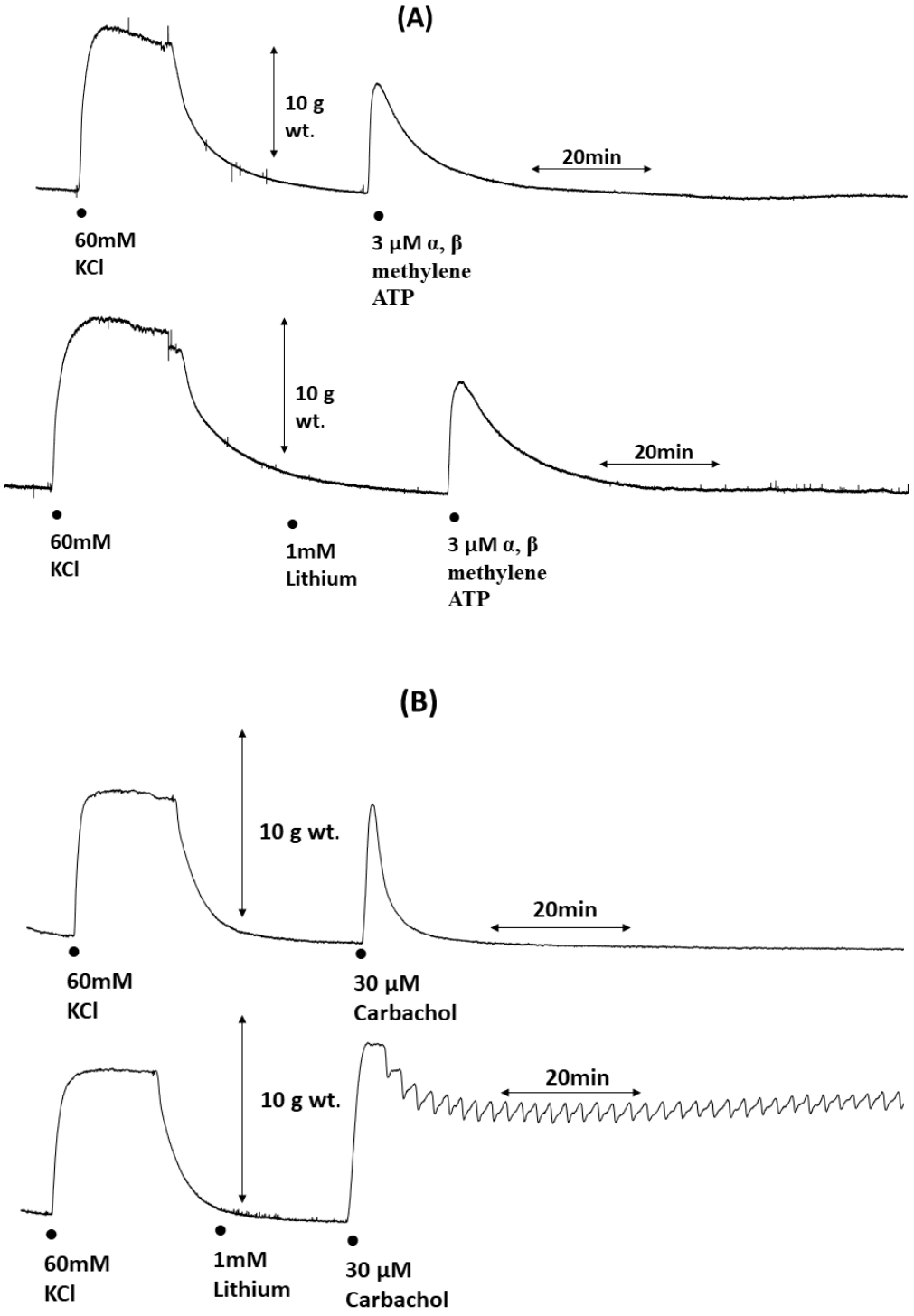

Figure 3-19 Representative traces recording of the porcine isolated splenic artery paired segments to the single additions of (A) α,β methylene ATP 3µM and (B) carbachol 30µM in the absence and presence of 1mM LiCl.

3.3.3.2 The effect of adding LiCl on the time course of contraction induced by carbachol in the porcine splenic artery

In order to check the effect of LiCl on the time course of cholinergic agonist, carbachol 30μM was added in a single dose in the presence and absence of 1mM LiCl. Significant changes were observed in the time course curve of carbachol-induced vasoconstriction in response to contraction to 60mM KCl following the addition of LiCl. LiCl increased the initial maximum response from 93.4±13.2% to become 129.3±11.4% (n=11) and maintained it after 30 minutes of exposure at 79.6±11.0% in comparison with the control in which the percentage of response was less than 6.6±1% (n=11). Using a paired Student's *t*-test. See Figure 3.19 (B) and 3.20

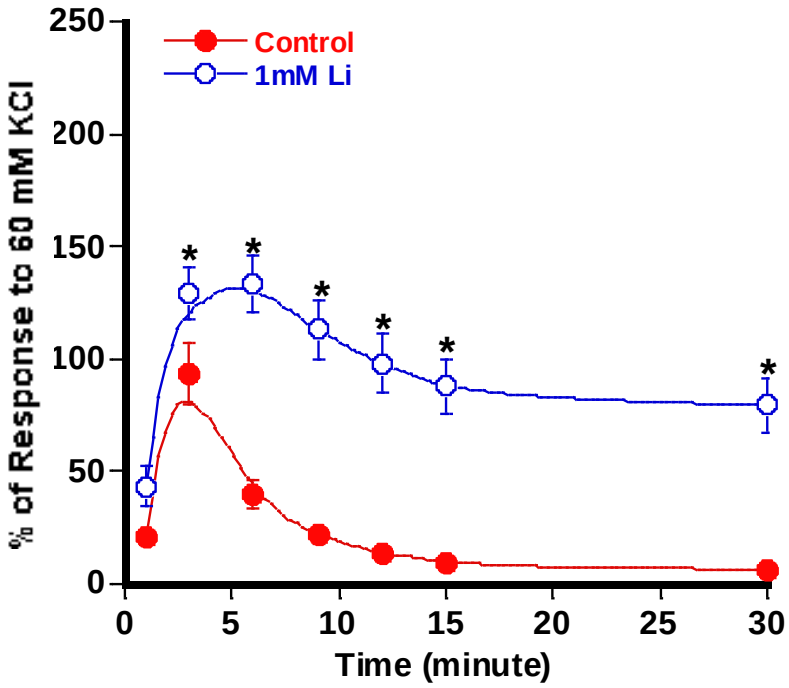

Figure 3-20 Time course for carbachol 30μM -induced contraction in a porcine splenic artery in the absence and presence of 1mM LiCl. expressed as a percentage to contraction induced by 60mM KCl. Data represented as mean± SEM (n=11). * - Denotes a statistically significant difference ($p < 0.05$) between responses using a paired Student's *t*-test.

3.3.3.3 The effect of adding LiCl on the time course of contraction induced by angiotensin II and vasopressin in U46619 precontracted porcine splenic artery

In order to enhance the contractility in the tissue and following the failure to induce a recordable contraction after direct application of both agonists, pre-contraction of tissue with U46619 to about 60% of the KCl preceding the incubation with 1mM LiCl. Both agonists failed to induce large response following the cumulative addition. In spite of the use of high concentration of both agonists 1μM, LiCl failed to produce a statistical difference in the time course of angiotensin II and vasopressin (n=5). The results of LiCl addition on the time course of both agonists are shown in Table 3.3

Table 3-3 The effect of LiCl on the time course of angiotensin II and vasopressin-induced contraction in porcine splenic artery expressed as a percentage to contraction of 60mM KCl. Data represented as mean± SEM (n=5).

Agonist	Response 3 min	Response 60 min
1μM Angiotensin II	25.3±6.2	13.5±4.8
1μM Angiotensin II + 1mMLi	18.4±4.2	12.2±3.3
1μM Vasopressin	28.4±7.2	10.1±3
1μM Vasopressin+ 1mMLi	22.2±5.2	13.3±4.1

3.3.3.4 The effect of adding LiCl on the relaxation induced by isoprenaline and dobutamine in porcine coronary artery

In order to investigate the effects of the LiCl on β-adrenergic receptor system and due to the presence of a high population of β-adrenoceptor in the porcine coronary artery tissue. Our results showed that the addition of 60mM KCl produced a sustained contraction equivalent to 8.5±1.1 g wt. (n=13) in the porcine coronary artery and the addition of (2-10nM) U46619 produced a slow-developing stable contraction, equivalent to 6.1±0.5 g wt. (n=13).

Figure 3.21 shows that both (A) isoprenaline and (B) dobutamine produced a concentration-dependent inhibition of U46619 induced contractions in the porcine coronary artery. Incubation the tissue with 1mM LiCl did not change tissue response to these agonists and did not change the rate of relaxation induced by either isoprenaline or dobutamine.

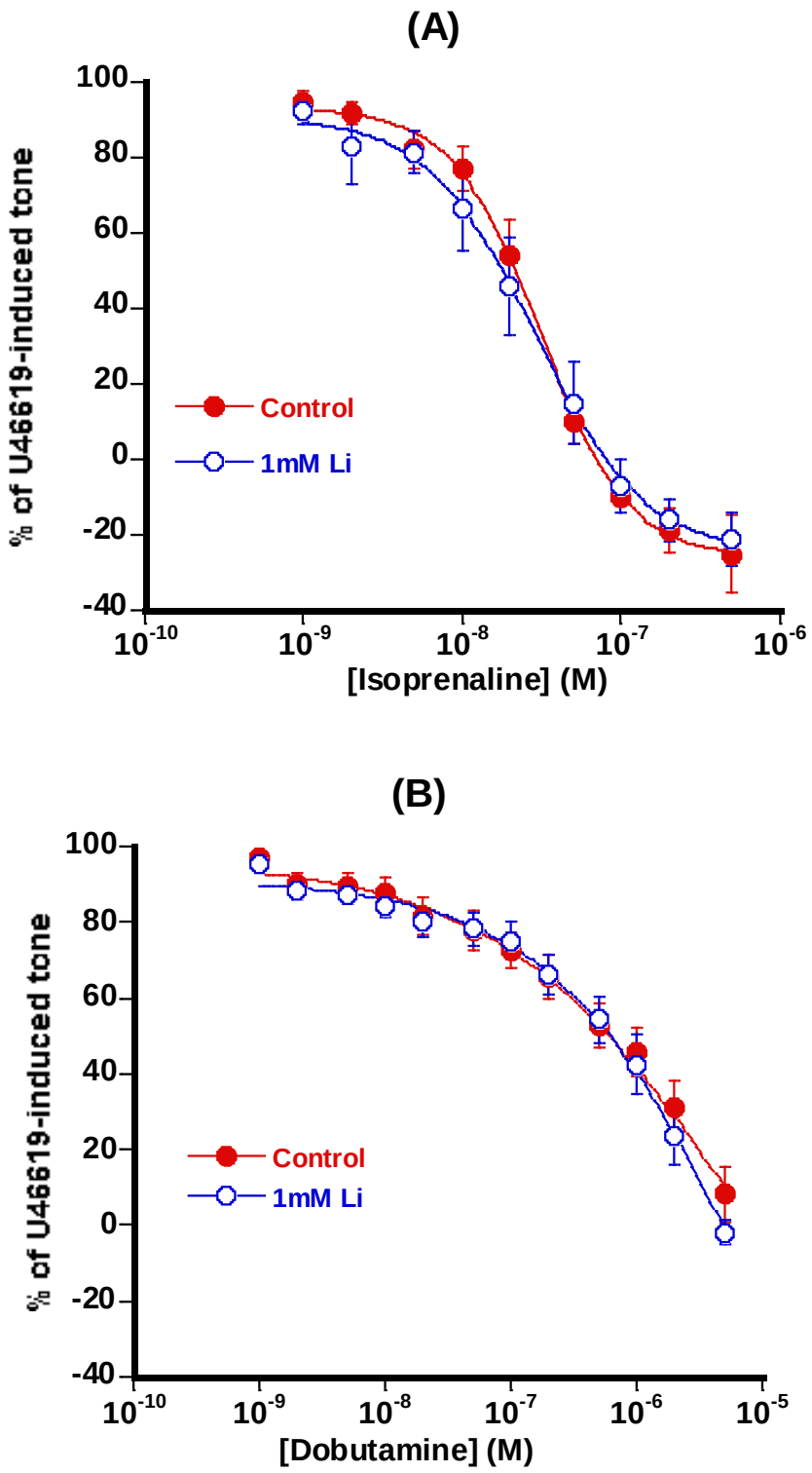

Figure 3-21 Cumulative addition of (A) isoprenaline (n=9) and (B) dobutamine (n=6) in the presence and absence of 1mM LiCl on the porcine coronary artery expressed as a percentage to contraction induced by U46619. Data represented as mean± SEM. P* value <0.05.

3.3.3.5 Role of rubidium, concentrations and type of lithium salts involved in the synergistic interaction between LiCl and L-erythromethoxamine in the porcine splenic artery

Figure 3.23 (A) and Table 3.4 show that the presence of lithium in three different salts (sulphate, chloride and carbonate) produced a significant enhancement in the concentration-response curve of L-erythromethoxamine in porcine splenic artery. This was accompanied by significant changes in the potency of the agonist.

In a different set of experiments, three concentrations of LiCl were used to examine their effect on the concentration-response curve of L-erythromethoxamine in porcine splenic artery. Both 1mM and 0.3mM LiCl significantly enhanced the maximum response to contraction induced by L- erythromethoxamine. In contrast, 0.1mM LiCl failed to initiate the synergistic interaction with L-erythromethoxamine. See Figure 3.23 (B) and Table 3.4.

Another set of experiments was conducted to compare the action of lithium with rubidium. Figure 3.22 and Table 3.4 show that the presence of rubidium did not enhance the concentration-response curve of L-erythromethoxamine in the porcine isolated splenic artery and the presence of this monovalent ion did not change the maximum response nor the potency for the agonist.

Table 3-4 Shows the changes in the maximum response and the potency following incubation the tissue with different salts and concentrations of lithium and rubidium followed by cumulative addition of L-erythromethoxamine agonist. Data expressed as mean± SEM of contraction to 60mM KCl * - Denotes significant differences between mean responses ($p < 0.05$; paired Student *t*-test).

Agonist	**R_{max}**	**pD_2**	**n**
L- erythromethoxamine	55±7.3	6.2±0.1	6
Li sulphate	158.9±11*	5.6±0.08*	6
Li chloride	148.6±6.3*	5.7±0.1*	6
Li carbonate	153.4±10.6*	5.8±0.01*	6
Set 2			
L- erythromethoxamine	42.3±12.7	6.4±0.02	4
0.3mM LiCl	195.7±10.4*	5.6±0.01*	4
1mM LiCl	189.5±3.4*	5.7±0.04*	4
0.1mM LiCl	52.9±1.6*	5.6±0.2*	4
Set 3			
L- erythromethoxamine	63.4±6.8	6.4±0.2	7
1mM LiCl	150.7±10.2*	5.8±0.18*	7
1mM Rubidium	70.4±2.9	6.3±0.12	7

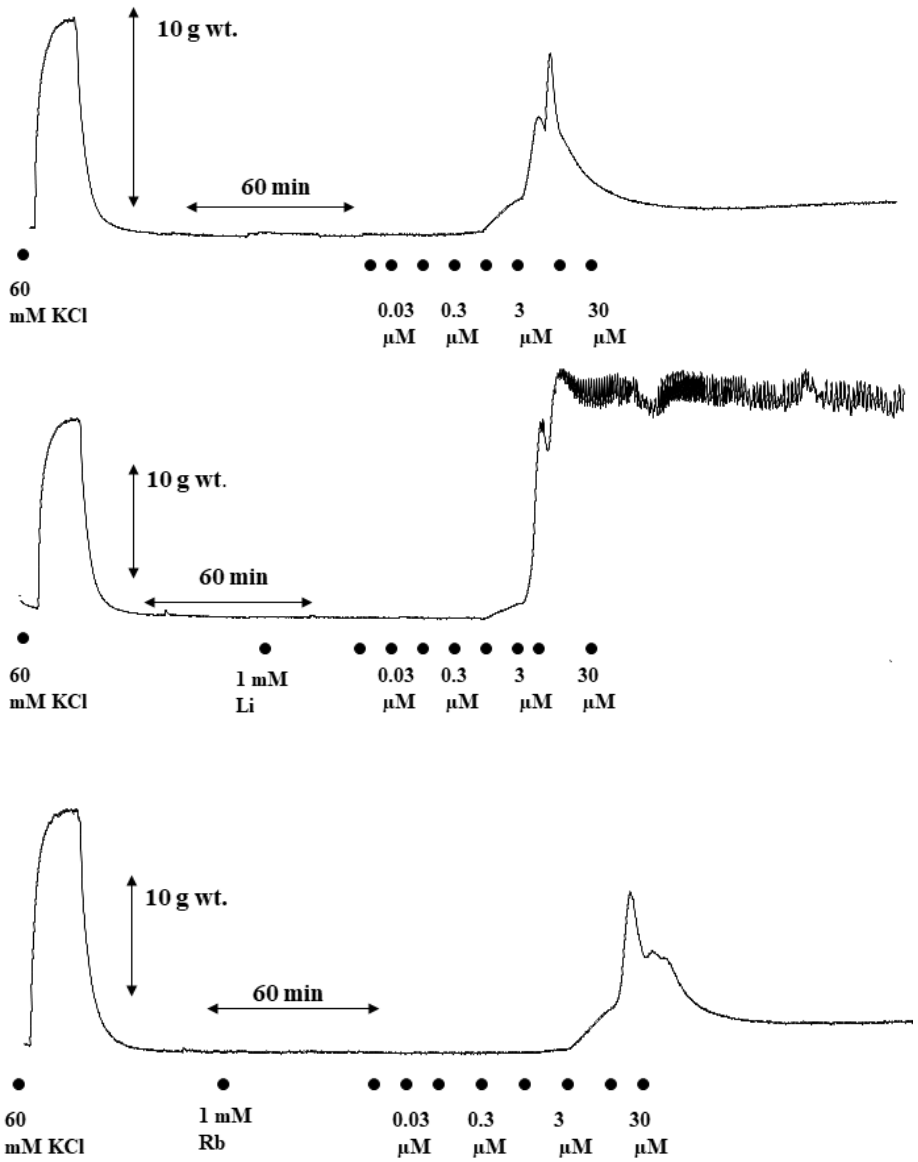

Figure 3-22 Representative traces for vasoconstriction effects following the cumulative addition of L-erythromethoxamine in the presence and absence of 1mM of LiCl and rubidium (Rb). Data are expressed as the percentage of contraction to 60mM KCl in the porcine splenic artery.

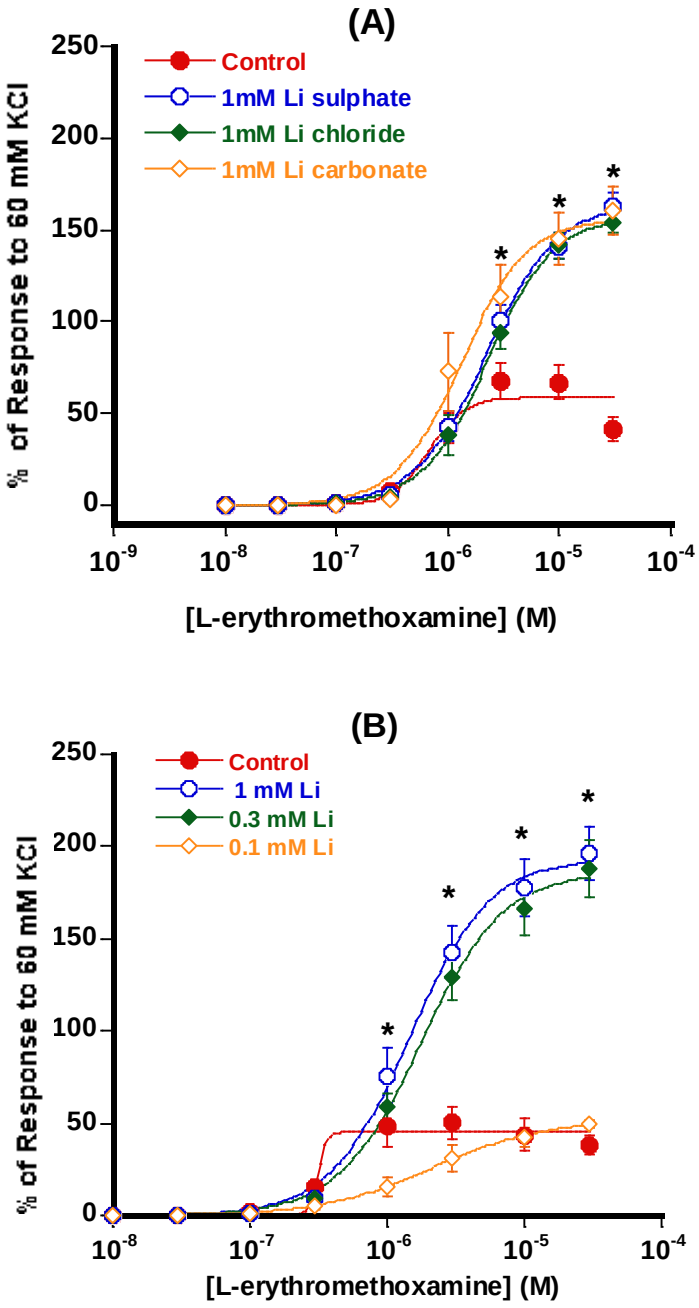

Figure 3-23 The concentration-response curve following the cumulative addition of L-erythromethoxamine in the porcine splenic artery after (A) the presence of 1mM lithium salts (sulphate, chloride and carbonate) n=6 and (B) the presence of 1, 0.3 and 0.1 mM LiCl n=4. Data are expressed as a percentage of contraction to 60mM KCl and are mean ± SEM.* - Denotes significant differences between mean responses ($p < 0.05$; paired Student *t*-test).

3.4 Discussion

The main aim of this chapter was to show the contractile response of porcine smooth muscles in different vascular and non-vascular tissues to stimulation produced by different agonists. Five different α_1-adrenoceptors were tested in this chapter including noradrenaline, phenylephrine, L-erythromethoxamine, metaraminol and cirazoline. In addition to other agonists that can produce contraction when binding with the same GPCR subunit $G_{q/11}$ and these are histamine, 5HT, α, β methylene ATP, angiotensin II, vasopressin and carbachol (Cotecchia 2010). All these agonists produced a contraction in isolated porcine vascular tissues, the time course of this contraction was not stable (except for cirazoline) and faded after a period which differs by different agents and tissue. After decades of the use for these agonists in general and phenylephrine in specific, only limited research highlighted this important observation that is associated with the high concentration of phenylephrine and less common with the low concentration. In order to provide better insight into this phenomenon, five different porcine vascular tissues were used for the purpose of this study including a porcine splenic artery, splenic vein, renal artery mesenteric artery and pulmonary artery. While the porcine urethral smooth muscles represent the non-vascular model. The second purpose of the work in this chapter was to compare the contraction induced by these agonists if the tissue preincubated with a therapeutic concentration of LiCl and highlight the effect of this interaction on the magnitude and the duration of action for these vasoactive substances.

The key observations from this study are first, in porcine splenic artery segments incubated with LiCl the response to stimulation by phenylephrine, which is a selective α_1-adrenoceptors agonist, was significantly enhanced in magnitude and duration. This enhancement was reported to be in the sustained response rather than the peak response following the addition of a high concentration of phenylephrine. This finding highlighted the fact that lithium (but not rubidium) at a therapeutic concentration (0.8-1.2) mM, which used to treat bipolar disorder (Bauer and Gitlin 2016), enhanced the action of phenylephrine, L-erythromethoxamine and metaraminol but not noradrenaline or cirazoline. Although all are α_1-adrenoceptor agonists and act through the stimulation of $G_{q/11}$ subunit on the cell membrane to produce contraction of vascular blood vessels (Cotecchia 2010).

Secondly, this positive interaction between LiCl and some α_1-adrenoceptors was reported in different preparations of porcine vascular tissues including the porcine splenic artery, splenic vein, renal and mesenteric artery while we could not confirm such interaction in the pulmonary artery. In non-vascular tissue of porcine urethra smooth muscle, lithium enhanced the time course of the contraction induced by phenylephrine. Thirdly, LiCl has no effect on the relaxation induced by stimulation of β_2-adrenoceptors in porcine coronary artery segments precontracted with U46619. Finally, LiCl has no effect on the contraction induced by other agonists, which bind to GPCR at cell membrane but act on different receptors systems including: histamine, 5HT, α, β methylene ATP, angiotensin II and vasopressin except carbachol that showed similar positive interaction to what we previously reported with phenylephrine, L-erythromethoxamine and metaraminol.

The discussion of this chapter is divided into two parts. The interaction between LiCl and α_1-adrenoceptors agonists in different porcine vascular and non-vascular tissues is the first part, while the second part emphasises the interaction with other vasoactive receptor systems.

3.4.1 The interaction between LiCl and α_1-adrenoceptors agonists in different porcine vascular tissues.

Nearly 40 years ago, Sherman and colleagues, suggested that at least part of lithium activity in managing the symptoms of psychosis disorder could be accredited to its ability to interfere with the phosphoinositide metabolism. Lithium non-competitively inhibits the enzyme inositol monophosphatase that is responsible for the degradation of inositol monophosphate and the production of myoinositol. This results in the inhibition of intracellular recycling of inositol triphosphate (IP_3) (Sherman *et al.*, 1981). Thus lithium and in addition to its role in psychosis also played an important role in researches involved the study of phosphoinositide metabolism (Nahorski *et al.,* 1991).

The α_1-adrenoceptors are a subgroup of GPCR located on the cell membrane. The binding of catecholamine agonists (adrenaline or noradrenaline) stimulates a variety of physiological responses, such as contraction of the smooth muscle in bladder, prostates, activation of cardiac muscles by activation of PLC enzymes and the production of IP_3 that resulted from the hydrolysis of phosphoinositol -bisphosphates

(PIP_2). IP_3 is responsible for the mobilization of Ca^{2+} from storage sites in SR and initiation of contraction (Wang *et al*., 2007).

Since there is no previous study which investigated the interaction between LiCl and α_1-adrenoceptors agonists in the porcine splenic artery in term of magnitudes or the time course for 30-60 minutes following the addition of the agonist. It could be argued that this study will provide light on this important interaction. The importance of this interaction comes from the fact that both groups have an essential role in the treatment of many current clinical conditions, and they are suggested to share similar cellular pathway to produce their effect through interference with phosphoinositide metabolism in the cells.

Our results suggested that the cumulative addition of phenylephrine, L-erythromethoxamine and metaraminol (phenethylamine-based selective α_1-adrenoceptors agonists) produced maximal contractions that were significantly less than those to noradrenaline and cirazoline (imidazoline base α_1-adrenoceptors agonist), proposing that they act as 'partial agonist' at α_1-adrenoceptors in this tissue.

The incubation with 1mM LiCl increased the maximum response to phenylephrine, L-erythromethoxamine and metaraminol without affecting responses to noradrenaline and cirazoline. These changes were associated with a significant reduction in the potency (pD_2) without reducing the effectiveness of the contraction. These finding suggested that key parameters defining α_1-adrenoceptors agonism in different porcine vascular tissues might be altered by the presence of LiCl. Comparable results were obtained in a porcine splenic vein, porcine renal artery, porcine mesenteric artery and porcine urethral smooth muscle.

The second interesting observation in this chapter was the single addition of phenylephrine agonist produced a contraction that was stable for more than 60 minutes at low concentration (<1μM) however, with increasing the agonist concentration the magnitude of the contraction increased but lost its stability after less than 10 minutes. The presence of LiCl did not interfere with the action at a low concentration of phenylephrine. However, with higher doses, LiCl significantly enhanced the maximum response and prolonged the duration of contraction induced by phenylephrine in porcine splenic artery. Similar enhancement in the time course was reported with L-erythromethoxamine and metaraminol but not with noradrenaline or cirazoline. In line with our results, The initial observation was made in our lab that the addition of LiCl enhanced and better-maintained the contraction of isolated sheep

internal anal sphincter in response to phenylephrine and L-erythromethoxamine (Rayment *et al.,* 2007).

As mentioned in the introduction of this thesis, the interaction between LiCl and α_1-adrenoceptors was reported in a large number of published studies. For more than 20 years Dehpour and his group focused on the effect of incubating the tissue with different concentration of LiCl in different vascular and non-vascular tissue of rats and rabbits. they highlighted the interaction with different receptor systems including cholinergic, adrenergic, vasopressin and angiotensin agonists (Dehpour *et al.*, 1993;1995;1995b; 2000).

In line with these results, another research conducted by Skomedal and his group in 1991, described the synergistic interaction between lithium ions and the inotropic effect of phenylephrine in rat heart. They have emphasised that the use of (0.25-3 mM) Li enhanced the contraction of the isolated papillary muscle to the contraction induced by phenylephrine following an incubation period of 30 minutes *(Skomedal et al.*, 1991). Molderings and Schiimann, (1987) reported a similar increase in inotropic action produced after the addition of phenylephrine in the guinea pig heart following the incubation with 7mM LiCl. Contrary to previous results Mantelli *et al.*, (1988) demonstrated that the chronic exposure to LiCl antagonises the inotropic effect produced by α_1-adrenoceptors in ventricles of guinea- pig. They attributed this to the antagonism to the ability of lithium to inhibit the concentration of *myo*inositol in the CNS. The ambiguity between the results of these studies could be better explained if the dual-action theory was adopted *i.e.* even in the same system and conditions lithium can produce two contradicted effect. For example, 0.5mM lithium was reported to inhibit the endothelial-derived relaxing factor (EDRF) while the use of 2mM was found to increase EDRF in rat aorta following the addition of acetylcholine (Dehpour *et al.*, 1995). Another supported study for this theory was reported with acetylcholine-induced relaxation in rat mesenteric artery incubated with different concentration of LiCl, which show that lower doses reduced the relaxation. In contrast, the use of higher concentrations of lithium enhanced acetylcholine-induced relaxation (Rahimzadeh-Rofouyi *et al.*, 2007). This enhancement in the action of phenylephrine was attributed to the ability of LiCl to inhibit IMPtase enzymes. Another possible explanation to limits, this phenomenon to the three phenethylamine-based selective α_1-adrenoceptors agonists could be due to some structural specifications. This could be supported by my colleague finding as she also tested LiCl interaction with guanfacine, A61603 and xylometazoline (α_1-adrenoceptors agonist) in porcine splenic artery. She reported that incubation the tissue with LiCl significantly changed the pD_2 from 6.11±0.08 to become

5.73±0.05 (n=8) with the former, while the interaction with the A61603 and xylometazoline were not statistically significant. It is reported that guanfacine has phenethylamine structure and produce its action via stimulation of α_2-adrenoceptors. While both A61603 and xylometazoline have imidazoline like structure and they act by stimulation of both α_1 and α_2 adrenoceptors (Knepper *et al.*, 1995; Mehrotra *et al.*, 2007). So further experiments will be conducted to highlight the mechanism in chapter 5 of this thesis.

Our results in the non-vascular smooth muscle of porcine urethra showed that this tissue responded differently to the stimulation by KCl. The addition of KCl initiated a contraction that reached its peak response within 3-4 minutes. This contraction and unlike that previously reported in vascular tissue in this thesis is not well maintained. The tissue relaxed even in the continuous presence of KCl. Another peculiar observation was washing the KCl induced a contraction in the urethral smooth muscle rather than relaxation. In addition to that, the successive response to the stimulation with KCl seems to be weakened.

This peculiar response of porcine urethral smooth muscle could be explained as a sort of desensitisation for continuous exposure to the stimuli (KCl) or successive exposure to the same stimuli. It also could be explained by phenomena named "auto-regulatory escape". This phenomenon was reported following the continuous infusion of noradrenaline in cat intestinal vessels. The tissue contracted by the action of noradrenaline for a short period before it returns back to pre-noradrenaline level (Ross 1975). This action could result from the assumption of presences of a special system that interfere with the concentration of free Ca^{2+} in porcine urethral smooth muscle and reduce the level of contraction to baseline. It seems difficult to generalise this phenomenon in the non-vascular tissue outside the general circulation; however, this could be a reason to explain this response but further studies are warranted. Another putative explanation of this peculiar response to stimulation by KCl could be the presence of pacemaker-like cells. In contrast to contraction induced following the neuronal stimulation with the adrenergic or cholinergic system, the myogenic tone can be generated in the absence of neuronal input. In 2000, researchers described for the first time special self-firing cells, which can generate myogenic tone in the urethral of the rabbit. They term these cells as interstitial cells of Cajal. Similar pacemaker cells previously reported in the gastrointestinal tract and human bladder (Shafik *et al*., 2004). This finding could partially explain that the myogenic tone generated in porcine urethral smooth muscle originated from the pacemaker cells rather than from the increase in the Ca^{2+} level following the opening of L-type Ca^{2+} channels.

In this study, we have looked at the effect of LiCl on the relaxation induced by stimulation of β_2-adrenoceptors in a porcine coronary artery after a stable contraction by thromboxane mimetic U46619 was initiated. It was reported that isoprenaline can stimulate relaxation in the porcine coronary artery (Kameyama *et al*., 1985). No changes in relaxation speed or potency of isoprenaline or dobutamine were reported following the exposure to LiCl. The effect of lithium on the relaxation was studied in rat aortic ring precontracted with phenylephrine before exposed to relaxation by Ach and sodium nitroprusside. It was reported that acute administration of 2mM LiCl enhanced the maximum relaxation induced by Ach an action credited for the ability of lithium to increase the concentration of IP_3 inside the cell, in contrast, the endothelium-independent relaxation induced by sodium nitroprusside through direct stimulation of cGMP did not affect by LiCl(Dehpour *et al.,* 2000). This suggested that the interaction of LiCl and Ach was due to an increased in the synthesis of NO. In addition to that, we further tested the role of NO in the interaction with phenylephrine using L-NAME. it was previously reported that the use of 100μM of L-NAME inhibits the relaxation which results from the production of NO in overnight stored segments of the porcine splenic artery (Lot and Wilson 1994). Our results (Appendix IV) showed that using in the presence of L-NAME the contraction induced by phenylephrine was further enhanced when tissue preincubated with LiCl. This suggested that NO pathway was not involved in this interaction.

3.4.2 The interaction between LiCl with various receptors agonists in different tissue

After we have reported the interaction between LiCl and α_1-adrenoceptors in multiple vascular tissues, we conducted several experiments in which we investigated the potential interaction of LiCl with other vasoactive agents acting by binding to GPCR and specifically $G_{q/11}$ subunits. Our results revealed that 5HT, histamine or α, β methylene ATP induced - contraction that was not sustained and decline after a single addition of high concentration of agonist. Furthermore, LiCl failed to prolong the duration to contraction except for carbachol in which, LiCl significantly enhanced the time course for more than 120 minutes and showed similar synergistic interaction to what we have reported with α_1-adrenoceptors.

A study conducted by Dehpour *et al.*, (1997) examined the effect of adding LiCl (0.5, 1, and 10mM) to muscles of rat urinary bladder and study its effect on contraction induced by vasopressin. It was found that tissue became more sensitive to the action of the agonist in the presence of LiCl. This could be attributed to the fact that at least

part of lithium activity results from its ability to interfere with the phosphoinositide pathway. This finding was consistent with other research conducted by the same group who reported that the neuromuscular blocking effect of aminoglycoside antibiotics was reduced if tissue incubated with LiCl. This could be due to the ability of lithium to interfere with the release of acetylcholine at the neuromuscular junction (Dehpour *et al.*, 1992).

These results are in agreement with those obtained by Ullian group (1995) who demonstrated that incubation the rat aortic segments with LiCl enhanced the contraction induced by angiotensin II with no effect on the time course of the contraction. On the cellular level and contrary to expectation, the interaction between angiotensin II and 10mM lithium in adrenal glomerulosa cells produced inhibition in the response of the agonist (Balla *et al.,* 1984). What could be considered as a limitation for the above study is, it is well documented that the use of vascular smooth muscle in the cell line may not match the vascular smooth muscle in the renal tissue due to the ability of the cells to dedifferentiation beside the use of high concentration of lithium ions.

According to our results, which showed that neither vasopressin nor angiotensin II was able to produce a response due to the absence of their receptor in porcine splenic artery. In which most of the contractile response resulted from activation of α_1-adrenoceptors (Wright *et al.,* 1995a) with the minimum effect of α_2-adrenoceptors (Hamza Denfria, 1999). We tried to enhance tissue contractility to theses agonists by submaximal contraction with U46619 (Shatarat *et al.*, 2014) however, no significant changes were reported.

Rubidium and lithium are soft alkali metals located in the same group in the Periodic Table. In order to check if the interaction with α_1-agonists is not due to chemical properties of lithium, tissue was incubated with 1mM rubidium before cumulative additions of L-erythromethoxamine were added. Our results showed that rubidium did not change the concentration-response curve of the agonist in the porcine splenic artery. These results are in agreement with Rayment *et al*., (2007), who compared the effect of incubation segments of sheep internal anal sphincter in 1mM of Li and rubidium. They reported that only lithium was able to attenuate the reduction in muscle tone following the addition of L-erythromethoxamine.

Three different types of lithium salts were tested (chloride, carbonate and sulphate) it was clear that the action of lithium-ion is attributed to the ion itself with no role of the salt's moiety in this interaction. Another important observation in this chapter was this synergistic interaction occur at therapeutic and subtherapeutic concentrations of lithium, even with 0.3 mM similar synergistic interaction with α_1-adrenoceptors agonist was reported (Bosche *et al*., 2016). The importance of this type of interaction between LiCl, phenylephrine and metaraminol may have therapeutic potential because these agents currently used in the clinical field. We could enhance the decongestant effect of phenylephrine (Johnson and Hricik 1993) or its ability to increase the blood pressure in patients with hypotension (Hoen *et al.*, 2005) as well as its role in control urinary and faecal incontinence (Jones *et al.*, 2003) if combined with clinically acceptable levels of LiCl. Evaluation of the effect of LiCl using *in vivo* model is reasonable at some point of this study.

In conclusion, the results from this chapter showed that the time course following a single addition of a high concentration of α_1- adrenoceptors agonist can produce a rapid contraction in different porcine isolated vascular tissue. This contraction is not sustained and weakened with time. Synergistic interaction between LiCl and subgroup of α_1-adrenoceptors-induced contraction in different vascular and non-vascular tissue was reported. This synergism appeared as an enhancement in the maximum response as well as a prolongation of the time course for the contraction. Furthermore, this interaction was reported following the use of a therapeutic or even, a sub-therapeutics concentration of lithium, but not rubidium. In the next chapters of this thesis, I will further investigate the decline in the time course of noradrenaline and highlight the factors which can interfere with noradrenaline-induced contraction in porcine vascular tissue. Then followed by discussing the possible mechanisms behind this interaction in chapter 5.

Chapter 4: Internal and External Factors Affecting Time Course of Noradrenaline-Induced Contraction in the Porcine Isolated Splenic Artery

4.1 Introduction

In the previous chapter, I demonstrated that in the porcine isolated splenic artery contractile responses elicited by phenylephrine and noradrenaline exhibited notable differences in the time course of responses. While both agonists activate postjunctional α_1-adrenoceptors (Wright *et al.*, 1995), cumulative concentration responses curves to noradrenaline revealed that low concentrations were not sustained, but declined 5 min after attaining a peak contraction, responses to high concentrations appeared to be better-maintained. In contrast, while responses to low concentrations of phenylephrine were relatively stable, contractions elicited by higher concentrations were characterised by rapid attainment of the peak response followed a marked decline. This profile for the responses to phenylephrine was qualitatively similar to that noted in both the sheep isolated internal anal sphincter (Rayment *et al.*, 2007) and porcine isolated urethral smooth muscle. Another notable difference between these two α_1-adrenoceptor agonists is that while the presence of 1mM LiCl enhanced contractions to phenylephrine and ensured responses to high concentrations were stable, the magnitudes and time course of contractile responses to noradrenaline were not altered.

Noradrenaline is a catecholamine derivative and this profile for contractile responses in smooth muscle preparations could arise from either tissue's factors or chemical-related factors *per se*. For example, it is well documented that smooth muscle cells are endowed with neuronal and extraneuronal metabolising systems for catecholamines (Gillespie *et al*. 1970; Iversen. 1971; Rahman *et al.*, 2008). Specifically, noradrenaline can be metabolised in smooth muscle cells by both monoamine oxidase and catecholamine oxidase and produce inactivate metabolites This effect of uptake and subsequent metabolism of noradrenaline could account for the observation that contractions to low concentrations do not maintain an equilibrium response See Figure 4.2, (Grohmann *et al.*, 1986; Grohmann and Henseling, 1988).

Alternatively, failure to maintain an equilibrium response to noradrenaline could represent either desensitisation of α_1-adrenoceptors during exposure to the agonist (Lurie *et al*., 1985; Seasholtz *et al.*, 1997) or the impact of endothelial-derived vasodilators released by activation of endothelial α_2-adrenoceptors (Miller *et al.*,1991; Liao and Homcy, 1993).

In terms of chemical factors contributing to the observations, the stability of noradrenaline as a sterile intravenous solution has been calculated to be 4 hours at room temperature (Haggendal and Johnson 1967), However, it is recognized when used for isolated tissue bath experiments at 37 $^{\circ}$C, pH 7.4 and oxygenated physiological salt solution noradrenaline is more labile. Various strategies have been used to improve the stability of noradrenaline for isolated organ bath experiments, including the inclusion of EDTA, to prevent catalytic auto-oxidation by a low concentration of heavy metal ions, or ascorbic acid (Hughes and Smith 1978). The inclusion of these agents has been shown to modify the contractile response to noradrenaline and adrenaline in hamster cremaster arterioles and the rat aorta (Maxwell *et al.* 1983). Maxwell and colleagues in (1983) argued that failure to include these agents in physiological solutions meant that catecholamines lose their effectiveness within minutes.

The main aims of this chapter are:

I. Examining in detail the influence of intrinsic tissue factors including uptake and metabolism on the concentration-response curve and time course of noradrenaline in isolated porcine tissue.
II. Examining the chemical instability of noradrenaline in Krebs Henseleit solution on the time course of contractile responses for the porcine isolated splenic artery.
III. Highlight the role of reducing oxygen tension on the stability of noradrenaline.
IV. Investigating the biological activity of noradrenaline after exposing to experimental conditions in the absences of tissue and check the desensitisation of the receptors in this preparation.

4.2 Methods and materials

4.2.1 Method

Porcine spleen from both genders was obtained from a local abattoir in iced boxes filled with Krebs Henseleit solution. Upon arrival to the lab coarse dissection performed by dissecting out 8 cm length of the porcine artery with the surrounding tissue to be stored in Krebs Henseleit solution overnight in a refrigerator at 4° C. On the next day, fine dissection was performed by removing the connective tissue and set-up the artery segments with a 4mm length in a 20ml organ bath. The segment was suspended between two steel wires; the upper attached to Force transducer while the lower was attached to a glass rode. The organ bath was filled with warm Krebs Henseleit solution at 37°C and gassed with either, 95% oxygen and 5%CO_2 or air (16% oxygen, 1% hydrogen and 78 nitrogen).

4.2.2 Protocols

Unless it is stated before, all responses were expressed as % to 60 mM KCl. After the viability of tissue being tested with three additions of KCl, cumulative additions of noradrenaline to induce a contraction were added to isolate porcine splenic artery segments. In this chapter, and following the incubation of the tissue with different concentrations of chemicals for at least 30-40 minutes, four protocols were used to record the changes in tissue responses after the addition of noradrenaline.

The first protocol was conducted by the cumulative addition of noradrenaline to the tissue starting from 0.01μM until the maximum concentration of the agonist was added at 30μM. The increase in the concentration after each addition was approximately threefold the previously concentration. The next concentration was added after equilibrium was established between the organ bath concentration and the tissue. This represents a standard way of the cumulative addition of the agonist and the majority of researchers follow this pattern as it required less time and produced acceptable response curve. In another set of experiments, another approach was followed for the cumulative addition of the noradrenaline, in which, the next addition of noradrenaline to be added only after the response to the first concentration disappeared and the tone reached the baseline.

In both of these cumulative methods, maximum response and potency of noradrenaline were calculated in different conditions. The third protocol used in this chapter was to expose the tissue to a single high concentration of the noradrenaline

and record the changes in tissue response at different time points to obtain the time course curve for the agonist responses. Finally, the fourth method was used to describe the changes in the response across different time points after the final concentration of the cumulative addition of the agonist was reached.

4.2.3 Chemicals and solution

The following concentrations of chemicals were used in this chapter: (10nM-30μM) noradrenaline tartrate tolcapone hydrochloride as catechol-O-methyl transferase (COMT) inhibitors, pargyline hydrochloride as monoamine oxidase (MAO) inhibitor, EDTA as chelating agent ascorbic acid as anti-oxidant, cocaine as inhibitors for reuptake$_1$, corticosterone as inhibitors for reuptake$_2$, propranolol hydrochloride as inhibitor for the β- adrenoceptor, L-NAME as inhibitor for nitric oxide synthase enzyme and KCl. The source and solubility of these chemicals were mentioned in chapter two, Table 2.1

4.2.4 Data analysis

The contractions elicited by drugs were expressed as a gram weight (g wt.). All the tissue responses after a cumulative addition of agonist were recorded either as a percentage of the 3rd (60mM) KCl contraction or as a percentage of the maximum response produced using Microsoft Excel software. Logistic equation (Kaleidagraph version 4.5.2 Synergy software) was used to draw the best fit curve from which the following parameters were obtained: R_{max} (maximum response to cumulative addition of an agonist), EC_{50} (concentration causing 50% of maximum responses), pD_2 (–log EC_{50}). All the results were expressed as mean ± standard error (SEM). The number of observations in different animals for each experiment was expressed as (n) and this usually ranged from 4-14 for each experiment. In all experiments, 2-tailed paired Student's *t*-test was used to compare differences between 2 groups and considered significant if the P-value was <0.05%.

4.3 Results

In this chapter, different sets of experiments were conducted to focus on the time course of noradrenaline-induced contraction in the isolated porcine splenic artery.

4.3.1 Time course for the action of some vasoconstrictors in the isolated porcine splenic artery

In order to show that the contractile response to KCl is stable and can be considered as a reference to check the tissue responses in the following experiments, an experiment was designed to add high concentration of three commonly used contractile agents, KCl 60mM, U46619 100nM and noradrenaline 30μM; the tissue responses at different time points were recorded for the following 180 minutes. As Figure 3.1 shows exposing the porcine splenic artery to KCl, U46619 and noradrenaline resulted in a contraction in which the time course for KCl and U46619 was stable over 3 hours period. In contrast, the contractions to noradrenaline declined by more than 50% over 80 min and completely disappeared by 180 min. The responses of six experiments to noradrenaline, KCl and U46619 over 80 min were 76.95±15.0%, 115.2±16.0% and 73.4±4.3 respectively of the response to 60mM KCl.

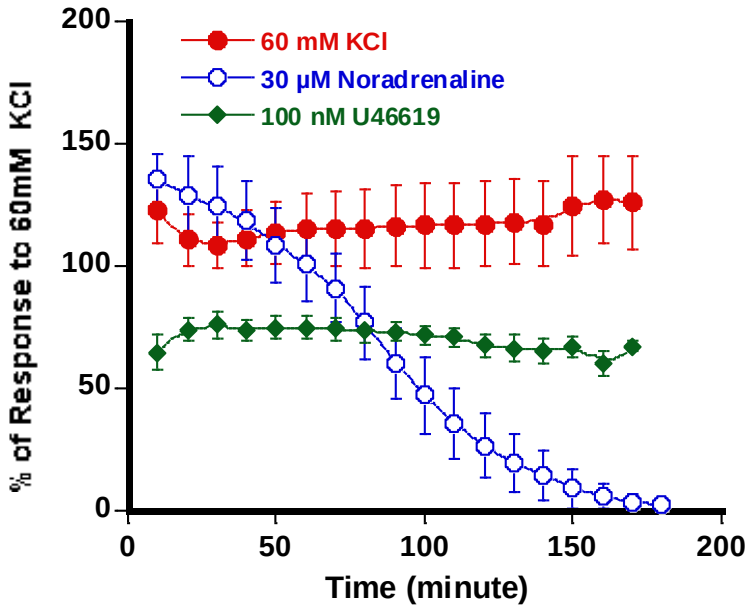

Figure 4-1 The time course of the contractile responses to KCl, U46619 and noradrenaline in the porcine splenic artery as a percentage to 60mM KCl. The magnitude of the contraction was measured at a different time point following the addition of the agonist. The value expressed as mean ± SEM (n= 6).

4.3.2 Comparison of cumulative and non-cumulative responses to noradrenaline in the porcine splenic artery

The aim of this experiment was to compare the tissue responses to stimulation with noradrenaline in two different protocols. The first was the cumulative addition of an agonist when the equilibrium was reached between drug and tissue. The second method which we used herein, was to add noradrenaline and allow it to produce the response (if there is any) and add the next concentration only when the response to the added concentration returns to baseline. Figure 4.3 shows that both protocols produced similar maximum response and potency, however, the time course for the response was markedly different in shape. See Figure 4.2 and Table 4.1

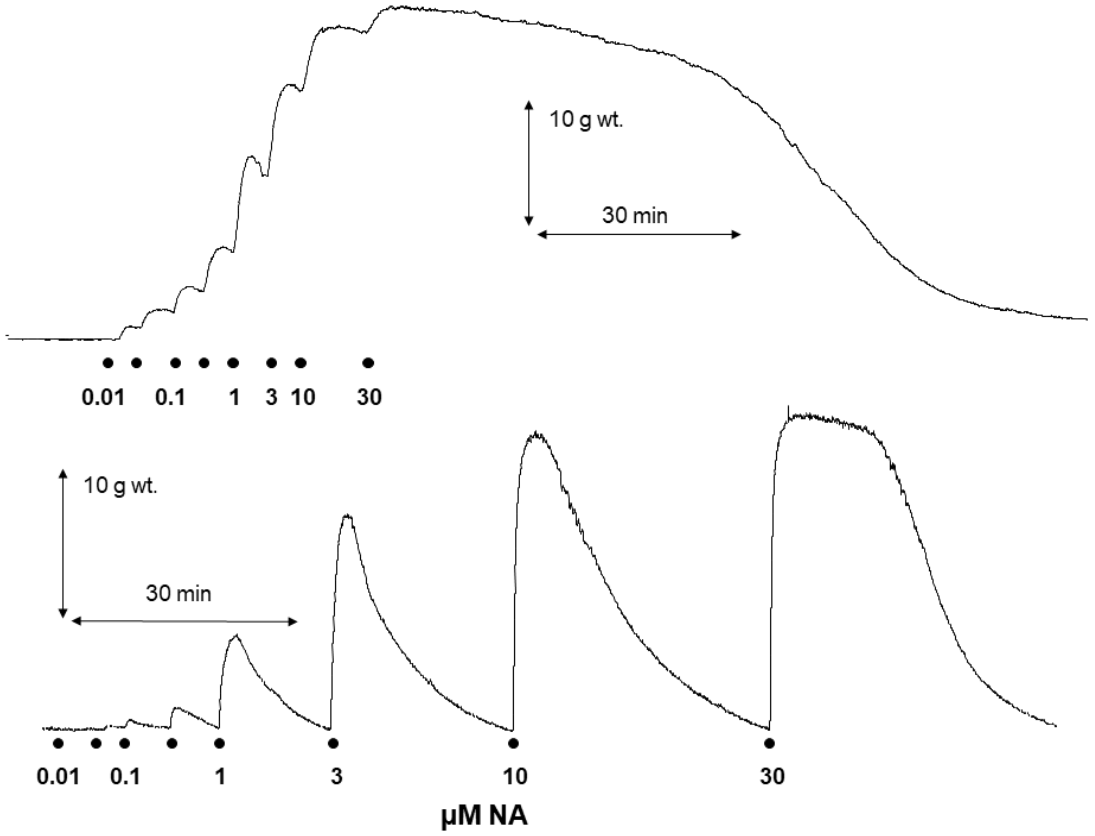

Figure 4-2 Representative traces for two different approaches to adding noradrenaline cumulatively to the isolated organ bath containing segments of the porcine isolated splenic artery. The upper trace shows the traditional method while the lower traces show the alternative method which required more time to get the response back to the baseline before the addition of the next concentration of the agonist.

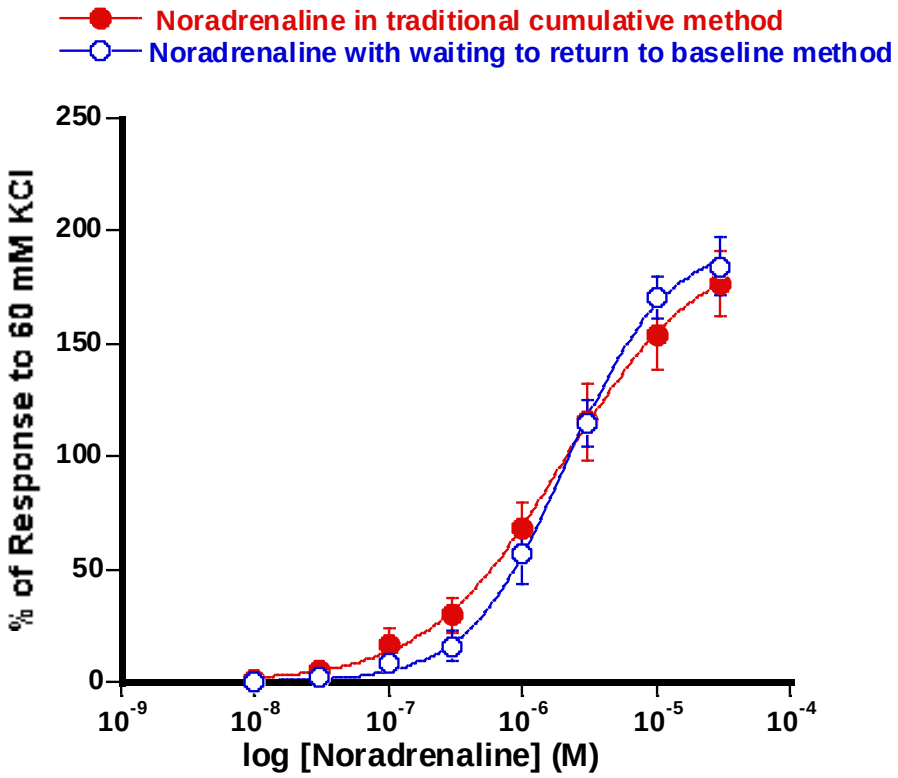

Figure 4-3 The concentration-response curve for noradrenaline-induced contraction in the porcine splenic artery following the use of two different cumulative methods to add noradrenaline. Responses expressed as % to 60mM KCl and are mean ± SEM (n=6). Please note that the concentration in the second method resulted from the addition of noradrenaline based on the notion that all of the previous drugs have been destroyed.

4.3.3 The effect of adding reuptake inhibitors on the time course of noradrenaline induces contraction in the porcine splenic artery

The cumulative addition of noradrenaline, in the presence of cocaine 10μM alone or in combination with corticosterone 30μM and propranolol 1μM produced significant changes in the concentration-response curve of noradrenaline and shifted the curve to left, in response to contraction to 60mM KCl (n=13). The potency for noradrenaline significantly changed from 5.64 ±0.09 to 5.9±0.06 with P-value <0.05 using paired Student's *t*-test following the addition of reuptake inhibitors. However, the maximum response did not change significantly. See Table 4.1 and Figure 4.4 (A)

In the same set of experiments, in the presence of cocaine 10μM alone and in combination with corticosterone 30μM and propranolol 1μM, did not produce significant changes in the time course of noradrenaline following the addition of 30μM noradrenaline. The three curves declined to less than 5% of their peak responses after 120 minutes. Responses were measured as % to 60mM KCl (n=8). See Figure 4.4 (B) and Table 4.1

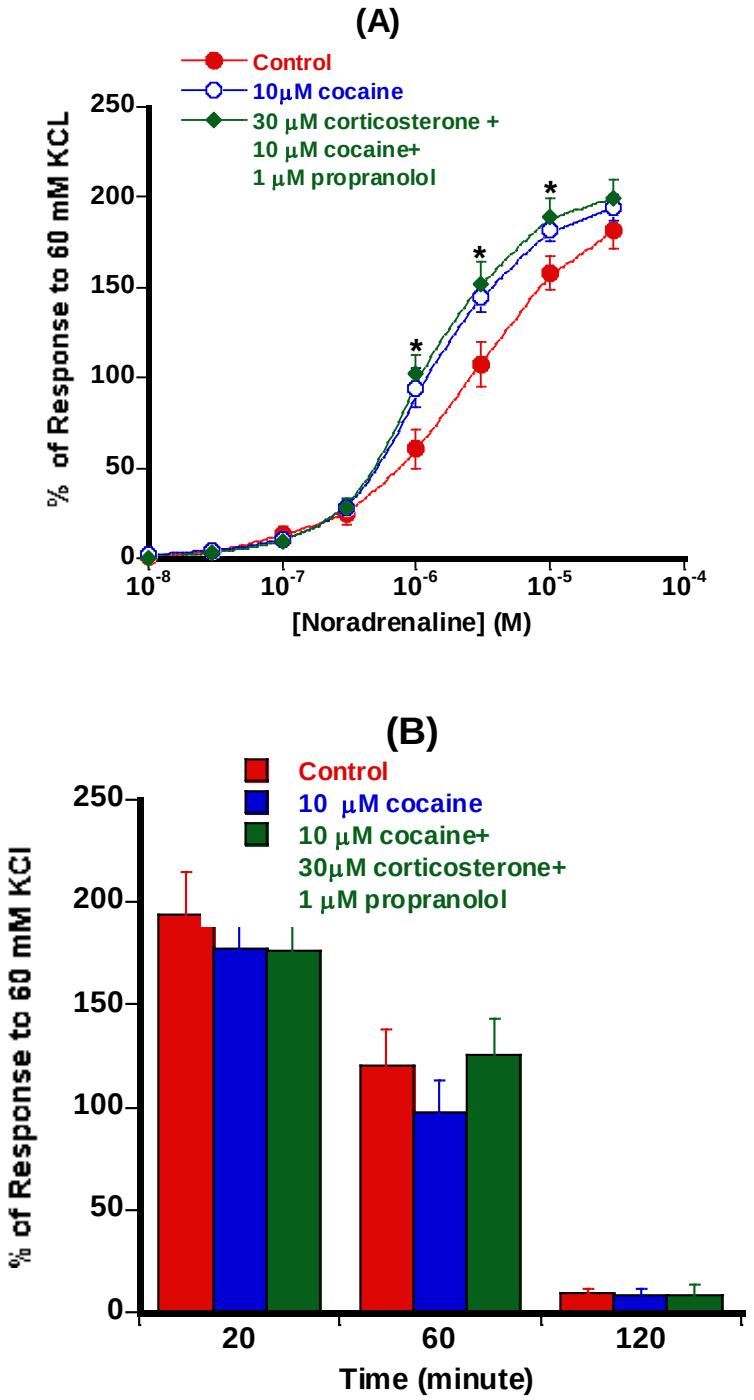

Figure 4-4 (A) The cumulative concentration-response curve (n=13) to noradrenaline in the porcine isolated splenic artery in the presence and absence of cocaine, corticosterone and propranolol. (B) The time course (n=8) of the contractile response after addition of the final concentration of noradrenaline 30μM under these conditions. Responses have been expressed as a percentage of the contraction to 60mM KCl and are shown as the mean ± SEM. The P-value was <0.05; using paired Student's *t*-test.

4.3.4 The effect of inhibiting the metabolising enzymes on noradrenaline-induced contraction in the porcine splenic artery

In the isolated porcine splenic artery Figure, 4.5 (A) shows that the presence of 50μM pargyline affected neither maximum nor the potency of response to noradrenaline (see also Table 4.1). However, the decline in contractile force after attainment of the maximum response Figure 4.5 (B) was reduced by the presence of 50μM pargyline at all-time points examined over 2 hours. The P-value was <0.05; using paired Student's *t*-test (n=14). See Table 4.1

On the other hand, incubation the tissue with 1μM tolcapone (catechol- O-methyltransferase) inhibitor did not statistically change either the concentration-response curve or the time course of contraction induced by noradrenaline in response to 60mM KCl contraction. After 9 minutes of exposure to agonist, the max responses were 152.7±6.4% (n=8); 158.3±6.4% (n=8) for the control and the tolcapone treated segments respectively. The response declined to less than 20% for both the control and the treated segments after less than120 minutes. See Figure 4.6 and Table 4.1

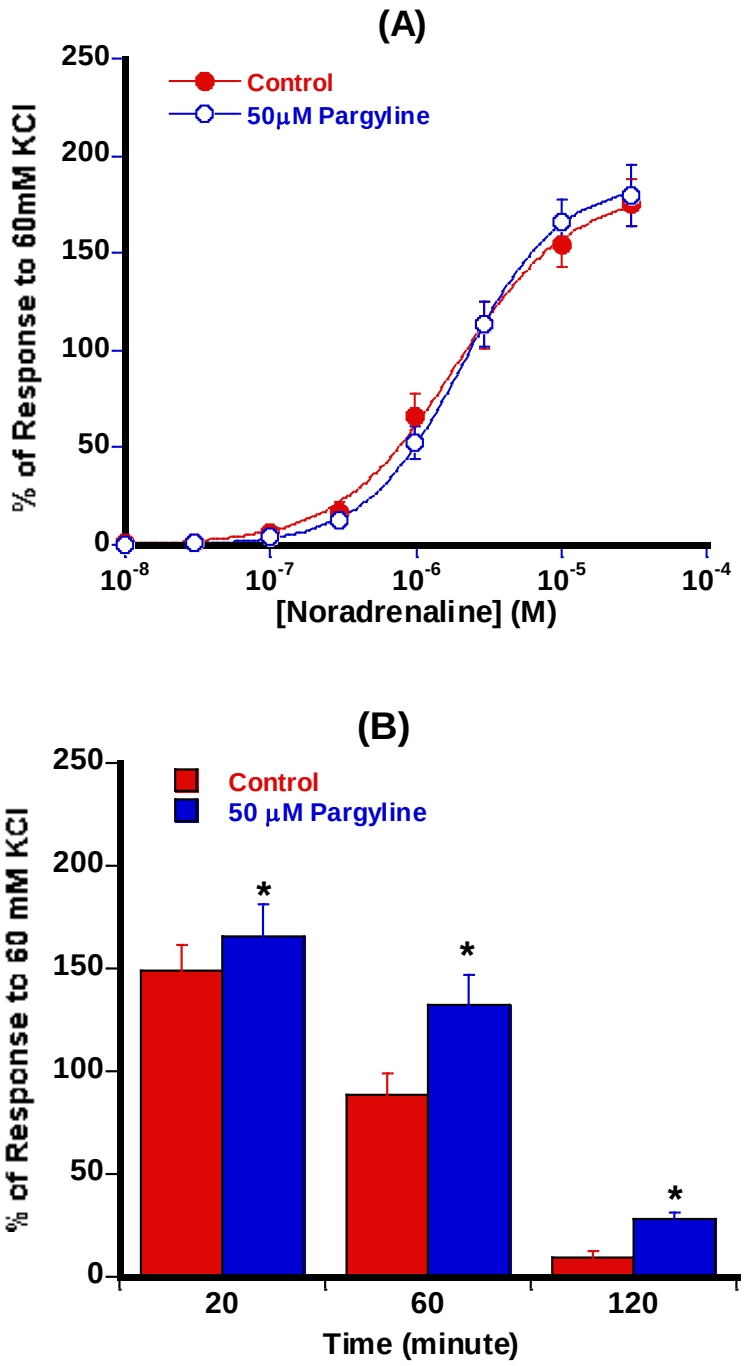

Figure 4-5 (A) Cumulative concentration-response curves to noradrenaline in the porcine isolated splenic artery in the presence and absence of 50μM pargyline. (B) The time course of the contractile response after addition of the final concentration of 30μM noradrenaline under these conditions. Responses have been expressed as a percentage of the contraction to 60mM KCl and are shown as the mean ± SEM of 14 observations with and without pargyline. The P-value was <0.05; using paired Student's *t*-test.

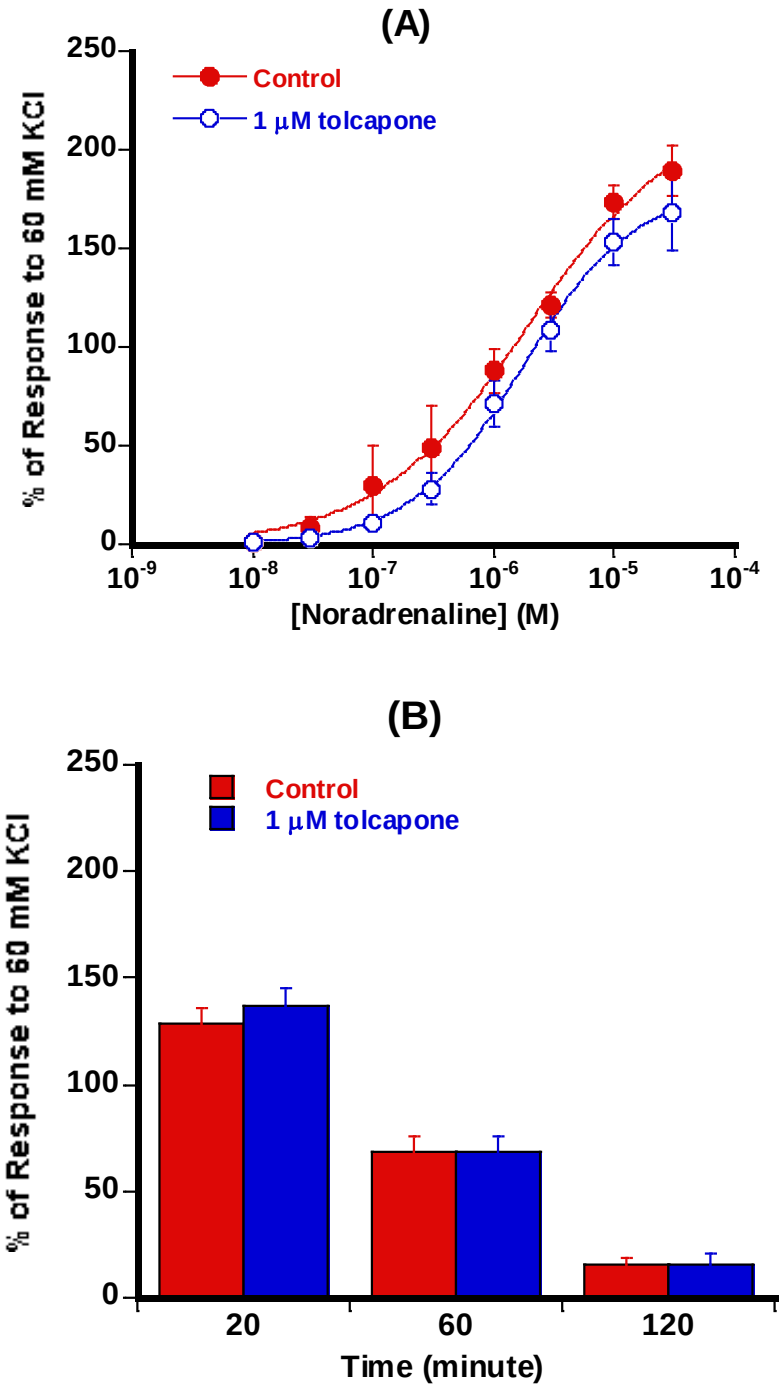

Figure 4-6 The cumulative concentration-response curve to noradrenaline in the porcine isolated splenic artery in the presence and absence of tolcapone 1μM (n=10). (B) The time course of the contractile response after addition of the final concentration of noradrenaline 30μM under these conditions (n=8). Responses have been expressed as a percentage of the contraction to 60mM KCl and are shown as the mean ± SEM with and without tolcapone.

4.3.5 The effect of adding ascorbate and EDTA on the time course of contraction induced by noradrenaline in the porcine splenic artery

The presence of EDTA 10μM or ascorbic acid 50μ did not produce significant changes in the concentration-response curve of noradrenaline. Neither the potency nor the maximum response was affected by the presence of these agents. See Table 4.1 and Figure 4.7 (A).

The measurements of the responses were taken after the tissues reached the maximum effect in response to 60mM KCl. The results of 6 experiments showed that after 10 minutes the responses were 194.7±16.1%, 189.1±10.7% and 183.2±18.5% for the control, EDTA and ascorbic acid respectively. While after 140 minutes, ascorbate only maintained the response in statistically significant value at 118.7±14% in comparison with control and EDTA in which the responses were 70.2±6.5%, 75.4±6.7% respectively. Using a paired Student's *t*-test. See Figure 4.7 (B) and Table 4.1

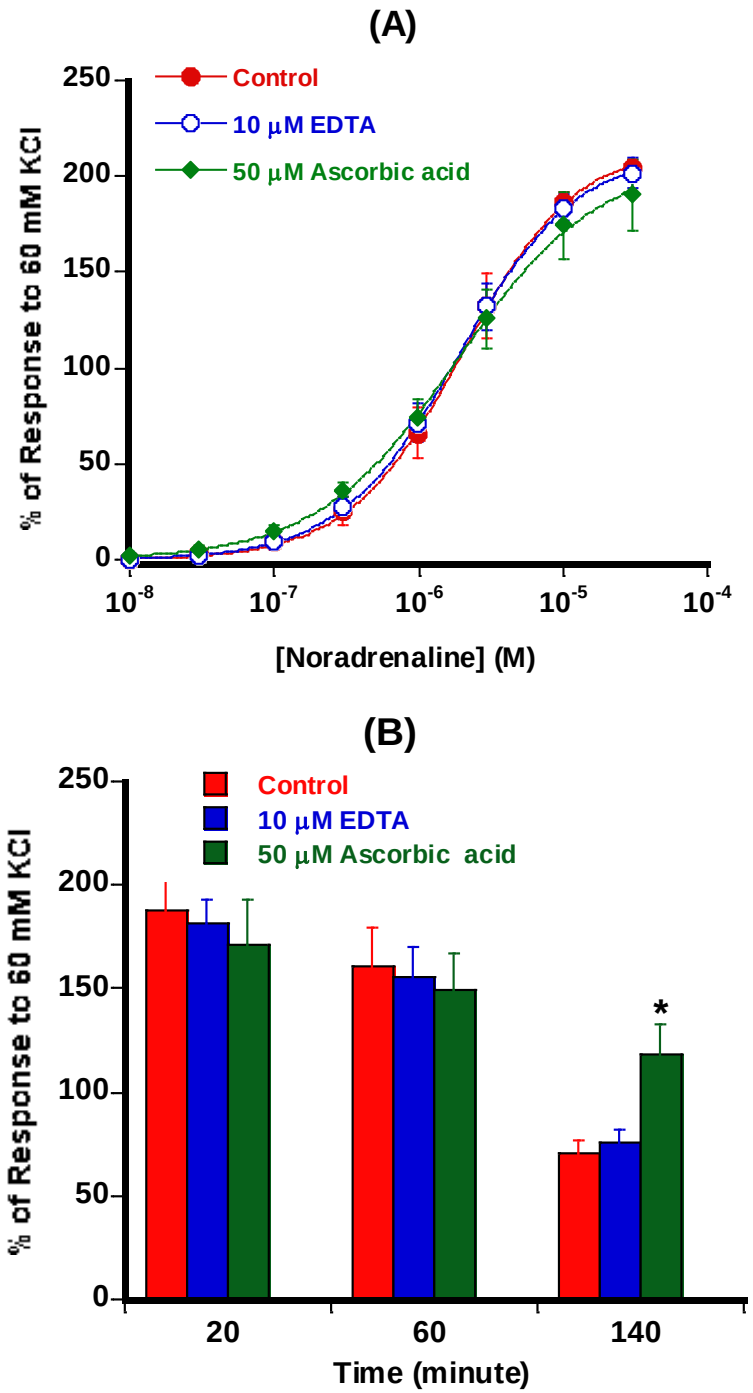

Figure 4-7 (A) The cumulative concentration-response curve to noradrenaline in the porcine isolated splenic artery in the presence and absence of EDTA 10µM and ascorbate 50µM. (B) The time course of the contractile response after addition of the final concentration of noradrenaline 30µM under these conditions. Responses have been expressed as a percentage of the contraction to 60mM KCl and are shown as the mean ± SEM of 6 observations. The P-value was <0.05; using paired Student's *t*-test.

4.3.6 Check the desensitisation and bioassay of noradrenaline in the organ bath after the concentration of noradrenaline return to baseline

In this experiment, we have examined the bioactivity of noradrenaline where it was incubated within the bath chamber without tissue and test its biological activity on the tissue if added afterwards. Taking 2ml volume (i.e. 3μM) from the bath where 30μM noradrenaline previously added and gassed with 95% oxygen for 120 minutes failed to induce any biological response in the tissue while adding a fresh 3μM noradrenaline from a fresh stock induced reproducible responses comparable to the responses induced before regarding its magnitude and duration. It was noticed that the colour of the organ bath solution was changed from colourless to pink after two hours in the presence and absences of the tissue. See traces in Figure 4.8.

In a different experiment, a comparison in the solution colour was conducted following gassing the system with two different concentrations of oxygen (95% and 16%), the results of this experiment demonstrated that the higher the concentration of the oxygen the darker coloured in the bathing solution if similar concentrations of the drug was used initially. see Figure 4.9.

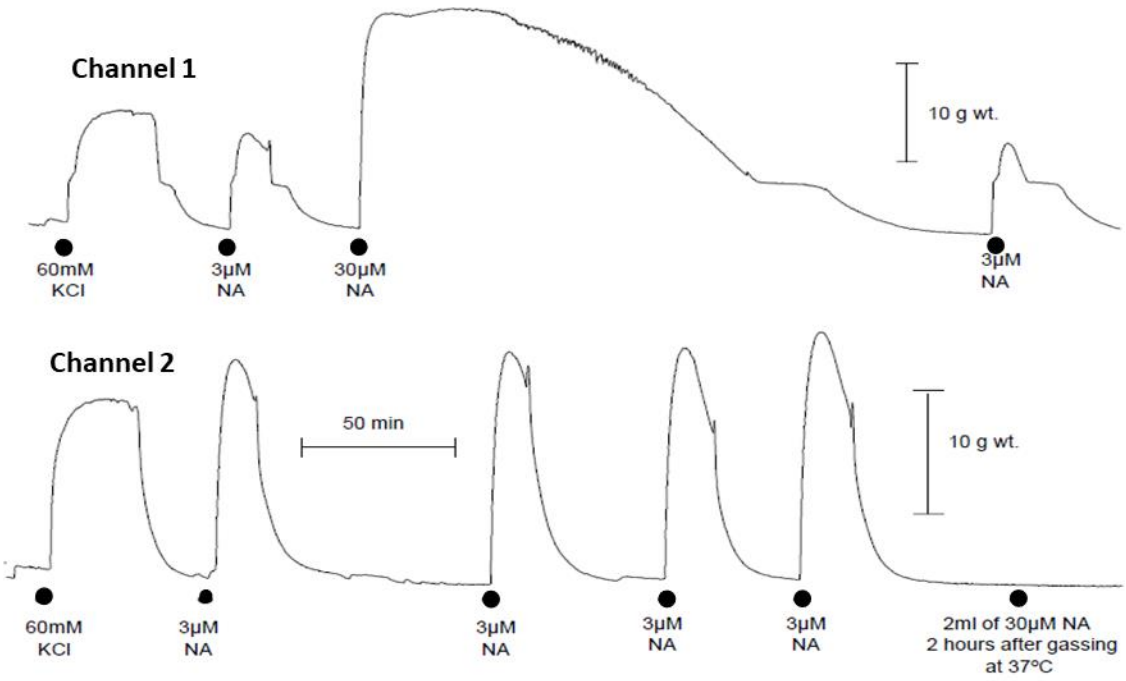

Figure 4-8 Channel 1, shows a standard response to 60mM KCl, followed by a contraction elicited by 3μM noradrenaline. Noradrenaline was washed from the bath after 10 minutes. Thereafter, the preparation was exposed to 30μM noradrenaline for 180 minutes during this time the large contractile response declined back to baseline. 15 minutes later the addition of 3μM noradrenaline, produced a response similar in magnitude to that produced by at the beginning of the experiment. Prior to the addition of 3μM noradrenaline to channel1, 2ml of Krebs Henseleit solution was removed and kept at 37°C. Channel 2, shows that intermittent exposure of porcine splenic artery to 3μM noradrenaline over 2 hours elicited a response. However, the addition of 2 ml of Krebs Henseleit solution (containing 30μM noradrenaline) to Channel 2 failed to elicit a response.

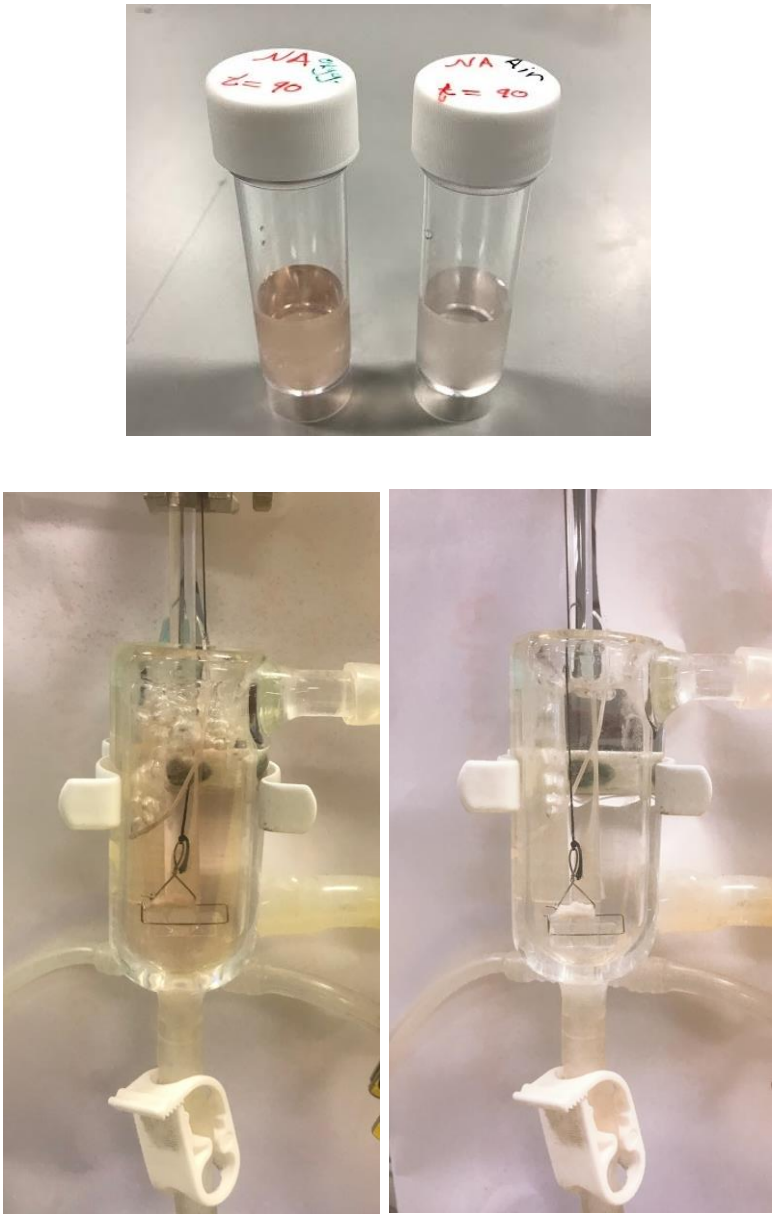

Figure 4-9 Pictures show the difference in the colour of the buffer solution after being gassed with oxygen 95% (left) and air which contained 16% oxygen (right) over 90 minutes following the addition of 30μM noradrenaline to segments of the porcine splenic artery.

4.3.7 The effect of changing oxygen tension of the time course of contraction induced by noradrenaline in the porcine splenic artery

In this group of experiments, we examined the concentration-response to noradrenaline in the presence of 95%O_2; 5%CO_2 and/or air (78% N_2; 16%O_2; 5%CO_2). Figure 4.10 (A) and Table 4.1 show the concentration-response curve for noradrenaline did not significantly change by changing the gassing system except when the maximum concentration of noradrenaline was used (n=8).

On a different group of experiments, the concentration of noradrenaline after 120 minutes was 80.5±8.9%, 10.7±1.8% of the response to 60mM KCl contraction in the air, and oxygen aerated system respectively following the single addition of 30μM noradrenaline. The time course significantly changed. The use of air mix prolonged the duration for the contraction induced by noradrenaline, the P-value was <0.05; using paired Student's *t*-test (n=6). See Figure 4.10 (B) and Table 4.1

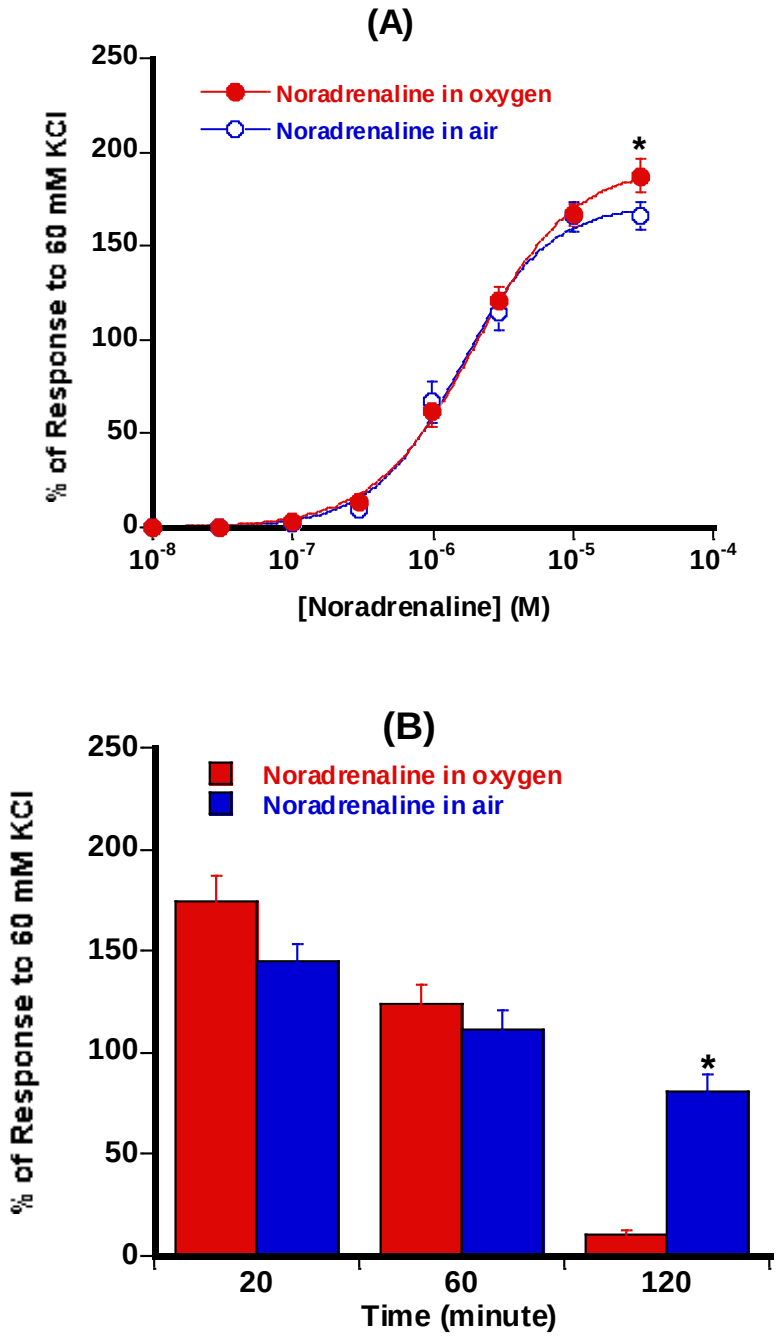

Figure 4-4 (A) The cumulative concentration-response curve to noradrenaline in the porcine isolated splenic artery gassed with oxygen and air (n=6). (B) The time course of the contractile response after addition of the final concentration of noradrenaline 30μM under these conditions (n=8). Responses have been expressed as a percentage of the contraction to 60mM KCl and are shown as the mean ± SEM in oxygen and air gassed system. The P-value was <0.05; using paired Student's *t*-test.

Table 4-1The maximum response and pD_2 values for noradrenaline in different experimental conditions at different time point. The values shown represent the mean ± SEM. * represent a statistically differences from control and P-value <0.05 while ** indicating a P-value < 0.001 using paired Student's *t*-test.

Condition noradrenaline (NA)	**Potency - pD_2**	**R_{max} (% of 60mM KCl)**	**50-60min (% of 60mM KCl**	**120-140min (% of 60mM KCl)**
NA cumulative (n=6)	5.69±0.16	211.4±13.6	Not available	Not available
NA non-cumulative	5.60±0.07	200.4±10.2	Not available	Not available
Control (n=13)	5.64±0.09	205.4±12.7	120.1±17.3	9.7±1.5
10μM Cocaine	5.9±0.06*	196.8±7.4	97.7±15.7	7.0±2.75
10μM Cocaine 30μM Corticosterone	5.9±0.06*	200.1±9.7	125.2±18.4	5.04±3.4
Control (n=6)	5.7±0.11	212.8±4.3	161.2±18.2	70.2±6.5
50μM AA	5.7±0.06	210.3±16.5	149.2±17.7	118.7±14.1*
10μM EDTA	5.7±0.09	210.5±5.7	155.8±14.5	75.4±6.7
Control (n=14)	5.81±0.13	181.2±13.1	88.6±10.6	9.4±2.7
50μM Pargyline	5.76±0.06	186.3±15.5	132.0±14.6*	27.7±4.1*
Control (n=10)	5.88±0.07	204.1±14.4	68.1±7.6	4.4±1.2
1μM Tolcapone	5.84±0.10	194.5±15.8	68.3±7.9	9.5±3.9
Control (n=8)	5.82±0.08	194.4±11.9*	124.1±9.0	10.7±1.8
95% Air/5%CO_2	5.71±0.08	169.3±8.6	111.1±9.5	80.5±8.9 **

4.4 Discussion

The basis of the work in this chapter was the observation in porcine vascular smooth muscle that contractions elicited by noradrenaline and phenylephrine, agents thought to work at the same target, α_1-adrenoceptors, produce a response with markedly different time courses. In light of the known propensity of noradrenaline to the breakdown in aqueous solution, or to be metabolised by smooth muscle and nerves in blood vessels, we examined in more details the effect of various interventions designed to shed light on the surprising lability of responses to noradrenaline.

The main finding in this chapter is that noradrenaline elicits a contraction in the porcine splenic artery through the stimulation of α_1-adrenoceptors. This contraction is not sustained and declines with time in contrast to the contraction induced by KCl or thromboxane mimetic agonist U46619. This decline is due to the auto-degradation of the noradrenaline with a minimal role of the external factors which can interfere with the magnitudes or the duration of action to noradrenaline. The effects of inhibiting the metabolism or oxidation did not prolong the duration of action significantly. Furthermore, the blocking of reuptake mechanisms significantly shifted the concentration-response curve to the left and only the use of low oxygen tension in the experimental procedures was found to delay the degradation of noradrenaline which loses its biological activity without causing desensitisation to α_1-adrenoceptors in the tissue.

The finding that contractions to 60mM KCl and U46619, agents thought to work by opening voltage-sensitive calcium channels or activating thromboxane receptors, respectively (Coleman *et al.*, 1981; McKenzie *et al.*, 2009; Ratz *et al.*, 2005; Kawaguchi *et al.*, 2010). Responses were stable over a three-hour period illustrates that the decline in response to noradrenaline is not simply a function of the smooth muscle. Rather, it is something specific to noradrenaline, either in terms of 'handling' by the blood vessel or the chemical properties in solution.

Our results showed that the contractions induced by U46619 and KCl are stable. Hence, we have used KCl as a reference to standardise the contraction induced by all contractile agents. In addition, the noradrenaline produced a contractile response which wanes with time (Figure 4.1), this is in line with previous studies (Denfria., 2015) which demonstrate that the contraction induced by cumulative addition to noradrenaline was not sustained.

In this study, we used two different methods for the cumulative addition of noradrenaline and compared between them. The first was the standard method of cumulative addition, to generating concentration-response curve in which after adding the concentration of the drug we wait 5-10 minutes, or what we assumed as equilibrium point, and then add the next concentration. In the alternative non-cumulative method and because we could not detect a stable response following the addition of low concentration of noradrenaline, we waited for the response to go back to baseline before we added the next concentration (Figure 4.2). The advantage of the second method is that you can observe the stability and the time of onset for each concentration. Many researchers tend to use the standard method to generate an attractive curve without paying attention to the other facts that result from investigating the trace of the response such as the onset and time course to the response. In contrast, in this thesis, we could argue that the involvement of the traces can help to better understand the response of the tissue following stimulation with different agonists.

These results are in contrast to what was previously reported with (Lurie *et al.*, 1985). They have reported that prolonged exposure to a high concentration to α_1-adrenoceptors agonist resulted in desensitisation of the receptors and loss of response. However in this study, the exposure time was about 7 hours during it, the bathing solution was replaced three times and fresh stock of the agonist was included in every time so we can predict that the receptors in the tissue were overstimulated with a high concentration of the agonist and for a prolonged period.

Different factors can affect the noradrenaline response in porcine splenic artery. Some of these factors are tissue related, such as the neuronal and extraneuronal uptake (Gillespie *et al.* 1970; Iversen. 1971; Rahman *et al*. 2008) which can in addition to the metabolism mechanism affecting the response to noradrenaline in *in vitro* study in smooth muscle (Grohmann *et al.,* 1986; Grohmann and Henseling 1988).

In this study, we have found that blocking the uptake mechanisms by using cocaine, corticosterone and propranolol (the latter use to block the possible activities of β-adrenoceptors by noradrenaline) significantly shifted the concentration-response curve to the left and enhanced the potency of noradrenaline (Figure 4.3). This inline with other studies which reported the same (Denfria 2015). In addition to that, blocking the metabolism of noradrenaline by monoamine oxidase and catechol –O-methyltransferase enzymes by using pargyline and tolcapone respectively (Trendelenburg 1974; Truong 2009) only produced significant enhancement in the

time course following the addition of high concentration of noradrenaline, no changes in the pD_2 nor R_{max} were reported (Figure 4.3 and 4.4).

In order to confirm the intrinsic role of noradrenaline and to enhance its stability in isolated organ bath solution EDTA (Dillon *et al.,* 2004) and ascorbic acid *(*Maxwell *et al.,* 1983) were added to the solution. According to the previous studies, these agents act as protectors from the chemical degradation of noradrenaline solution as they are binding with the heavy metals present in the medium and reduce their detrimental effect on the stability of catecholamines.

Our results showed that the use of EDTA has no role in maintaining the stability of noradrenaline and changed neither the concentration-response curve nor the time course of the contraction induced in porcine splenic artery. In addition to that, the presence of ascorbic acid only enhanced the time course and maintained the response after 140 minutes (See Figure 4.6). Similarly, Maxwell and his group in 1983, reported that without the addition of ascorbate, the catecholamine solution loses its effect within minutes (Maxwell *et al.,* 1983). Moreover, Dillon and his group, in 2004 reported that ascorbate has a profound effect on the magnitude of contraction initiated by catecholamines solutions in rabbit aorta strips. They reported that the use of ascorbate in a concentration> 15μM enhanced the contraction induced by 100nM noradrenaline by more than two-fold while the use of ascorbate failed to enhance the contraction induced by angiotensin II and solution of KCl (Dillon *et al.,* 2004).

The other possible reason behind the decline in response following the stimulation with catecholamines was the auto-oxidation of the compound which is previously reported in many types of research (Furchgott 1956; Hughes and Smith 1978; Alvarez-Diduk and Galano 2015). It was suggested that the stability of noradrenaline solution is about 4 hours at room temperature, however, the stability is markedly reduced in isolated tissue bath experiments at 37°C, pH 7.4 and oxygenated physiological salt solution. This could be the fact that continuous gassing the system with high oxygen tension can accelerate the rate of chemical degradation (Hughes and Smith 1978). In their study, Kitto and Bohr measured the biological activity of catecholamine in bathing solution in the presence and absence of tissue. They have reported that incubating adrenaline in Tyrode's solution gassed with oxygen at 37°C in the absences of tissue preserved the biological activity for less than three minutes without the using of potentiators (Kitto and Bohr 1953).

Stimulation of α_1-adrenoceptors with a sympathomimetic agonist such as noradrenaline can produce contraction in smooth muscle cells through stimulation of phosphatidylinositol turn over (Tsujimoto *et al.*, 1987). The desensitisation of α_1-adrenoceptors was previously reported in many studies (Docherty *et al*., 1981; Seasholtz *et al*., 1997). This type of desensitisation is not related to AC activity and hence other suggested mechanisms were proposed such as blunting of the agonist activity to stimulate the α_1-adrenoceptors or reducing the number of α_1-adrenoceptors at the cell membrane (Lurie *et al*., 1985).

Our results showed that the addition of noradrenaline 3μM was still able to induce reproducible contractions in porcine splenic artery segments even if previously exposed to a maximum concentration of the agonist noradrenaline 30μM for 120 minutes. This might exclude any role of receptor desensitisation in the system. In addition to that, we also reported that the addition of noradrenaline 3μM which was incubated within a bath and gassed with oxygen 95% at 37 C° failed to initiate a response. This suggested that noradrenaline undergo a chemical degradation and lost its biological activity with time and can be noticed by the change in the colour of the bath to pink. See Figure 4.7 (C).

In line with our results, Lurie *et al*. (1985), reported that noradrenaline failed to initiate contraction in the smooth muscle of rabbit aorta previously incubated with adrenaline for 7 hours before being washed. The EC_{50} for noradrenaline was increased by 10-fold in the pre-treated segments 310±190nM compare with the control segments which have an EC_{50} value at 35±14nM. However, they attributed this failure to induced contraction to the desensitisation of α_1-adrenoceptors to the action of noradrenaline following the prolonged exposure rather than to chemical degradation of noradrenaline. This could be due to the fact; they replaced the buffer solution in the bath every two hours and added a fresh concentration of noradrenaline with it. The other difference was the exposure time in our protocol was 120 minutes while in their experiment 7 hours was used to incubate the tissue with the noradrenaline.

All the preceding experiments with a maximal concentration of noradrenaline have shown that contractile responses to this agonist decline to baseline over two hours and this were associated with a colour change in the bathing solution. This suggested that the agonist is destroyed rather than desensitisation of the receptors. Confirmation of this suggestion is provided by the results in Figure 4.8 Channel 2, which shows that even after prolonged exposure to noradrenaline 30μM and the response declined to

baseline, subsequent addition of noradrenaline 3μM elicited a response comparable to that at the beginning of the experiment.

In order to confirm that the loss in the activity of noradrenaline was due to degradation of the drug rather than a desensitisation in α_1-adrenoceptors, we design an experiment in which we gassed a bath that contained a high concentration of noradrenaline with oxygen for 90 minutes in the absence of tissue and then used this solution to initiate a contraction to the isolated porcine splenic artery. Our results showed that no biological activity was detected in the isolated segment following the addition of the solution. In contrast, the use of fresh solution from stock was able to produce a contraction even following the previous exposure to the high concentration of noradrenaline (Figure 4.8) which exclude any possible role for desensitisation of α_1-adrenoceptors in this preparation.

Interestingly, our results showed that using less oxygen tension by replacing the gassing system with air rather than 95% oxygen significantly enhanced both the time course and the maximum response to noradrenaline. The reduction in the rate of noradrenaline destruction can be easily detected by comparing the changes in the colour in the bath after 90 minutes. This change in the colour known as auto-oxidation, which was previously reported as an indicator of the chemical degradation of catecholamines (See Figure 4.10).

In summary, in this chapter we have reported that the response to noradrenaline was not stable and declined with time, this decline less affected by tissue factors and it was more related to auto-oxidation. The use of cocaine and corticosterone as reuptake inhibitors enhanced the potency for noradrenaline without affecting the time course for the contraction. In addition, no role for the addition of EDTA was detected while the presence of ascorbate maintained the time course slightly. Furthermore, the stability of noradrenaline solution was reduced in the experimental condition involving high oxygen tension to gas the system. This suggests that using air mixture or lower the oxygen tension could probably decline the destruction and prolong the time course for the contraction induced by noradrenaline in porcine splenic artery.

Chapter 5: Examining the Suggested Mechanisms for the Interaction of LiCl and α_1-Adrenoceptor Agonists in Porcine Isolated Splenic Artery

5.1 Introduction

In the preceding chapters, I have established that contractions produced by phenethylamine-based agonist selective for α_1-adrenoceptors are either enhanced or maintained by the presence of lithium ions. The concentrations at which this happen is within the therapeutic range for lithium when used for the treatment of bipolar conditions. Surprisingly this effect of lithium was not observed against the endogenous ligand for α_1-adrenoceptors, noradrenaline or the imidazoline derivatives cirazoline. In the majority of experiments conducted in this chapter, phenylephrine was used while in some of the experiments L-erythromethoxamine or metaraminol were used interchangeably.

The characteristic feature of the response to α_1-adrenoceptor agonist enhanced by lithium is that under control condition the response to high concentrations was not sustained and decline by more than 70% over an hour or less. This type of contractile response is qualitative with other GPCR agonist e.g. carbachol, 5HT, angiotensin II, histamine. Though only the former there is any evidence that lithium produces a similar enhancing effect, one possible explanation for this type of response is a result of receptor desensitisation (Perez *et al.*, 2013) involving receptor internalisation (Lurie *et al*., 1985).

Perez and colleagues (2013) reported that exposure of HEK293 cells to 100μM phenylephrine was associated with the internalisation of fluorescence labelled α_1-adrenoceptors a process that commenced with 5 minutes. This time course of this movement of receptors coincided with a similar reduction in the associated signalling mechanisms. This effect was seen with all α_1-adrenoceptors subtypes but in the case of the α_{1A-} exposure to concanavalin A inhibitors of endocytosis, receptors internalisation was inhibited (Perez *et al.*, 2013). In light the report of Barbieri, that the porcine splenic artery possesses predominantly α_{1A}-adrenoceptors (Barbieri *et al.*, 1998). I have examined in more details the characteristic of the response to phenylephrine and whether there is potential to influence responses to concanavalin A. As far as I am aware no previous study has examined the effect of concanavalin A, on contractile responses in vascular smooth muscle.

Numerous early reports concerning the action of lithium on excitable tissue have focused on membrane-related effects, particularly in relation to the activity of Na^+ /K^+ ATPase (Dunham and Senyk 1977), thus I have examined the time course of the effect of lithium ions in modulating α_1-adrenoceptor agonist with emphasis on whether the

action is principally intracellular or extracellular. Lithium influx into the cell through two major pathways, Na^+-proton exchanger and epithelial Na^+ channels both are sensitive to amiloride (Bedford *et al.*, 2008)

Finally, as detailed in the introduction of this thesis, some of the early targets for lithium are enzymes especially inositol monophosphatase (IMPtase) (Berridge *et al*., 1982) and glycogen synthase kinase (GSK) (Klein and Melton 1996). These potential targets are generally inhibited in an uncompetitive way by lithium mainly by displacing the divalent cation (Corbella and Vieta 2003). I have explored whether the effect related in chapter3 is mimicked by organic inhibitors of (IMPtase) and (GSK) in isolated porcine vascular smooth muscle.

In order to better understand this interaction, and since lithium influx into the cell through two major pathways, Na^+-proton exchanger and epithelial Na^+ channels both are sensitive to amiloride (Bedford *et al.*, 2008). We plan some experiment that aimed to detect the site of action of lithium ions. Does it need to be inside the cell in order to enhance the magnitude and the time course for some α_1-adrenoceptor agonist?

The work described herein is mainly focused on the following points:

I. Examine the role of desensitisation and internalisation of receptors following the stimulation with phenylephrine in porcine splenic artery.
II. Examine the effect of adding amiloride on the interaction of LiCl with phenylephrine.
III. Compare the effect of lithium with Na^+-K^+ pump ATPase inhibitor (ouabain) on the response of the porcine isolated splenic artery to phenylephrine.
IV. Compare the effect of lithium with IMPtase and GSK3 inhibitors on the time course response of the porcine isolated splenic artery to L-erythromethoxamine and phenylephrine.
V. Examine the effect of LiCl on the potency and magnitude of contractile agents in porcine splenic artery.

5.2 Materials and methods

5.2.1 Methods

Porcine spleen from both genders was obtained from a local abattoir in iced boxes filled with Krebs Henseleit solution. Upon arrival to the lab coarse dissection was performed by dissecting out 8 cm length of the porcine artery, with the surrounding fat and connective tissue and then stored overnight in Krebs Henseleit solution at 4°C. On the next day, fine dissection was performed by removing the connective tissue and set-up the artery segments with a 4mm length in a 20ml organ bath. The segment was suspended between two 0.4 mm steel wires; the upper attached to Force transducer connected to AD Instruments Quad Bridge pre-amplifier unit while the lower was attached to a glass rode. The organ bath was filled with warm Krebs Henseleit solution at 37°C and gassed with 95% oxygen and 5% CO_2.

5.2.2 Protocols

After 40-50 minutes equilibration in Krebs Henseleit solution, segments were placed under 10g wt. tension and allowed to relax over 40 minutes. Unless it is stated before, all responses were expressed as a percentage to the contraction to 60mM KCl. After the viability of tissue being tested with three additions of KCl, cumulative additions of an agonist to induce a contraction were added to isolate porcine splenic artery segments. In this chapter, and following the incubation of the tissue with different concentrations of chemicals for at least 30-40 minutes, three protocols were used to record the changes in tissue responses after the addition of an agonist.

The first protocol was conducted by the cumulative addition of the agonist to the tissue started from 0.01μM until the maximum concentration of the agonist was added at 30μM. The increase in the concentration after each addition was three logs of the previously added concentration. The next concentration was added after equilibrium was established between the organ bath solution and the tissue. In some experiments associated with desensitisation, another concentration-response curve was produced using the same tissue following wash and waiting for 60 minutes before applying either similar or different contractile agent.

The second protocol used in this chapter was exposing the tissue to a single high concentration of the agonist and record the changes in tissue response at different time points to obtain the time course curve for the agonist responses.

The third protocol involved, keeping the tissue after it reached its maximum response to the cumulative addition of the agonist and recorded the response at different time points for the following 60-120 minutes.

5.2.3 Chemicals and solution

The following chemicals were used in this chapter: noradrenaline tartrate, L-erythromethoxamine hydrochloride, metaraminol bitartrate, phenylephrine hydrochloride, cirazoline hydrochloride. In addition to L690, 488 (1-[(4-Hydroxyphenoxy) ethylidene] bis [phosphinylidyne bis (oxymethylene)]-2, 2-dimethylpropanoate) and L690, 330 ([1-(4-Hydroxyphenoxy) ethylidene] bisphosphonic acid) as inositol monophosphatase inhibitors. SB216367 (3-(2, 4-Dichlorophenyl)-4-(1-methyl-1H-indol-3-yr)-1H-pyrrole-2, 5-dione as glycogen synthase 3 inhibitor. Rubidium chloride and lithium chloride. Amiloride hydrochloride as Na^+ channel blocker. Concanavalin A from *Canavalia ensiformis* (Jack bean) as internalisation blocker. Ouabain octahydrate as Na^+/K^+-ATPase ion pump blocker. A61603 hydrobromide, U46619 as thromboxane receptor stimulant, Egtazic acid, Ethylene-bis tetraacetic acid, glycol ether diamine tetraacetic acid (EGTA) as calcium chelating agent. The manufacture and solubility of these chemicals were previously described in Table 2.1.

5.2.4 Data analysis

The contractions elicited by drugs were expressed as a gram weight (g wt.). All the tissue responses after a cumulative addition of agonist were recorded either as a percentage of the third 60mM KCl contraction or as a percentage of the maximum response produced using Microsoft Excel software. Logistic equation (Kaleidagraph version 4.5.2 Synergy software) was used to draw the best fit curve from which the following parameters were obtained: R_{max} (maximum response to cumulative addition of an agonist), EC_{50} (concentration causing 50% of maximum responses), pD_2 (–log EC_{50}) or potency. All the results were expressed as mean ± standard error (SEM). The number of observations in different animals for each experiment was expressed as (n) and this usually ranged from 4-14 for each experiment. In all experiments, 2-tailed paired Student's *t*-test was used to compare differences between the two groups and considered significant if the P-value was <0.05%.

5.3 Results

5.3.1 The effect of LiCl on the desensitisation of adrenergic receptors in vascular smooth muscle

In this group of experiments, we identified the effect of adding LiCl before double exposure to the cumulative addition of phenylephrine. Therefore, we used a Krebs solution that contains LiCl to wash in the LiCl treated segments before the second exposure to the cumulative addition of phenylephrine.

Table 5.1 and Figure 5.1 show that the second exposure of the tissue to the concentration-response curve of phenylephrine produced slightly drop in the R_{max} in the absence of LiCl. In contrast, the presence of LiCl enhanced tissue response to the second stimulation of tissue with a significant drop in the potency.

Table 5-1 Tissue responses to two cumulative addition of phenylephrine (PE1, PE2) in the presence and absence of 1mM LiCl in a porcine splenic artery, n=10. * - Denotes significant differences between mean responses ($p < 0.05$; paired Student *t*-test).

	R_{max}	**pD_2**	**n number**
PE 1	134.3±16.5	6.5±0.15	10
PE 2	113.2±17.6	6.4±0.1	10
PE 1+Li	221.4±19.9*	6.2±0.1*	10
PE 2+Li	244.7±18.8*	6.2±0.09*	10

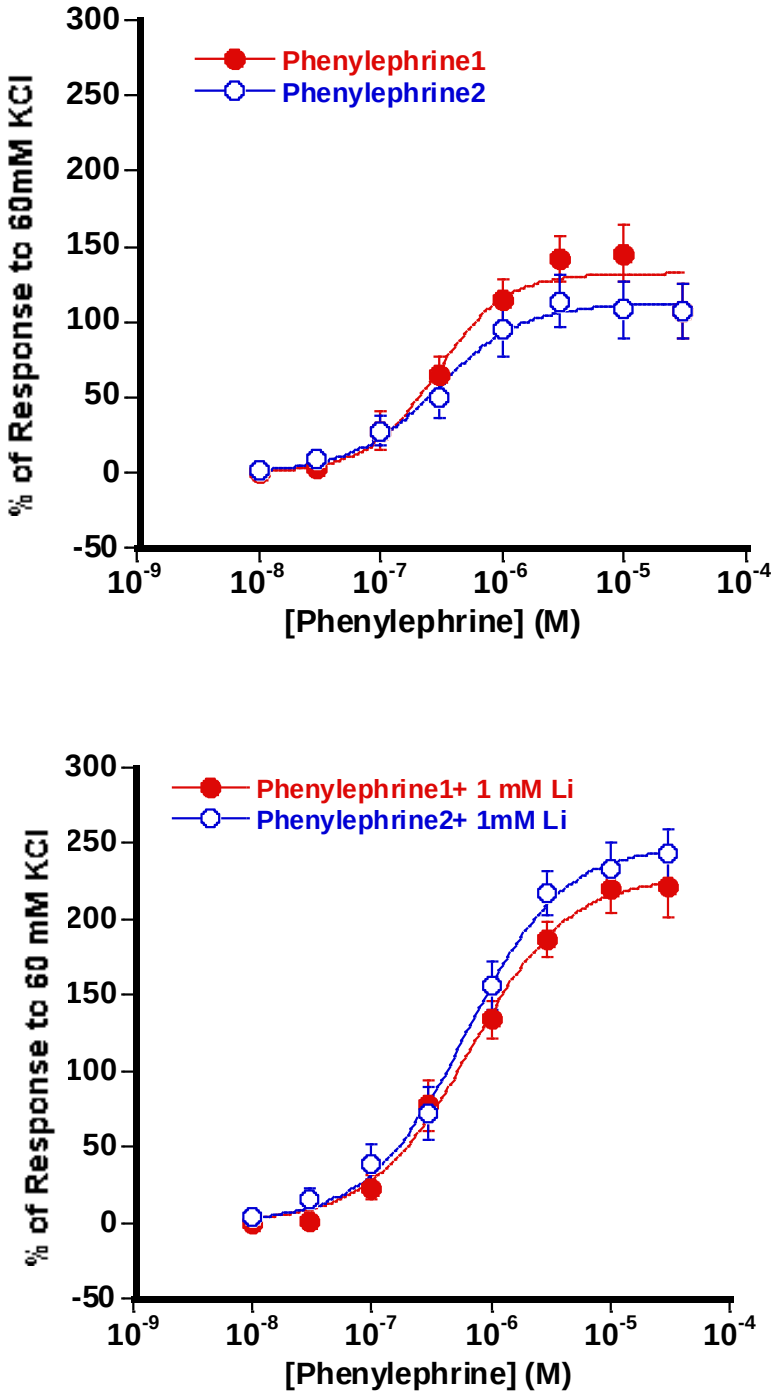

Figure 5-1 The concentration-response curve following the cumulative addition of phenylephrine in the absence (upper) and the presence (lower) of 1mM LiCl in porcine splenic artery. Data are expressed as a percentage of contraction to 60mM KCl and are mean ± SEM, (n=10).

In another set of the experiment, we looked at what will be the effect of LiCl if we used different agonist such as noradrenaline. Therefore, we exposed the tissue to phenylephrine 30μM in the presence and absence of LiCl 1mM. We washed and stimulated the tissue with noradrenaline 10μM in the presence and absence of LiCl 1mM. As seen in Figure 5.2, the previous exposure to LiCl+phenylephrine significantly enhanced and better-maintained the time course for the contraction induced by noradrenaline in compare with the control in which slight contraction was elicited. The n number for this experiment was 14.

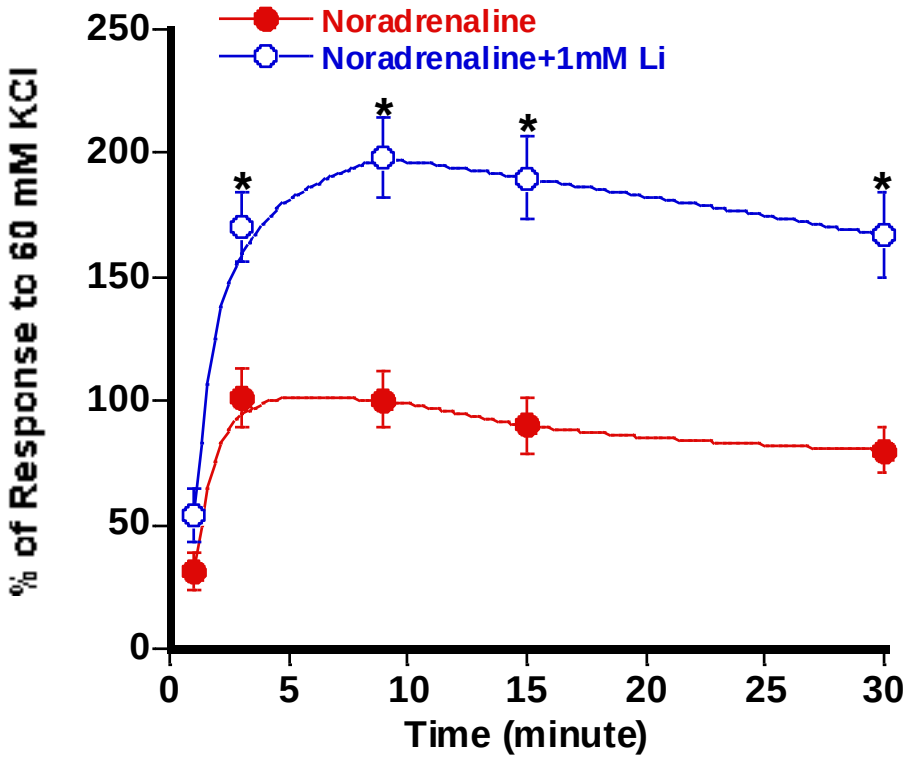

Figure 5-2 The time course of noradrenaline in a porcine splenic artery following the previous exposure to phenylephrine 30μM in the presence and absence of LiCl 1mM. Data are expressed as a percentage of 60mM KCl and are mean ± SEM.* - Denotes significant differences between mean responses ($p < 0.05$; paired Student *t*-test).

5.3.2 The suggested role of receptor internalisation in the synergistic interaction of LiCl with α_1- agonists in the porcine splenic artery

Figure 5.3 (A) and (B) show that the addition of 250µg/ml Con A significantly reduced the magnitudes of the tissue response to phenylephrine in comparison with control. In addition to that, the presence of Con A did not block the enhancement produced by LiCl to the time course of contraction induced by phenylephrine 30µM. in the same experiments exposing the tissue to 10µM noradrenaline, the presence of LiCl before the addition of phenylephrine better-maintained the time course for noradrenaline 10µM in the presence and absence of Con A, see Figure 5.3 (C)

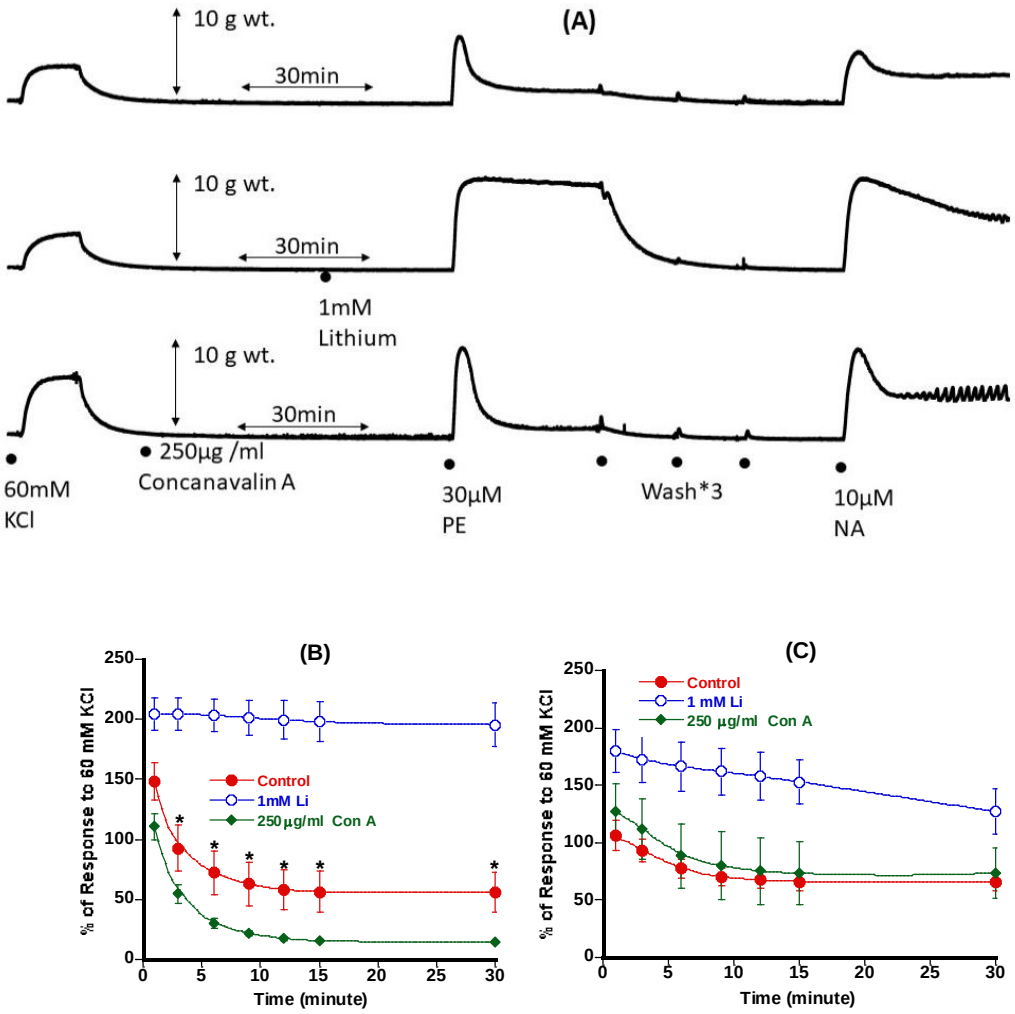

Figure 5-3 (A) Traces and the time course curve for the contractile response in the porcine splenic artery at different time points after the addition of phenylephrine (PE) 30µM (B) and the addition of noradrenaline (NA) 10µM (C) in the presence of 1mM Li, 250µg/ml Con A. Data expressed as mean± SEM of contraction to 60mM KCl (n=8). * - Denotes significant differences between mean responses ($p < 0.05$; paired Student *t*-test).

5.3.3 Role of Na^+ channels in the synergistic interaction between LiCl and phenylephrine in the porcine splenic artery

In this group of experiments, I aimed to detect the site of action of lithium ions. Does it need to be inside the cell in order to enhance the magnitude and the time course for some α_1-adrenoceptor agonist? Amiloride 10μM was used in these experiments (higher concentration can affect the contraction induced by the agonist). As Figure 5.4 shows, blockage the Na^+ channels with amiloride did not affect the synergistic interaction of LiCl with phenylephrine following the single addition of phenylephrine 30μM. This may suggest that LiCl can induce its effect extracellularly or it enters the cell by another mechanism rather than Na^+ channels.

In a different set of experiment and in order to better understanding if LiCl is required to present all the time after potentiating the contraction induced by phenylephrine another procedure was followed. In these experiments, LiCl was added for 10 minutes before being washed with Krebs solution. In order to prevent the LiCl from influx into the cell in the other channels amiloride was added before LiCl and then washed with amiloride containing Krebs before the addition of phenylephrine. It was noted that LiCl although being washed, it enhanced the magnitude of the contraction to phenylephrine and as expected amiloride lower the amount of LiCl being involved in the interaction but did not prevent it completely. However, the time course still markedly different from both the control and LiCl treated segments, see Figure 5.5

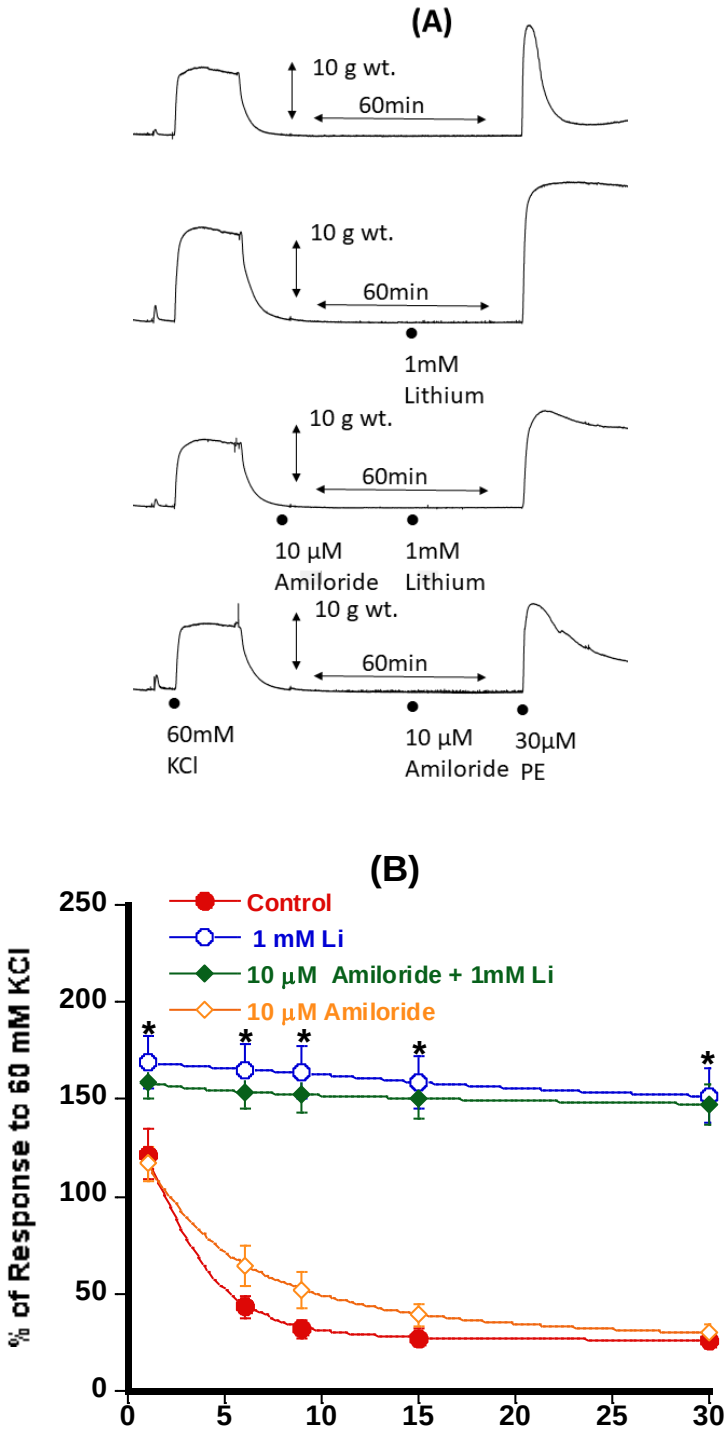

Figure 5-4 (A) Traces recording and (B) the contractile response curve in the porcine splenic artery at a different time point after the addition of phenylephrine 30µM in the presence of amiloride 10µM and LiCl 1mM. Data are expressed as the percentage of contraction to 60mM KCl. Data are mean ± SEM (n=6). * - Denotes a significant difference between mean responses ($p < 0.05$; paired Student *t*-test).

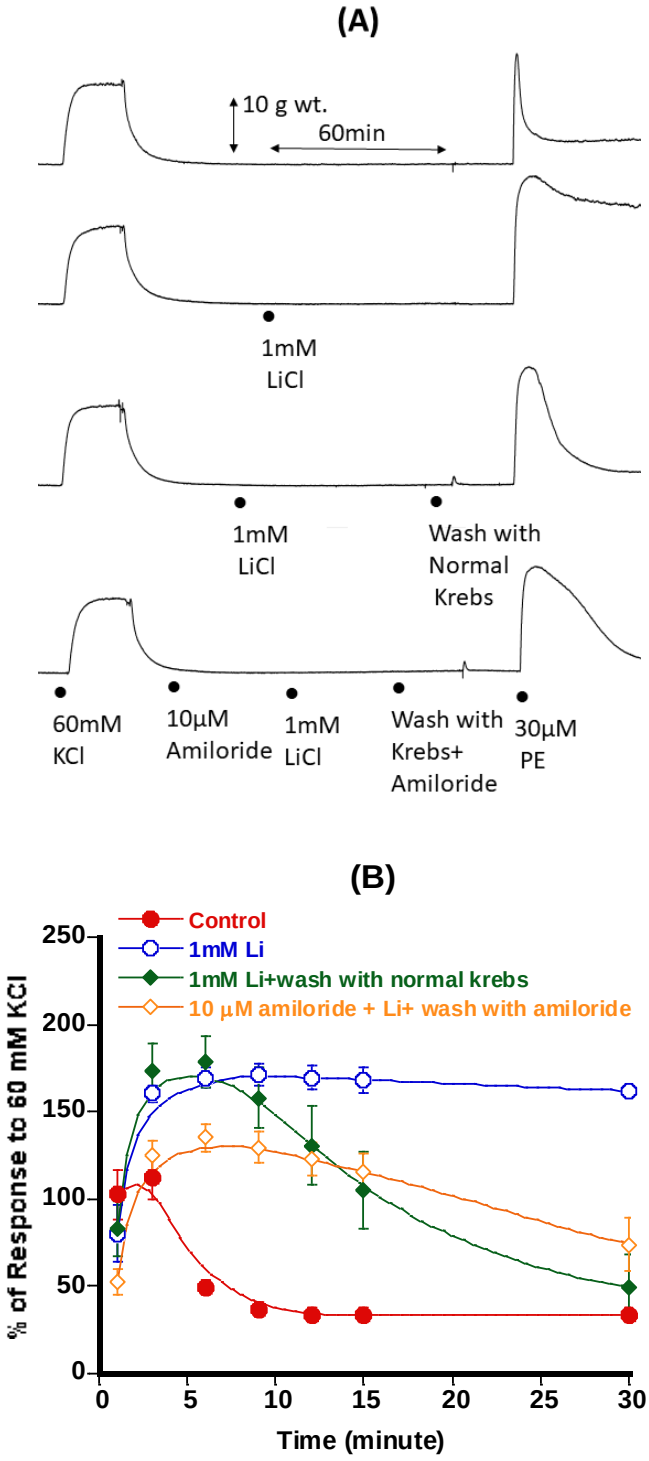

Figure 5-5 (A) Representative traces and (B) time course for the contractile response in the porcine splenic artery after the addition of phenylephrine 30µM in the presence of amiloride 10µM and LiCl 1mM. LiCl was washed with either normal Krebs or amiloride containing Krebs before inducing contraction with phenylephrine 30µM. Data are expressed as a percentage of 60mM KCl and are mean ± SEM (n=8).

5.3.4 Role of Na^+/K^+ ATPase pump in the synergistic interaction between LiCl and phenylephrine in the porcine splenic artery

In this set of experiments, I aimed to check this pump role in the interaction of LiCl with phenylephrine. Because the use of ouabain produced a contraction which interferes with the interpretation of the data following the addition of the pressor agent (phenylephrine), so I decided to use a lower concentration of the inhibitor that provides the inhibition to the pump with minimal interference with the baseline tone of the tissue. Hence, two concentration of 30nM and 100nM ouabain were used in these experiments. As Figure 5.6 shows that the addition of LiCl and low concentration of ouabain significantly enhanced and better-maintained tissues responses, 6 minutes following the addition of the agonist phenylephrine 10μM, the responses were 99.3±10 and 145.6±7.1 in the control and LiCl treated segments respectively. Using ouabain, the responses were 135.8±15.8, 142.1±6.1 in the (100, 30) nM incubated segments respectively. After 60 minutes, the responses were 41.7±5.9 %, 138.1±8.5 % for the control and LiCl treated segments and were 116.9±16.2 %, 102.3±7.6 % for 100nM and 30nM ouabain treated segments respectively. P-value < 0.05; paired Student *t*-test n=8.

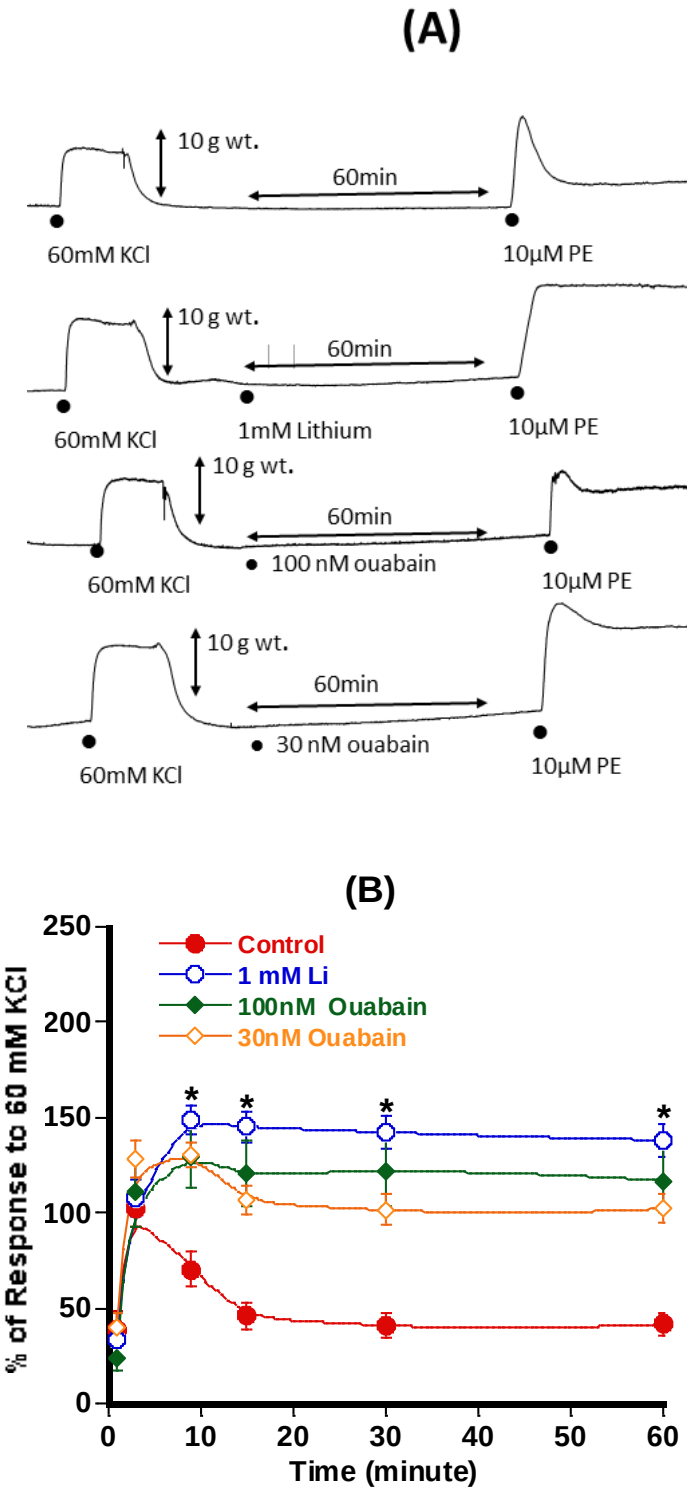

Figure 5-6 (A) Representative traces and (B) the time course curve for the contractile response in the porcine splenic artery at different time points after the addition of phenylephrine 10µM in the presence of 30nM, 100nM ouabain and LiCl 1mM. Data are expressed as a percentage of 60mM KCl and are mean ± SEM. Using a paired Student's *t*-test (n=8). * - Denotes a significant difference between mean responses ($p < 0.05$).

5.3.5 Inhibition of the Inositol monophosphates (IMPtase) in normal Krebs

After the application of L-erythromethoxamine 30μM and as seen in Figure 5.7 both IMPtase inhibitors failed to mimic the action produced by LiCl. Sixty minutes after the addition of L-erythromethoxamine 30μM the time course curve for the control declined to 15.8±4.7 % (n=5) of the response to 60mM KCl. Neither the presence of L-690,330 300μM nor L690, 488 10μM inhibitors of IMPtase, affected the time course of contractions of the porcine isolated splenic artery to L-erythromethoxamine and their responses were 17.5±9% and 20±8.0% of 60 mM KCl (n=5) respectively. While the response in the presence of 1mM LiCl enhanced and better-maintained even after 60 minutes at 212.0±27% (n=5) of the contraction to 60mM KCl. The P-value was <0.05 using a paired Student's *t*-test. See Figure 5.7 and 5.8 (A).

In a different set of the experiments we used another α_1-adrenoceptor agonist metaraminol, it is a commonly used drug in the treatment of acute hypotension and it is acting through the stimulation of α_1-adrenoceptors in the vascular tissue, again we compared the effect of LiCl with L690, 488. Figure 5.8 (B) shows that the use of IMPtase inhibitor did not mimic the action of LiCl in porcine splenic artery.

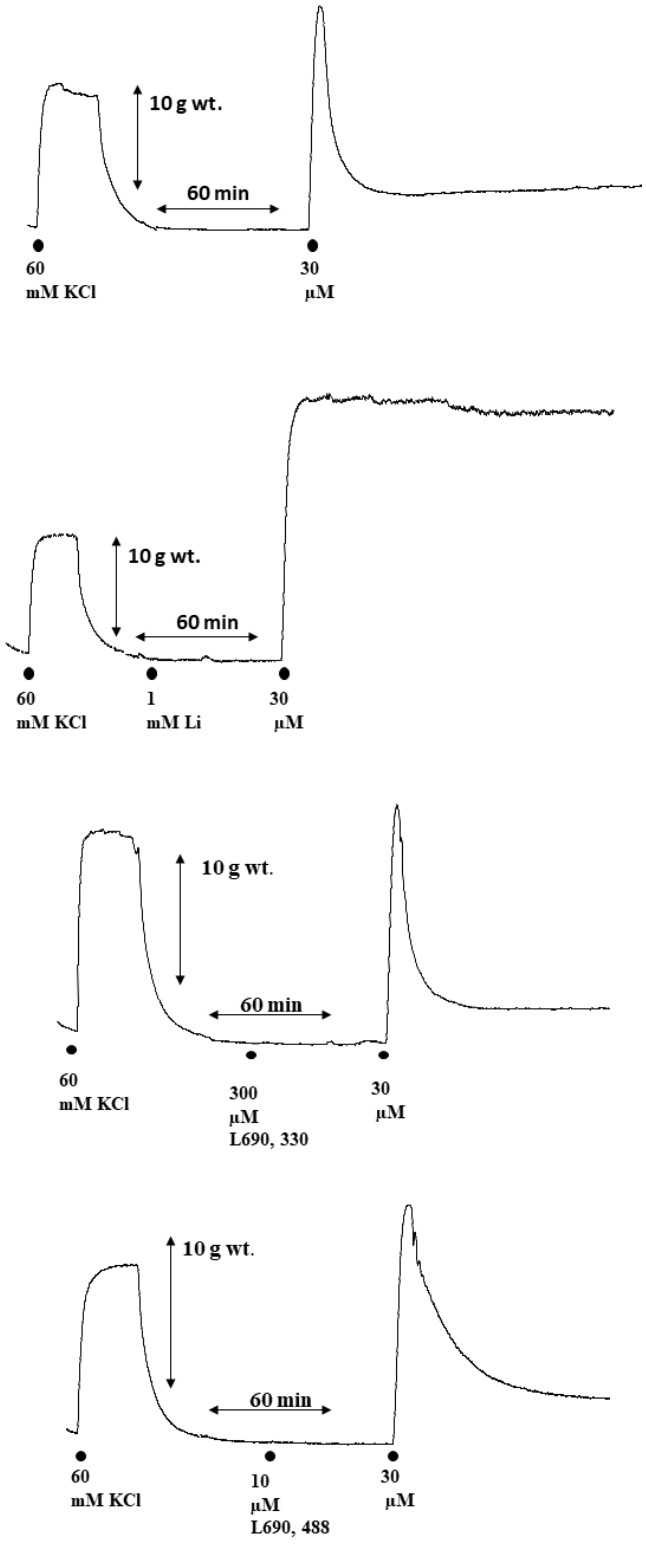

Figure 5-7 Representative traces for vasoconstrictor effects of L-erythromethoxamine 30µM in the presence and absence of 1mM LiCl, L690, 488 10µM and L690,330 300µM. Data are expressed as a percentage of contraction to 60mM KCl in porcine splenic artery.

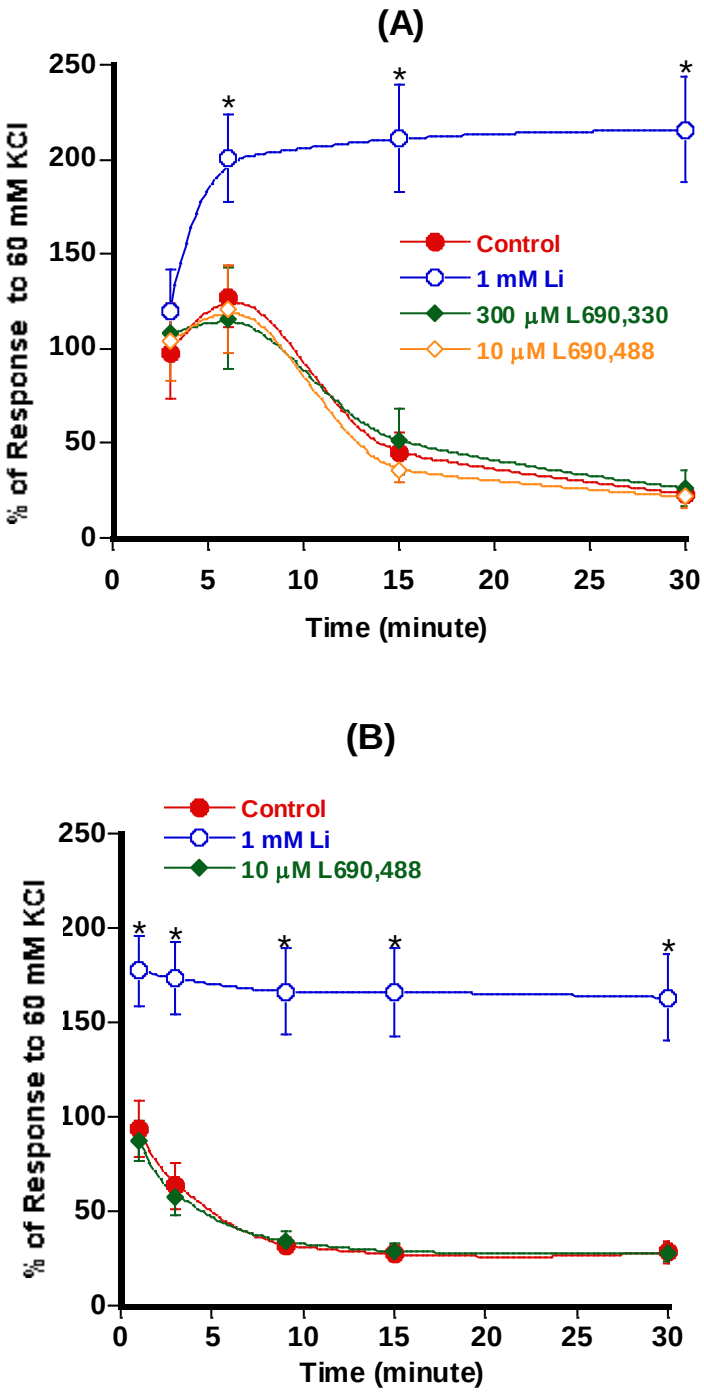

Figure 5-8 The contractile response in the porcine splenic artery at a different time point after the addition of 30µM of (A) L-erythromethoxamine (n=5) and (B) metaraminol (n=5) in the presence of IMPtase inhibitors and LiCl. Data are expressed as the percentage of contraction to 60mM KCl. Data are mean ± SEM. * - Denotes a significant difference between mean responses ($p < 0.05$; paired Student *t*-test).

5.3.6 Role of Ca^{2+} in the contraction induced by α_1-adrenoceptor agonists in the presence and absence of LiCl and another IMPtase inhibitor

In order to check the role of Ca^{2+} on the contraction induced by α_1-adrenoceptor agonists and the interaction with lithium ions. Ca^{2+} free Krebs was prepared with the addition of EGTA 100μM. In this experiment, metaraminol was used to initiating the contraction in porcine splenic artery. Figure 5.9 (A) and (B) shows that in the absence of Ca^{2+} ions there was a significant reduction in tissue response to contraction induced by metaraminol 30μM. In addition to that, the presence of LiCl 1mM and L690, 488 10μM did not produce significant changes in the time course of contraction to metaraminol.

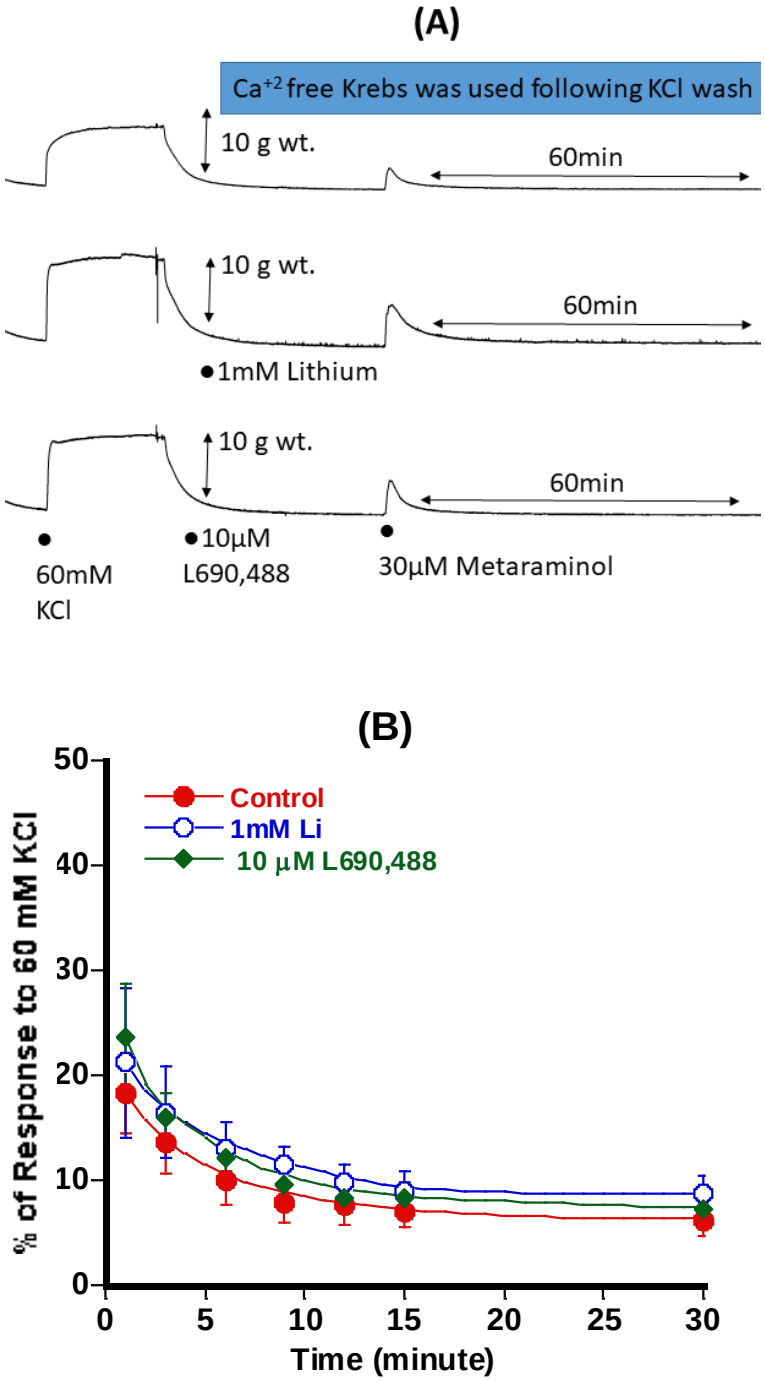

Figure 5-9 (A) The representative traces and (B) The contractile response curve in the porcine splenic artery at a different time point after the addition of metaraminol 30µM in Ca^{2+} free Krebs. Data are expressed as a percentage of contraction to 60mM KCl and are mean ± SEM (n=7).

5.3.7 Role of inhibition of glycogen synthase kinase enzyme (GSK3) alone and in a combination with IMPtase inhibitors

An assessment for the role of another important enzyme which also well known to have a role in the action of lithium ions was also performed, glycogen synthase kinase 3 (GSK3), so we used SB216367 as selective inhibitors. Two different sets of experiments were performed. In the first set, the cumulative addition of the agonist (L-erythromethoxamine) was added in the presence of LiCl 1mM, and SB216367 300nM. Only segments incubated with LiCl produced statistically significant changes in both R_{max} and pD_2 with a P-value<0.05 using paired Student's *t*-test (n=7), See Table 5.2 and Figure 5.10, 5.12

In the second set of experiments, we aimed to check what would be the effect of using a combination of inhibitors for both IMPtase and GSK synthase. The porcine splenic artery segments were incubated with L-690,330 300μM and of SB216367 300nM. Once more, segments incubated with LiCl 1mM produced significant changes in both R_{max} and $pD_{2;}$ while the inhibitors combination slightly increased the R_{max} without affecting the pD_2 of the agonist, Using paired Student's *t*-test for 6-7 observations. See Table 5.2 and Figure 5.11 and Figure 5.12

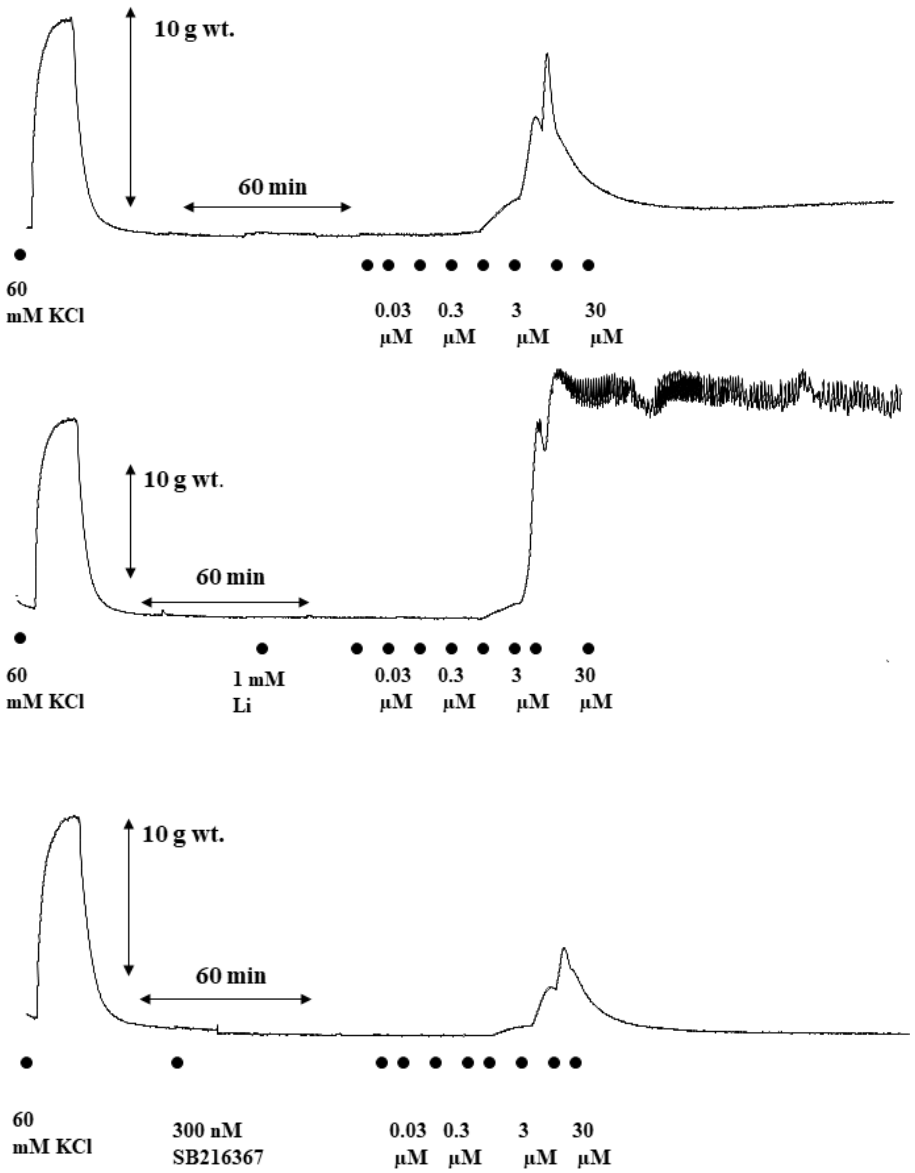

Figure 5-10 Representative traces for vasoconstriction effects of L-erythromethoxamine in the presence and absence of 1mM of LiCl and SB216367 300nM. Data are expressed as the percentage of contraction to 60mM KCl in the porcine splenic artery

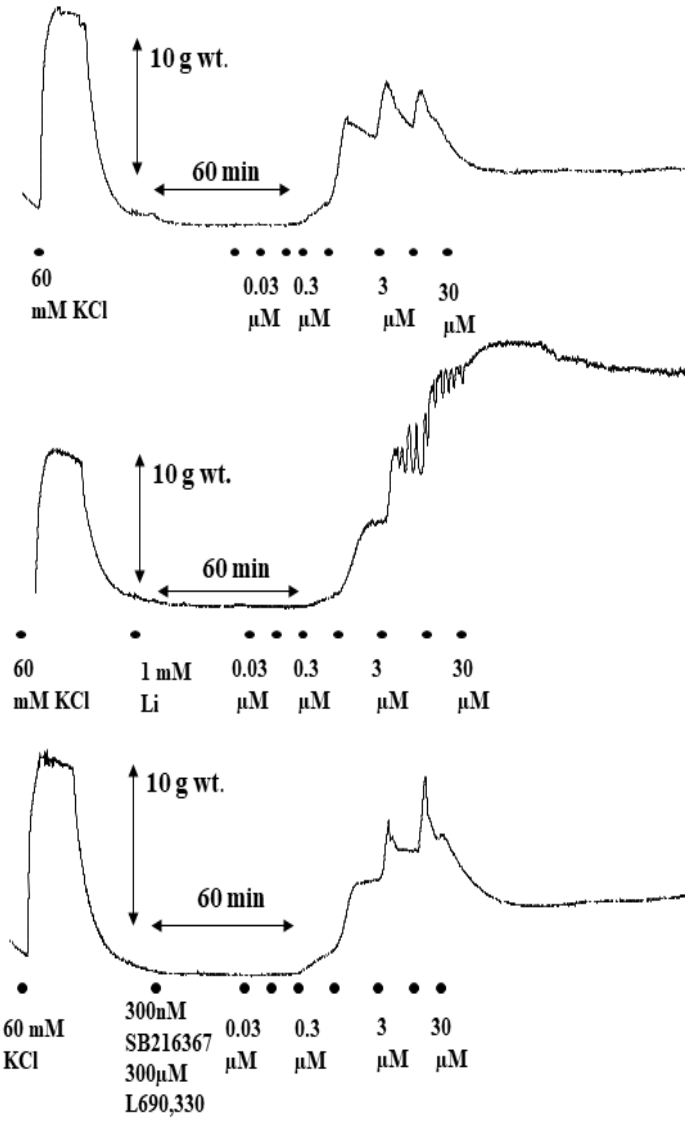

Figure 5-11 Representative traces for vasoconstrictor effects of L-erythromethoxamine in the presence and absence of 1mM LiCl and a combination of 300nM SB216367 and 300µM L-690,330. Data are expressed as the percentage of contraction to 60mM KCl in porcine splenic artery.

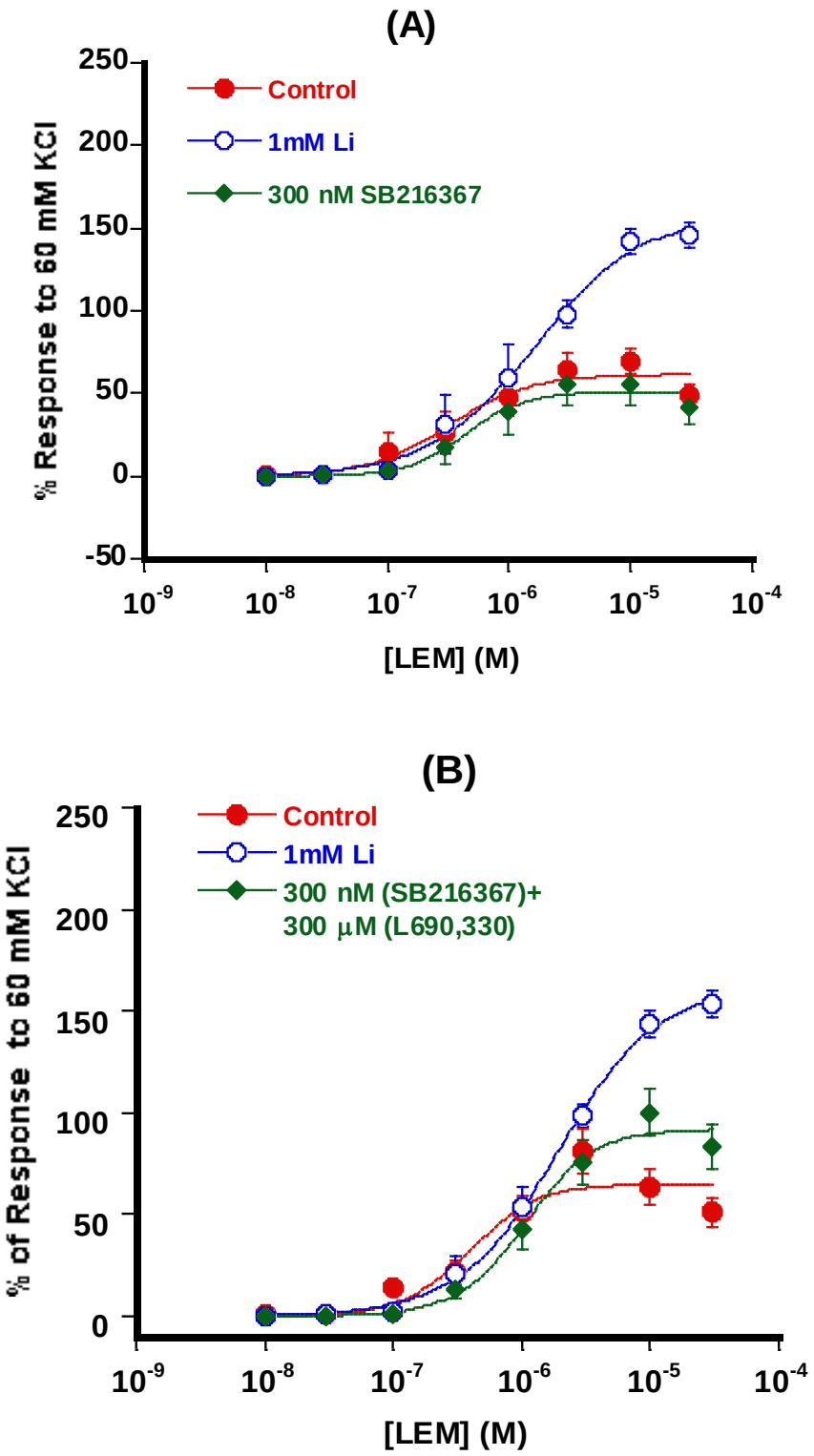

Figure 5-12 Concentration-response curve for vasoconstrictor effects of L-erythromethoxamine in the presence and absence of LiCl 1mM and (A) SB216367 300nM, n=7 or (B) combination of 300nM SB216367 300µM L-690,330 n=6. Data are expressed as the percentage of contraction to 60mM KCl in porcine splenic artery.

Table 5-2 Shows the changes in the maximum response and the potency following incubation the tissue with LiCl 1mM and 300nM of SB216367 either alone or in a combination with 300μM L-690,330, and the cumulative addition of L-erythromethoxamine agonist. Data expressed as mean± SEM of contraction to 60 mM KCl * - Denotes significant differences between mean responses ($p < 0.05$; paired Student t-test).

Drug	**R_{max}**	**pD_2**	**n number**
Set 1			
Control	63.4±6.8	6.4±0.2	7
Li	150.7±10.2*	5.8±0.18*	7
SB216367	60.29±10.6	6.18±0.16	7
Set 2			
Control	65.0±8.6	5.9±0.4	6
Li	161.1±7.5*	5.7±0.12*	6
SB216367+ L-690,330	91.7.4±7.6*	5.9±0.06	6

5.3.8 The effect of adding LiCl after contraction induced by a single addition of cirazoline and U46619

As seen in Figure 5.13 (A) the addition of 30μM of cirazoline produced a sustained contraction which remains maintained even after washing twice with fresh buffer. Interestingly and by serendipity, when 1 mM LiCl was added to the system it produced a relaxation that brings the tone back to baseline in less than 30 minutes.

in a different set of experiments, contractions in porcine splenic artery segments were initiated following the addition of a single concentration of two potent contractile agents cirazoline and the thromboxane mimetic U46619. Figure 5.13 (B) shows the contraction induced by these agents were stable for more than an hour. At time point=0, 1mM LiCl was added to the three segments. Interestingly, LiCl produced a quick drop in the contraction tone and reached the baseline within 30 minutes. On the other hand, U46619 induced contraction declined slightly before recovering from Li effects.

On another set of experiments, we tested a similar model of interaction using a different concentration of cirazoline. Interestingly, a similar type of interaction was reported with (3, 10 and 30) μM of cirazoline. The decline in response was comparable to that result from washing the highest concentration of cirazoline Figure 5.14.

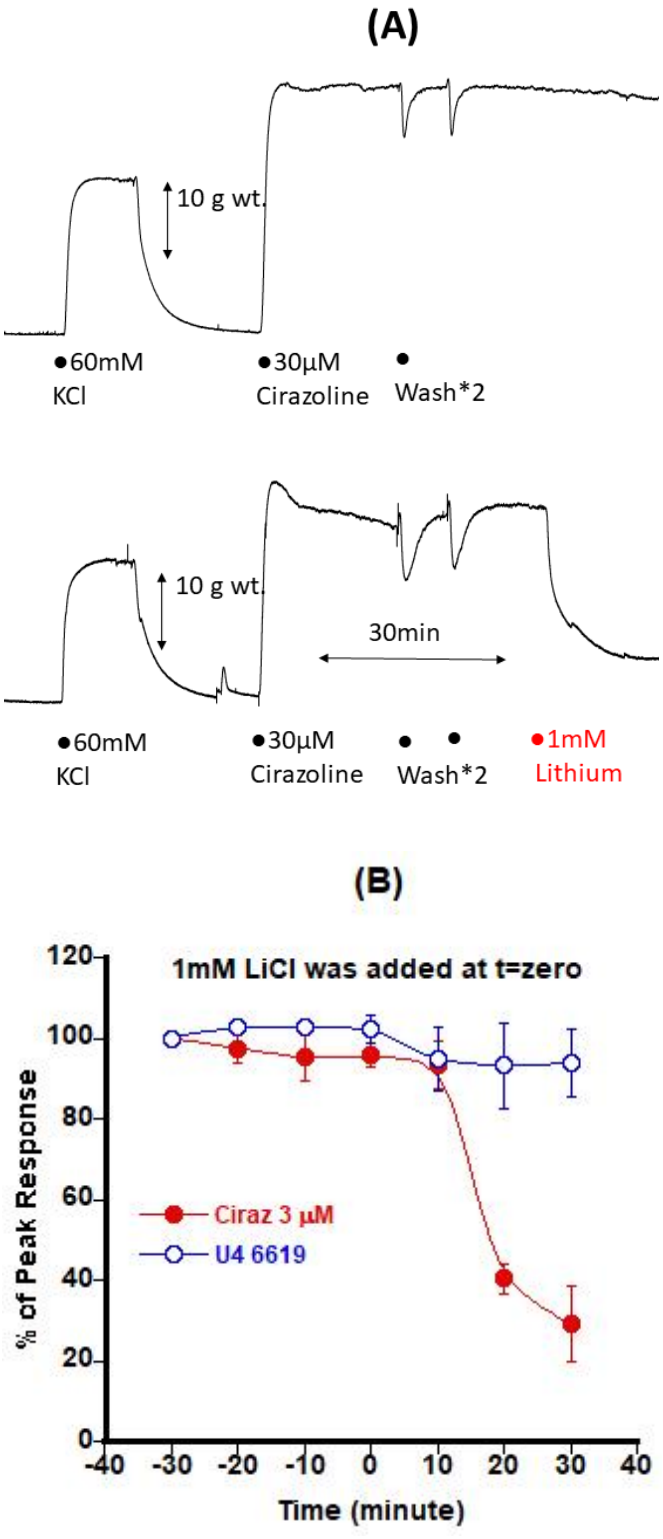

Figure 5-13 (A) The representative trace for the response to contraction induced by cirazoline 30µM and LiCl 1mM (B) The time course of contraction induced by cirazoline and U46619 following the addition of 1mM LiCl. Data expressed as mean± SEM of contraction to 60 mM KCl,n=4.

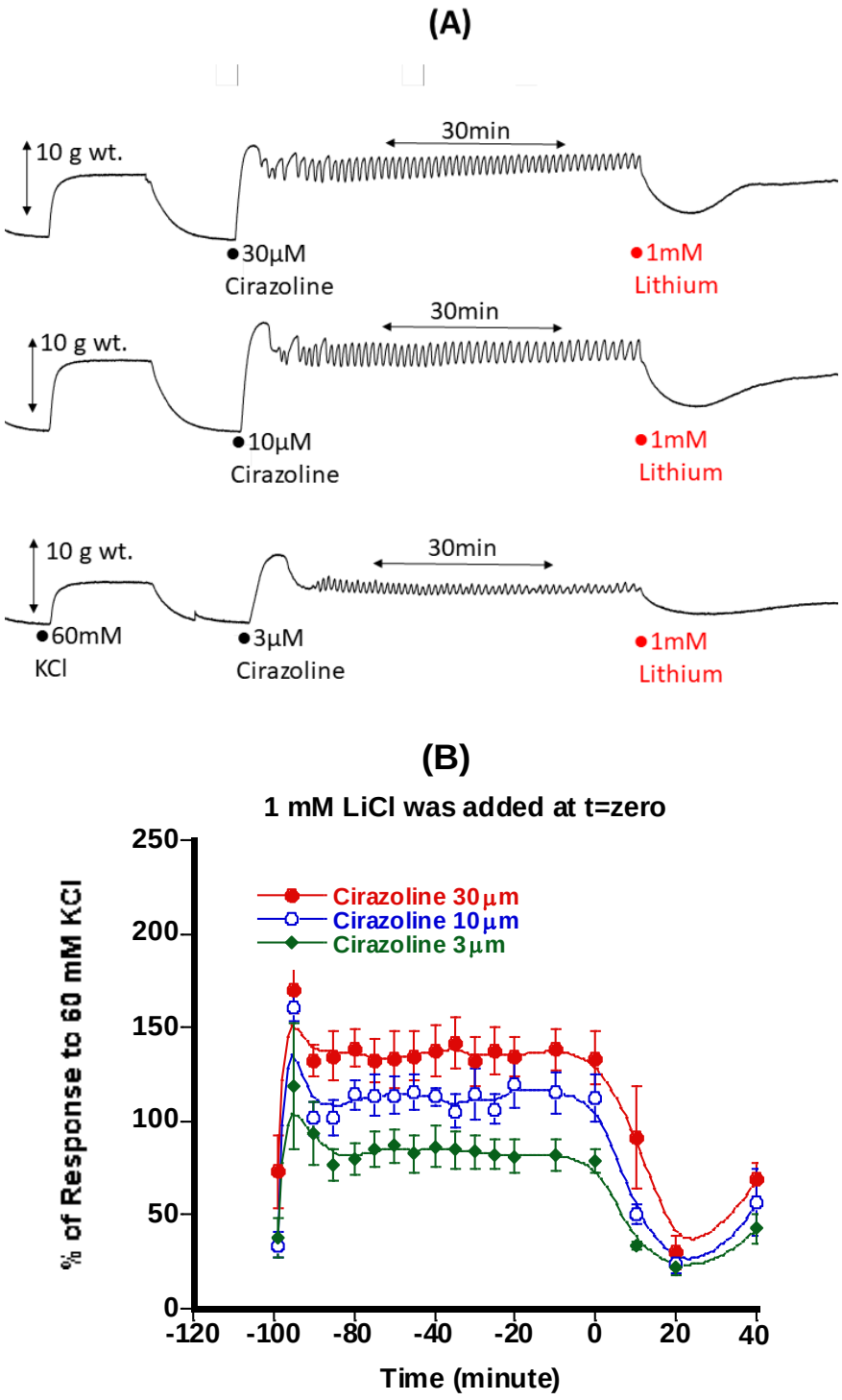

Figure 5-14 (A) Representative trace (B) and the time course curve for vasoconstrictor effects of (3, 10, 30) µM of cirazoline followed by the addition of 1mM LiCl. Data are expressed as the percentage of contraction to 60mM KCl in a porcine splenic artery (n=6).

5.4 Discussion

Results obtained from this chapter, using isometric tension technique in porcine isolated splenic artery confirm that the synergistic interaction between the therapeutic concentration of LiCl with α_1-adrenoceptors agonist is not related to desensitisation and receptor trafficking mechanism. It was important to check the desensitisation as a possible mechanism for this interaction by exposing the tissue to two cumulative addition of phenylephrine, although the desensitisation was developed after the second addition, the presence of LiCl prevent this desensitisation and enhances tissue responses to contraction induced by α_1-adrenoceptor agonists.

Furthermore, the involvement of α_1-adrenoceptors in this interaction necessities the need to highlight the receptor trafficking mechanism. Our results confirmed that the use of an internalisation blocker failed to prevent the synergistic interaction, which suggests no role of receptor internalisation in this interaction.

Another factor involved in this interaction is the movement of LiCl into and outside the cell; amiloride was used to block the entrance of LiCl into the cell by blocking the apical epithelial Na^+ channels. The results of this study suggested that even in the presence of amiloride LiCl was able to enter the cell by an alternative mechanism rather than Na^+ channels or Na^+- proton exchanger and induce the interaction with phenylephrine. The use of ouabain to block Na^+/K^+ ATPase pump enhanced the time course of the contraction induced by phenylephrine but in much smaller magnitude in compare to LiCl. Finally, we examined the ability of lithium to inhibit IMPtase or GSK3 enzymes or even a combination of them. The use of selective inhibitors to those enzymes failed to mimic the action of LiCl in this preparation.

5.4.1 Role of receptor desensitisation and internalisation

Activation of α_1-adrenoceptors in the vascular blood vessels will result in contraction of these vessels, the degree of responsiveness of these vessels can be modified and change in different conditions such as disease states and hormonal abnormalities. The responsiveness of the vessels to the subsequent stimulation with the same agonist can be altered and this can result in changes in the pathophysiology of the disease and drug responsiveness.

The excessive exposure to the high concentration of the agonist for long period can produce tolerance or what is also known as receptor desensitisation in which loss of sensitivity is the main feature (Lurie *et al*., 1985). This loss of responsiveness could be either homologous (specific) or heterologous (generalised) depending on the extent of dampening to the receptors systems (Yoshida *et al.*, 1982). In line with our results, cells response to noradrenaline was markedly decrease if pre-treated with oxymetazoline, due to desensitisation and phosphorylation of α1-adrenoceptors (Alcantara-Hernandez *et al*., 2017).

Our studies suggested that exposing the isolated porcine splenic artery to the cumulative addition of phenylephrine initiated a contraction, which is reproducible. The presence of LiCl enhanced tissue responsiveness following the first and second exposure. Furthermore, the finding that prior exposure of the isolated splenic artery to a high concentration of phenylephrine can suppress even noradrenaline -induced contractions. On the other hand, the presence of LiCl prevented that suppression in tissue response to the single addition of a high concentration of noradrenaline. This proposed an interruption to desensitisation mechanism in this receptor system that could result from an enhancement of coupling between α_1-adrenoceptors and their agonists, with the suggestion of minimal role of receptor downregulation or reduction in the affinity in such interaction (Munoz *et al.,* 1989).

The GPCR has been extensively investigated with regard to their involvement in receptor trafficking and internalisation, concanavalin A (Con A) was used as an internalisation inhibitor (Segura *et al.*, 2013). Interestingly, observations in cell-based systems provide support for short-term changes in cell surface receptor numbers in response to the presence of high concentrations of an agonist. Fluorescent labelled ligands for α_1-adrenoceptors has revealed the presence of βarrestin-mediated trafficking of the receptors from the cell membrane to intracellular organelles (Wang *et al.*, 2007; Stanasila *et al.*, 2008; Perez-Aso *et al.*, 2013).

Moreover, high concentrations of phenylephrine and oxymetazoline, but not noradrenaline, have been reported to cause time-dependent desensitisation of the receptor within 30 min of exposure to the agonists, that was associated with a reduction in cell surface receptors (Akinaga *et al.*, 2013).

Presently, it is not known whether lithium ions can interfere with this process, but it is noteworthy that this cation has been reported to disrupt β-arrestin-associated signalling events in striatal tissue (Beaulieu *et al*., 2008). Furthermore, a β-arrestin: α_1-adrenoceptor complex has been observed in human prostate smooth muscle (Chen *et al*., 2013), so it is feasible that this is an important target throughout the cardiovascular system.

Our results show that in the presence of Con A, the response of tissue to contraction induced by phenylephrine was decline. In addition to that, LiCl was able to enhance the response to contraction induced by phenylephrine. This suggested lacks for the role of receptor trafficking in the contraction with α_1-adrenoceptor in this blood vessel. It is noteworthy to mention that no previous study has tested the action of Con A using the isolated tissue bath recording technique.

5.4.2 Role of Na^+ channels and Na^+/K^+ ATPase pump on the pharmacokinetics of lithium ions

Lithium influx into the cell through two major pathways, Na^+-proton exchanger and epithelial Na^+ channels both are sensitive to amiloride (Bedford *et al.*, 2008). For a better understanding of the location of LiCl involved in the interaction with α_1-agonists, we used amiloride in this set of experiments. Amiloride blocks lithium entry to the intracellular through Na^+ channels and this channel represents the main entry site for lithium to the intracellular compartments (Kortenoeven *et al.*, 2009). Results show that blocking lithium influx into the cells did not inhibit the synergistic interaction with phenylephrine and the use of higher concentration was associated with the generation of tone, which interferes with the results of these experiments so 10μM was used. The results also suggest that lithium can influx into the cells and produce the interaction with phenylephrine using an alternative pathway.

On a different set of experiments, we have addressed the question regarding the time needed by tissue to be in contact with the LiCl to enhance the magnitude and duration of contraction induced by phenylephrine. It was clear from the results that washing LiCl after 10 minutes of contact with segments of porcine splenic artery enhanced the magnitudes for only 15 minutes before it starts to wane; response reached the baseline after 30minutes.

Interestingly preincubation of the tissue with amiloride and keeping it during and after washing the lithium reduces the enhancement in the magnitude and the time course of phenylephrine in compare to the presence of 1mM LiCl without washing, however, the enhancement is significant in comparison to the control. These results propose that amiloride partially block the influx of LiCl into the cell through Na^+-proton exchanger and epithelial Na^+ channel, and some other pathways might be involved in lithium movement across the cell membrane and act as an alternative route. Furthermore, lithium needs to enter the cell in order to produce the synergistic interaction with α_1-agonists.

The Na^+/K^+ ATPase is an enzyme located on the cell membrane, it is an active pump using ATP to provide energy to pumping Na^+ outside and importing K^+ to inside the cell against their concentration gradients. Ouabain is a non-selective inhibitor for Na^+/K^+ ATPase which can interfere with the inositol triphosphates pathways (Mains *et al*., 1990). In relation to the correlation between lithium and the activity of Na^+-K^+ ATPase, several *in vivo* studies showed that patient with bipolar disorder have low activity of this enzyme and there is a positive correlation between the plasma level of lithium and activity of this enzyme. In patients who are using a therapeutic level of lithium, the activity of Na^+-K^+ ATPase enhanced to 60-70% in comparison with patients do not receive lithium (Alexander *et al.*, 1986; Banerjee *et al.*, 2012). Similar sort of relation was reported with *in vitro* study using human embryonic kidney cells in which the activity of Na^+-K^+ ATPase was inhibited with ouabain.

In this study, results show that incubation the tissue with (30-100) nM ouabain produced an enhancement in the time course of contraction induced by phenylephrine in porcine splenic artery. Although the magnitude of enhancement is less than that reported with 1mM LiCl, it still considered as statistically significant. In line with our results regarding the effect of incubation the tissue with nano-molar concentrations of ouabain, it was reported that ouabain at low concentrations enhanced the contractility of rat vascular smooth muscle. This enhancement was attributed to the increase in the concentration of systolic free Ca^{2+} by increase transmembrane Ca^{2+} influx. The concentration of the influx Ca^{2+} could be affected by factors other than direct depolarization reported with the use of high concentrations ouabain; these factors could be deprivation of extracellular Na^+ that inhibits the Na^+-Ca^{2+} exchange or exposure to a high concentration of K^+ that induced depolarization (Zhu *et al.*, 1994; Zhu *et al.*, 1996). While other studies reported similar effect and highlighted ouabain effect to its ability to block the activity of nitric oxide enzyme in cultured vascular

smooth muscle and reduce the sensitivity of these cells to this agent (Callera and Bendhack 2003).

5.4.3 Role of inhibition of IMPtase and GSK3 enzymes in the interaction between LiCl and some α_1-adrenoceptor agonist in the porcine splenic artery

Previously in this thesis, we have reported that the synergistic interaction between LiCl and α_1-adrenoceptor agonists (L-erythromethoxamine, metaraminol and phenylephrine) in porcine vascular tissue associated with an enhancement in both the magnitude and the duration of response to these agonists. This finding underlines the point that the effect of LiCl is selective to a subgroup of α_1-adrenoceptors. One of the early-proposed mechanisms for the anti-manic effect of lithium is its ability to inhibit the metabolism of inositol through blocking the action of inositol monophosphatase (IMPtase) enzyme (Ackermann *et al.*, 1987; Berridge, 1989; Atac*k et al*., 1994)

In this study, we used two IMPtase inhibitors (L690, 488 and L690, 330) to try to mimic the effect of LiCl on the time course for the contraction induced by α_1-adrenoceptors agonists in the porcine splenic artery. The point is underlined by the finding that the use of these inhibitors of IMPtase enzyme failed to mimic the action of LiCl in this preparation. The other purported target for lithium in the management of bipolar disorder is the inhibition of glycogen synthase kinase (GSK3) enzyme (Klein and Melton 1996). While this enzyme has been reported to be associated with the development of hypertrophy of vascular and bronchial tissues (Deng *et al*., 2010). There is practically no information to suggest a role in the acute changes in the vasculature. Our results with the use of a selective inhibitor of GSK3 (SB216763) (Coghlan *et al.*, 2000) failed to produce an action comparable to what previously reported with LiCl. Because lithium ions are well documented to work with multiple mechanisms (Yu and Greenberg 2016) and because the majority of lithium activity belonging to its ability to inhibit two main enzymatic pathways. A combination of IMPtase and GSK3 inhibitors was tested to assess their role in the contractility initiated by L-erythromethoxamine in the porcine splenic artery and compare that with the ability of LiCl to increase the magnitude of the response to L-erythromethoxamine. Our results suggested that there is no role for the use of a combination of GSK3 and IMPtase enzyme inhibitors in the striking and selective action of lithium in porcine vascular tissues.

5.4.4 Relaxation after the addition of LiCl on the contraction induced by cirazoline

In line with the previous results and the theory of dual action for LiCl, our results showed that the addition of LiCl after initiating the contraction with cirazoline could produce relaxation. The tissue tone wanes off to baseline during a short period. The similar reaction did not report with other contractile agents such as U46619. It was difficult to find an explanation for this interesting effect especially after we have reported that LiCl has minimal effect on the time course of contraction induced by the same agonist (cirazoline). This interesting observation was discovered by serendipity Figure 5.13 (A). The aim of that experiment was to induce desensitisation in the receptors by exposing them to the high concentration of cirazoline followed by exposing the tissue to noradrenaline or phenylephrine. We have previously reported that cirazoline produced a stable response. Due to being impatient and due to the fact, that washing the system with fresh Krebs-Henselite did not reduce the tone of the contraction induced by cirazoline quickly; I decided to add LiCl without waiting for the response from coming back to baseline as planned. I noticed that following the addition of 1mM LiCl the response starts to drop rapidly and reached the baseline within less than 30 minutes. The use of sub-maximum concertation of cirazoline produced a similar form of interaction after the addition of LiCl Figure 5.14. Further experiments are needed to confirm such interaction with other contractile agents in both vascular and non-vascular tissue.

Chapter 6: The Effect of Physiological Interventions and Iontophoresis of Phenylephrine on Blood Flow in the Forearm of Healthy Volunteer

6.1 Introduction

Phenylephrine has been used topically to treat different clinical conditions for many years. When it is used topically a relatively high concentration of the drug is required to produce a response. It acts through selectively stimulating the α_1-adrenoceptors in different tissue including eye, nasal mucosa, anal sphincter and the skin. The topical application to the eye was reported to induce mydriasis. This action can be used in both diagnostic and therapeutic procedure (Alldredge *et al.*, 2013). In addition to that, the topical solution of phenylephrine was used in a combination with lignocaine to produce a nasal spray that applied locally to reduce pain associated with nasal gastric intubation in children (Craig *et al*., 2015). Another common use for phenylephrine was in the management of faecal incontinence which can result from different causes such as following exposure to pelvic radiation or post-surgical cases (Badvie and Andreyev 2005). Phenylephrine applied locally in the form of gel enhances the contractility of the internal anal sphincter (Park *et al*., 2007). It was also reported that phenylephrine topical solution can be used to control the symptoms related to urinary incontinence in stress women (Wein 2012). All these clinical uses besides the numerous studies which reported that topical application of phenylephrine solution of the skin using iontophoresis technique altered the blood flow and induced vasoconstriction through the stimulation of postjunctional α_1- and α_2- adrenoceptors in the cutaneous arteries in the human forearm (Drummond 2002).

All our results in previous chapters indicate that the contraction induced by a high concentration of phenylephrine is not sustained and decline with time and the preincubation of the tissue with a low concentration of LiCl enhanced and better maintained the duration. So, the aim of this chapter was to test the time course of the contraction induced in cutaneous blood vessels in forearm after exposure to phenylephrine and examined the effect of LiCl on that response.

Two types of interventions were design to challenge the skin blood flow in the forearm of human volunteers using laser Doppler flowmetry. The first set involved the physiological intervention by using cold pressor (CPT) and deep breath (DBT) tests. While In the second set of intervention low concentration of phenylephrine was applied to the skin of the forearm using iontophoresis technique.

6.1.1 Cold pressor test (CPT)

Is a widely used procedure to stimulate a sympathetic response in volunteers and it involves immersing the volunteer hand or foot in iced water around 4°C or below for 1-4 minutes. This will produce unpleasant stimuli leading to stimulation of the sympathetic system and release of adrenaline and noradrenaline as neurotransmitters. This release is associated with a wide range of changes in cardiovascular and haemodynamic parameters including blood pressure, temperature and heart rate (Zhao *et al.*, 2015).

In 1932 Hines and Brown developed CPT as a method to predict the future development of hypertension, they suggested that peoples who responded rapidly to stimulation with cold water are more prone to develop hypertension in the future (Hines and Brown 1933). Although other researchers argued with this hypothesis for years, Hines and Brown's theory is well established with the recent study which lasts for 9 years and included more than 1960 participants (Treiber *et al*., 2003). In the clinical field, CPT was used recently to measure cardiovascular function in pregnancy. In women who have or recently diagnosed with preeclampsia, were associated with greater response to CPT with an increase in both systolic and diastolic blood pressure with a rise in peripheral resistance in comparison to other normal pregnant women who have large circulating volume associated with low vascular resistance (Shelke *et al.*, 2014). CPT was used also to assess the pain and study its effect on the heart rate and blood pressure and investigate the differences in managing pain between the different individual (Brusselmans *et al*., 2015).

Several factors can affect the vasoconstriction of the blood vessels following CPT. In regards to the age, the response of the body is decreased with increasing the age of the participant, this could be due to a reduction in the ability of noradrenaline transmitter system or reduction in the transmitting the sensory nerve impulses (Greaney *et al*., 2015). The gender of participants can be attributed to changes in response to CPT. Most studies suggested that males have a greater rise in blood pressure than women, besides the interference produced by changes in the hormonal level during the menstrual cycle which can interfere with the results of CPT (Isii *et al.*, 2007).

The main advantages of using CPT as a useful tool in both performing the researches and also its use in the clinical field are being non-invasive, easy to perform with a reliable and reproducible result in a short time (Zhao *et al.,* 2012).

6.1.2 Deep breath test (DBT)

The DBT can also stimulate the sympathetic nervous system and produce haemodynamic changes. In this test, the volunteer asked to breathe normally before taking a deep inspiration and holding his breath for 60 seconds without trapping the air between his cheeks (Jagomägi *et al*., 2003). The previous technique repeated for three times (Allen *et al.*, 2013; Zheng *et al.*, 2012). Sympathetic and parasympathetic system can be affected by deep respiration process, which results in vasoconstriction and reduces systolic and diastolic blood pressure (Jagomägi *et al.*, 2003). During the DBT the glottis is closed, this is associated with a relaxation in the inspiratory muscles and enlargement in the thoracic cavity with an extension in blood vessels that result in a reduction in the volume of blood return to the heart and temporary reduction in both systolic and diastolic blood pressure (Markus and Harrison 1992). During the respiration, the changes in blood pressure can reach 20mmHg /cycle (Guyton and Hill 2013). Furthermore, the hypercapnia and hypoxia in the bloodstream during the deep breathing, stimulate chemoreflex stress to increase the activity of the sympathetic nervous system (Heusser *et al.,* 2009).

In a similar way to CPT, males are more affected by the reduction in blood flow following the DBT. According to Feger and Braune (2005), who measured the differences in blood flow in the index finger and found that the reduction was more pronounced in males than females and ascribing this to the differences in vital capacity between the genders. Although the use of DBT is less common that of CPT, it still has a diagnostic value for several neurovascular aspects associated with several pathological conditions including painful diabetic neuropathy, Raynaud's syndrome, leprosy and erythromelalgia (Wilson *et al.*, 1992; Abbot *et al.*, 1993; Wollersheim *et al.*, 1991).

6.1.3 Laser Doppler Flowmetry

Assessing the reactivity of microcirculation require the presence of special equipment that has the ability to record the changes in blood flow during the stimulation, in most cases the circulation needs to be stimulated or triggered by a stimuli such as exposing to sudden drop in temperature or oxygen level associated with CPT and DBT

respectively; or during the chemical application of drugs which can produce a vasodilation or vasoconstriction in skin blood flow. The vascular reactivity in large blood vessels such as femoral cerebral and brachial was measured using the ultrasound to "image" the in vivo vascular changes. It is difficult to predict the suitability of this method to measure the changes in blood flow in small blood vessels (Celermajer *et al.*, 1996; Cavill *et al*., 1998). Some researchers suggested the use of plethysmograph in microcirculation, however, the limitation was the absence of specificity to tissue beds (Allen *et al*., 2013).

Between 1970-1980 scientists start using laser Doppler flowmetry (LDF) as an experimental method to measure blood perfusion to various tissues including the skin, mucous membrane, the eye. LDF provides a simple, portable and non-invasive technique to measure the changes in blood flow, for this reason, in this chapter all the changes in the microcirculation of the forearm were measured by this device. LDF provides a very sensitive method to detect the effect of different stimuli including those resulted from physiological intervention such as localised cooling or heating, breathe holding as well as the local effect for some chemical that produce a reduction or increase in skin blood flow (Noble *et al.*, 2003; Morris *et al*., 1995; Kenney *et al.*, 1991; Hassan and Tooke, 1988).

6.1.3.1 How does LDF work?

A weak power light generated from diodes or Helium/Neon laser transferred by a probe with a wavelength 632nm and penetrate the skin. LDF measure Doppler shift as the probe emits a low power laser light that penetrates 1mm of the tissue on the surface of the tested area. The presence of red blood cells reflected some of the light while the surrounding tissue reflects the remaining light. The reflected light received by the optical fibre in the probe. The reflection extends can be affected by the velocity of blood and the number of blood cells in the area. The differences between the emitted and received laser light recorded on the screen of the unit and represent the changes in blood flow to that area. Some of the limitations of LDF is the inability to measure the precise value for blood flow due to the interference with some environmental factors such the light and heat in addition to being very sensitive to movement at the measurement site (Obeid *et al*., 1990). The advantage of using LDF to measure changes in skin blood flow also involved the ability to combine with other technical units which also used to investigate the effect applying heat and chemicals on the tested area (Bungum *et al*., 1996). A further explanation for these units will be discussed in more details in the methodology section of this chapter, see Figure 6.1

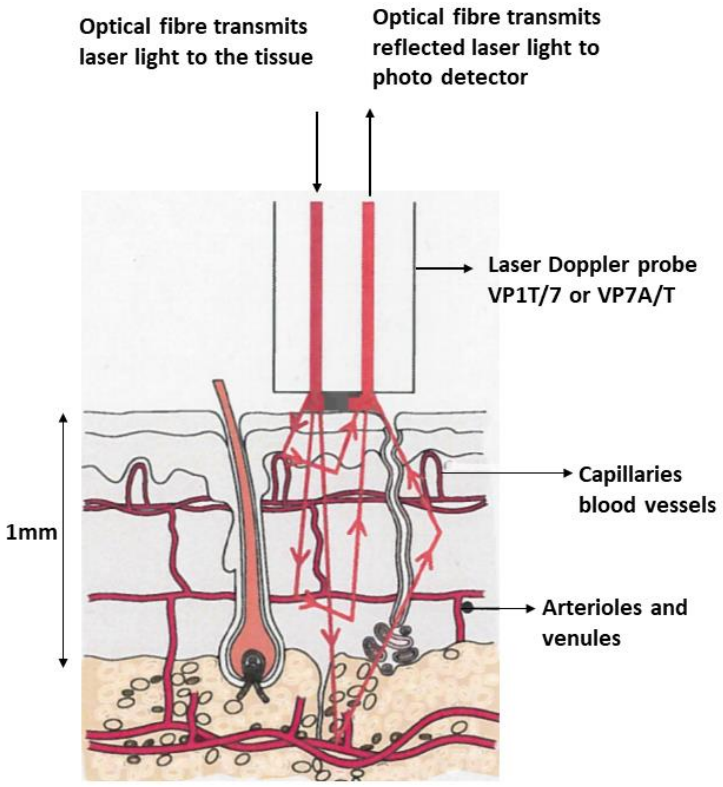

Figure 6-1 The mode of action for LDF in capillaries blood vessels.

6.1.3.2 The cutaneous microcirculation as a model of the systemic circulation?

In addition to the autoregulation which is the ability of microvascular tissue to provide a stable haemodynamic against changes in perfusion in response to various stimulus, the microvascular possess anatomical and neurovascular properties to make it a good model of the general circulation. The skin microvascular circulation consists of two groups of blood vessels, the upper network located in the papillary dermis where capillary loops arise. The second network is located lower in the dermis and consisted of ascending and descending arterioles and venules. The microvascular area is rich with nerve fibres which can control blood reactivity to different stimuli (Hill and Davis 2012). The presence of minimally invasive techniques to provide the required data regarding microcirculation and vascular function associated with many vascular disease such as hypertension, atherosclerosis, heart failure, diabetes and renal disease suggested the skin to be the perfect model to investigate the mechanism of these diseases (Carberry *et al.*,1992; Shamim-Uzzaman *et al.*, 2002; Stewart *et al.*, 2004; Cui *et al.*, 2005; Sokolnicki *et al.*, 2006).

6.1.4 Aim of this in vivo study

The primary aim of this chapter was to detect the time course for the vasoconstriction induced following the iontophoresis of phenylephrine. To our knowledge, no previous study reported the stability for the contraction induced using this drug in this preparation. In addition to that, I aimed to highlight the following points:

I. Detect the changes in microvascular blood flow following CPT and DBT in healthy volunteers.
II. Detect the effect of heating on microvascular blood flow.
III. Measure the changes in skin blood flow following the iontophoresis of phenylephrine.
IV. Investigate the effect of adding LiCl on the time course of phenylephrine iontophoreses into the skin.

6.2 Methodology

In this part of the study, the material and method related to *in vivo* study in the chapter will be outlined. The ethical approval, consent form, volunteer information sheet, questionnaire and the protocols were approved by the Ethics committee, based in Queen's Medical Centre, Nottingham. The experiments were conducted in the anaesthesia and intensive care department in the clinical research room.

6.2.1 Types of equipment

Three main units with different probes and chambers were used in this study. The main unit used for detecting the changes in the blood flow following both the physiological interventions as well as the iontophoresis of vasoconstrictors is LDF. There are two main types of this device: LDF imaging and LDF monitoring. LDF imaging acts by scanning a relatively large area of the tissue through sending laser light and generates images for the blood flow in that area with the presence of a time lag between the two ends of the area to be tested this lag is proportional to the size of the area being scanned. LDF monitoring provides the facility to monitor and record changes in blood flow on a small area of the skin using fibre optics probe that is fixed on the surface of the skin with a special probe holder and adhesive pads.

In our study LDF monitoring manufactured by Moor Instruments (Axminster, Devon, UK) was used to record the changes in microvascular skin blood flow known as MoorVSM-LDF2. This unit is compatible with other units designed for applying heat and chemicals which also used in this study. As mentioned in the introduction LDF produce laser light with a wavelength 785(±10) using a power of 0.5-1.5mW that allow 1mm tissue penetration without producing heat when applied to the skin area. Two main probes types were used in conjugation with MoorVSM-LDF to transmit laser light to the surface of the skin in the forearm, The DP1T/7 probe is a ring shape probe have centrally delivering fibre surrounded by 8 fibres to collect the reflected laser light. This probe mostly used in our study to detect the changes in blood flow during CPT and DBT. It is characterised by a low coefficient of variation due to its large surface and multiple receiving fibres. The second probe is DP12-V2, this has a needle-like a shape usually used with the heating probe (SHP2) it penetrates the heating probe and can measure the blood flow as well as the temperature through two fibres, present in the central part of the probe. According to manufacture instruction, those probes need to be cleaned and calibrated between subjects using the special solution provided by Moor company. Adhesive pads used to fix the plastic probe holder on the skin surface of the

forearm. The MoorVSM-LDF2 is very sensitive and several internal and external factors can interfere with the accuracy of reading during the procedure. These factors include internal factors such as

1- Mental state for the volunteer before and during the measurements. This can be avoided by allowing 30-40 minutes for each subject to acclimatise to the environment before starting the procedure
2- Breathing: The subject is advised breathing normally during the test because deep breathing can significantly reduce blood flow in the microcirculation
3- Avoid meals and drinks which contain caffeine, alcohol and nicotine in addition to the use of pain killers such as non-steroidal anti-inflammatory drugs and aspirin

The external factors include the following:

1- The movement of either the probe or the subject during the procedure. This can be avoided by supporting the arm in a comfortable position and the use a probe holder which is used to fix the probe in 90° angle to the skin surface with the avoidance of the superficial veins and the cubital fossa area to reduce the intervention with reading. The subject is discouraged from moving and talking during the procedure.
2- Direct light such as sunlight can interfere with LDF ambient temperatures and humidity of the place where the measurement takes place.
3- The force of applying the probe to the skin surface can reduce blood flow but the use of a proper probe holder can minimize this problem.
4- The presence of hairs in the area being tested can interrupt the reading of LDF2 so excess hair should be removed carefully using a new razor to avoid producing any wound.

The MoorVMS-LDF2 can provide a continuous reading for the following parameters

FLUX: the most common parameter usually reported in studies investigating blood flow. It represents the net effect of a number of red blood cells and the velocity of these cells in the measured area. Due to the wide variation in blood vessels, the colour of the skin and the structure of the skin in the tested area the use of the absolute unit to measure blood flow is inconvenient. It was agreed by both researchers and manufacturers to use perfusion unit (PU) to measure the changes in the microvascular circulation of the skin.

DC: represent the intensity of the laser light used measured by arbitrary unit (AU).

Temperature: the temperatures of skin in the tested area in Celsius degree (°C).

The second unit is the heating unit Moor SH02 used to control the temperature between 20-45°C at the site of interest when heating was used to induce hyperaemia. A special heating probe known as SHP2 used to connect the heating unit with the subject. This probe able to control the temperatures and usually used in combination with a VP12 probe that connected to the MoorVMS-LDF2 unit to record the changes in blood flow. In our study, we used the heating unit to raise the temperatures to 42°C and induce hyperaemia (increase blood flow to the area). This step facilitated the detection of vasoconstriction effect following the transdermal application of these vasoconstrictor agents. The area of interest in this study was the forearm, where the probe and chamber were fixed to measure the changes in blood flow resulting from either the physiological intervention with CPT and DBT or preheated the area and the iontophoresis of vasoconstrictors agents. The forearm has a large surface that provides the required area to fix the probes and chambers easily with minimal movements. Avoid placing the probe or iontophoretic chamber on superficial blood vessels or the cubital fossa area to reduce the interference with the reading.

The third unit used in this study was MIC2 which was used for transdermal drug delivery in a process known as iontophoresis. Iontophoresis is a simple process to use a small electrical current to enhance the penetration of ionic drug to the subcutaneous layer. MIC2 unit to provide the control of the iontophoresis and it is compatible with the MoorVMS-LDF2 which used to measure the changes in microcirculation during the iontophoresis process. MIC2 unit intended to be used in the research process rather than for treatment purpose. It has two electrodes (cathode and anode), one is attached to the chamber where the drug to be added and the second electrode is attached to inert wire has 4*4cm area rubber pad known as (MIC-CP) usually placed 10cm away from the chamber and fixed on the skin using adhesive conductive gel pad to close the electrical circuit. Different factors can affect the efficiency of the iontophoresis and affect the amount of drug delivered this include:

1- The used current and duration of the process (can be controlled either manually from the unit or remotely using the Moor software V4.1).
2- Solubility and ionisation degree of the drug.
3- The pH of the solution and concentration of the drug to be applied.
4- The thickness of the epidermis at different point of the skin surface.

Some clinical applications of iontophoresis include applying fentanyl which as an analgesic drug to manage pain. Furthermore, it is used to deliver H_2O to treat hyperhidrosis. Some other uses include reverse iontophoresis to measure the level of glucose in diabetic patients or to attract some ions from the bloodstream. The main advantage of iontophoresis over systemic administration of the drug by oral or parenteral route is that it provides a non-invasive method and reduce the side effects associated with the high doses of the drug and preventing the 1st pass effect that might reduce the efficacy of some drug. Moreover, the skin provides a large area and is easily accessible in both volunteer and patients. Two major limitations of iontophoresis are the exact amount of the drug being delivered by iontophoresis is not known. In addition to the use of high frequency can produce a galvanic effect or electrical vasodilation of the blood vessels due to the passage of a high electrical current which is usually reduced by using intermittent low current. It is also important to notice the polarity of the drug as the positive ions should be connected to the positive electrode while the negatively charged drug should be connected to the negative electrode. The drug intended to measure its effect on the blood flow is kept in a syringe without the needle and warmed before use. When a drug is injected into the chamber and to avoid trapping of the air allow the excess drug to leak from the other opening.

6.2.2 Drugs

The drugs used in this chapter were phenylephrine hydrochloride ampule 10mg/ml, which is an α_1-adrenoceptor agonist, Amdipharm UK Limited, London, UK. This diluted in 0.9%NaCl 10ml vial. The stock solution of phenylephrine was diluted on the day of use using 100µl of phenylephrine (1mg) to be added to 900µl NaCl then the total volume of the diluted solution was 1000µl. 100µl was added to the iontophoretic chamber before the iontophoresis of phenylephrine.

6.2.3 Protocols

Three protocols were used in this chapter to cover three main areas, the first was the physiological intervention with CPT and DBT and second, was the effect of heating on the skin blood flow using two types of probes while the third protocol was the iontophoresis of phenylephrine. These protocols were approved by the Ethics Committee at Queen Medical Centre (QMC), Nottingham in the Ethics No:21-1805 on 13th August 2018. See appendix 1

6.2.3.1 Protocol one: the CPT and DBT

Eleven healthy male volunteers were recruited for this study. After a screening visit to check the tolerance of the volunteers to the test conditions and filling the questionnaire form and signing the consent form, 9 healthy male volunteers agree to participate in the study, while one volunteer could not tolerate the pain after experience the CPT and withdrew from the study while the other volunteer showed irregular response. On the day of the test, blood pressure and heart rate were tested prior and after the CPT and DBT. The duration of the protocol was around two hours.

After the safety notice associated with the study was read by each volunteer, he was asked to sit down in a comfortable position. After 30 minutes of acclimatisation to the environment and room temperature, A VP1T/7 probe was fixed on the right forearm. This probe is connected to the MoorVMS-LDF2 unit. For the CPT 3 litres of iced water at 4°C in a container was prepared and the volunteer was asked to submerge his left hand up to the elbow in the water for one minute and to avoid touching the bottom of the container. After 1 minute the volunteer removed his left hand and the researcher dried it with a paper towel with minimum movement to avoid interpreting the reading which was recorded by LDF2. The volunteer rested between 5-8 minute before the DBT started. DBT involved asking the volunteer to take a deep breath and holding it for 60 seconds, followed by normal breathing. The volunteer was asked to avoid trapping the air in the mouth or between his cheeks. This was followed by another rest period for 5-8 minutes. Each test was repeated for three times and ending up with 6 intervention during the first day, see the protocol in Table 6.1. The screening and the test was conducted in the anaesthesia department in a temperature-controlled clinical study room with the presence of medical cover to be contacted in any emergency situations.

Table 6-1 The protocol for the physiological interventions followed by the 9 volunteers participated in this study

Rest	***CPT1***	***Rest***	***DBT1***	***Rest***	***CPT2***	***Rest***	***DBT2***	***Rest***	***CPT3***	***Rest***	***DBT3***
30	***1***	***8***	***1***	***8***	***1***	***8***	***1***	***8***	***1***	***-8***	***1***
min	***min***	***min***	***min***	***min***	***min***	***min***	***min***	***min***	***min***	***min***	***min***

6.2.3.2 The statistical calculation for physiological interventions

Due to the fact that LDF2 record the changes in blood flow each minute. The mean±SEM value for each test in each volunteer was calculated as 1 minute before the intervention and 1 minute during the intervention. The percentage of changes was calculated by comparing the mean values before and during the test. The graph which compares the changes in blood flow after each test was produced by expressing the mean±SEM two minutes before and two minutes after each intervention. The perfusion unit at -1 minute was considered a reference for the other responses. Paired *t*-test was used to compare the responses after each intervention. With P-value< 0.05 consider as statistically significant.

6.2.3.3 Protocol for localised heating

In order to reveal the action following the iontophoresis of vasoconstrictors, prior vasodilation was induced using local heating by SH02 unit and SHP2 probe. The changes in blood flow were recorded on MoorVMS-LDF2 unit using VP7A/T probe which penetrates the heating probe.

The heating procedure was applied to three groups. The first set of experiments used the same volunteer and measured the hyperaemia resulted from increasing the temperature to 42°C in three separate visits. In this set of experiments using these probes and a plastic holder to fix the SHP2 probe on the forearm away from cubital fossa and superficial blood vessels while the control probe was used to measure the blood flow at a distance around 5cm away from the first probe using VP1T/7 probe. The duration of the protocol was around two hours. After the volunteer read the safety notice associated with the study he was asked to sit down on in a comfortable position. After 30 minutes of acclimatisation to the environment and room temperature then the previously mentioned probes were fixed on the supported left forearm. 5-minute recording of the base blood flow followed by turning the heating unit on to start heating the area. Continued recording the changes in blood flow for about 120 minutes. In the second set of experiments, similar steps in the previous protocol applied to 4 different healthy men volunteers. Using the same probes and unit.

In the third group 10 healthy men volunteers, were recruited to be involved in this test. We used the same equipment and probes except the heating probe was not fixed directly to the forearm skin however it was fixed on the iontophoretic chamber MIC-ION3-P2. The latter provided a gap between the skin and the heating probe. The

heating was not effective unless a solution was added to the iontophoresis chamber to provide direct contact between the heating probe and skin surface.

6.2.3.4 Protocol three: the effect of localised heating and iontophoresis administration of phenylephrine

Sixteen healthy men were recruited for the iontophoresis study, ten volunteers have received the 1mg/ml concentration while the other six received different concentrations of phenylephrine. They spent 30 minutes to acclimatise the environment (heat and humidity) in the room. The area to be iontophorised was prepared by removing the hair using a razor (use cotton to clean it with ethanol) before MIC-ION3-P2 Iontophoresis chamber with SHP2 and VP12 probes being fixed on the right forearm using double adhesive discs. The other inert electrode was fixed 10cm away from the iontophoretic chambers using conductive gel pads. The temperature was set to 42°C using SH02 unit. The iontophoresis process conducted using MIC2 device (manufactured by Moor Instruments, UK) which was connected to the LDF (manufactured by Moor Instruments, UK) part using a USB cable. The current used in this study to deliver the drugs is 50μA for 60 seconds.

a stock solution of phenylephrine (1%) was previously diluted to 0.001 and located in a syringe without a needle to be applied in the iontophoresis chamber. All measurements began with a recording of skin perfusion for 10 minutes to get the baseline values. During the iontophoresis process, the heater was kept on to provide better vasodilation result with both phenylephrine and noradrenaline are delivered by anodal iontophoresis. On the volunteer left arm blood, pressure and pulse rate were recorded using OMRON electronic device before during and after the iontophoresis to exclude any systemic effect for the drugs. Usually, the procedure lasts for 60 minutes (30 minutes acclimatisation and 30 minutes of heating and iontophoresis).

Figure 6-2 Vascular Monitoring System MoorVMS-LDF2 (Moor Instruments)

Figure 6-3 VP1T/7 Combined large area optic and temperature probe (Moor Instruments)

Figure 6-4 VP7A/T large area laser Doppler probe with temperature (Moor Instruments)

Figure 6-5 VP12 small area optic probe used with SHP2 heating probe (Moor Instruments)

Figure 6-6 Moor Skin Heater Unit SH02 and SHP2 Heating probe (Moor Instruments)

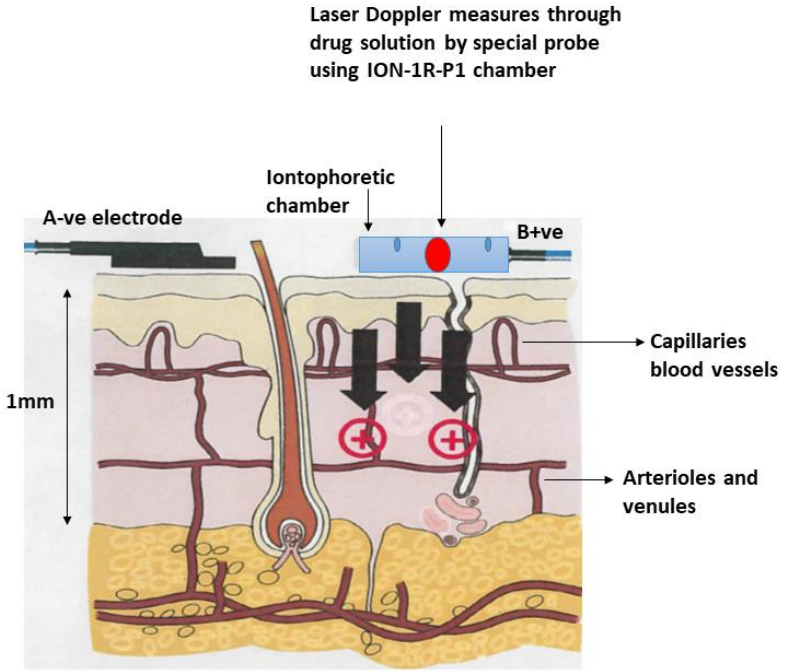

Figure 6-7 The mode of action of the iontophoresis in skin microcirculation

Figure 6-8 Iontophoresis Unit MIC2 and MIC-ION3-P2 Iontophoresis chamber (Moor Instruments)

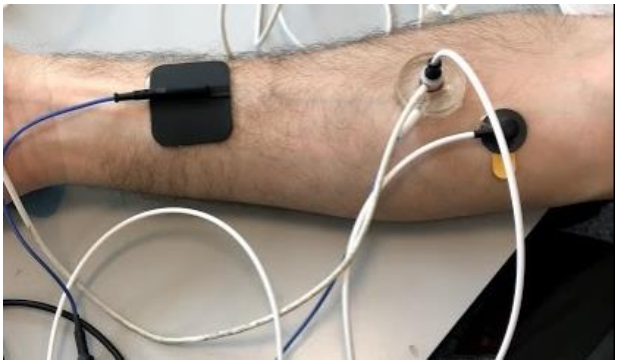

Figure 6-9 Picture shows the site of probes fixing on volunteer forearm during the iontophoresis with the prior heating process

6.3 Results

6.3.1 Protocol one: changes in the microcirculation of forearm following the physiological interventions with CPT and DBT

Eleven volunteers were recruited for this study. After the screening visit, one volunteer decided to withdraw after discomfort feeling during the CPT while other volunteer showed irregular responses to both tests and was excluded to reduce the margin of error. Nine male volunteers aged between 18 and 36 with a median age 26.

Figure 6.10 A & B showed that exposing the volunteer to either CPT or DBT resulted in a decrease in the perfusion unit. In addition to that, the first CPT produced the most noticeable effect when compared with the second and third tests. In contrast, the three DBT produced a similar drop in the blood perfusion.

Expressing the results of 9 volunteers produced a time course for the decrease in blood flow seen in Figure 6.11. The drop in skin perfusion produced by both tests was reversible, the body increased the blood flow after ceasing the intervention (each intervention last for 1 minute) within 1-2 minutes. Furthermore, our results showed that DBT produced a larger reduction in blood perfusion than the CPT and the difference was statistically significant with P-value<0.05, using a paired Student's *t*-test (n=9).

As seen in Table 6.2 the mean value for three interventions of each test was calculated using the reading of blood flow during one minute before the intervention. Comparing the mean for these reading shows the percentage of the drop in blood flow in each volunteer. Our results showed that the percentage of the drop in blood flow (% of change) due to CPT was (-19.6±1.9, n=9) While a larger drop was noticed with DBT (-29.8±2.6, n=9). The difference between the two tests was statistically significant with a P-value<0.05 using a paired *t*-test. Our results also indicate that the presence of inter-individual variation which was larger in magnitude to the differences in the same individual indicating the variability of blood flow even in healthy normotensive male participants.

Table 6-2 Compares the percentage of changes in the mean blood flow in 9 volunteers before and after experienced three CPT and/or three DBT, responses were expressed as a perfusion unit.

Volunteer	**Control**				**Cold Pressor Test (CPT)**				
	1ST	2ND	3Rd	Mean±SEM	1ST	2ND	3Rd	Mean±SEM	% Change
1	21	20	20.7	20.9±0.1	15.1	14.5	16.2	15.27±0.5	**-26.9**
2	17	12.8	15.9	12.5±0.6	13.5	11.3	12.9	15.2±1.2	**-17.5**
3	36	26.5	29.6	30.7±2.7	29	22	20	23.6±2.7	**-22.9**
4	18.5	16	16.6	17±0.4	14	12.5	13	13.1±0.4	**-22.7**
5	6.2	5.3	4.2	5.2±0.5	5.6	4.2	4.9	4.9±0.4	**-6.3**
6	25	27	26	26±0.5	20.2	22.3	21.7	21.4±0.6	**-17.6**
7	12.5	8.4	9.6	10.1±0.49	8.4	6.8	7	7.4±0.5	**-27.2**
8	22.3	26	27.1	25.1±1.4	17.8	24.5	19.5	20.6±2	**-18.0**
9	12.7	12.9	11.5	12.3±3.7	9.5	9	11.1	9.8±0.5	**-18.0**
Volunteer	**Control**				**Deep Breath Test (DBT)**				
	1ST	2ND	3Rd	Mean±SEM	1ST	2ND	3Rd	Mean±SEM	% Change
1	18.5	20.1	24.8	20.9±0.1	10.1	14.1	15.7	13.3±1.6	**-37.0**
2	14	12.6	14.9	13.8±0.6	10.1	9.7	10	9.9±0.1	**-28.4**
3	30.3	20.5	26.9	25.9±2.8	20.3	15.9	14	16..7±1.8	**-35.3**
4	17	16.4	18.6	17.3±0.6	14.7	11.5	13.2	13.1±0.9	**-24.2**
5	5.2	5.1	4.5	4.9±0.2	3.8	4	4	3.9±0.06	**-20.2**
6	21.3	31.2	25.5	26±2.8	18.8	26.6	14.9	20.1±3.4	**-22.6**
7	8.7	12.6	10	10.4±1.1	5	5.2	6.7	5.5±0.5	**-45.6**
8	20.3	20.6	23.4	21.4±0.9	13	16.7	13.2	14.3±1.2	**-33.2**
9	12.6	15	13	13.5±0.74	12.2	8.5	7	9.2±1.5	**-21.7**

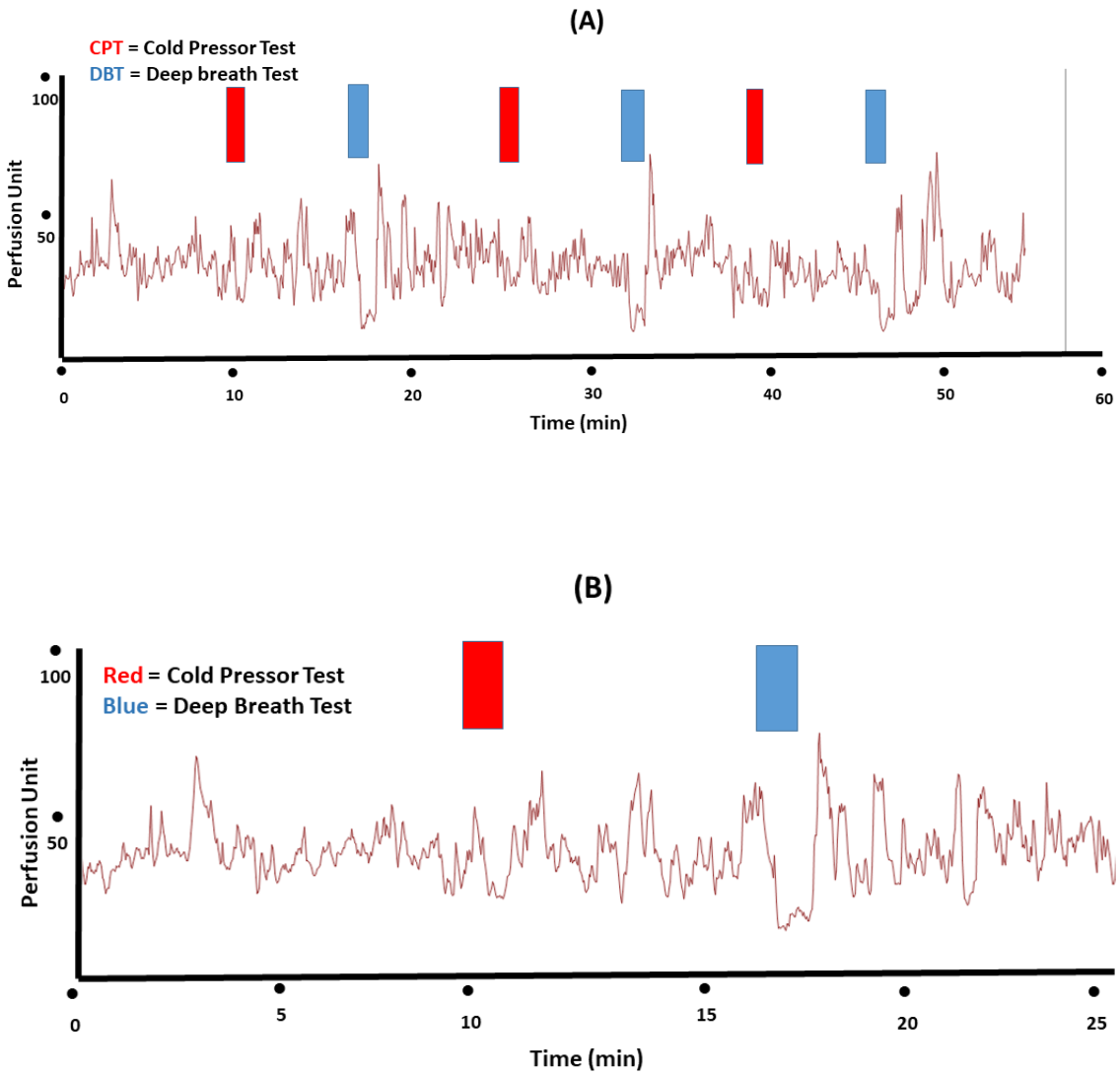

Figure 6-10 Traces from LDF emphasise the changes in blood perfusion after (A) six or (B) two, interventions using CPT and/or DBT in a healthy volunteer. Eight minutes resting interval separated each test. Data expressed as a percentage of perfusion unit.

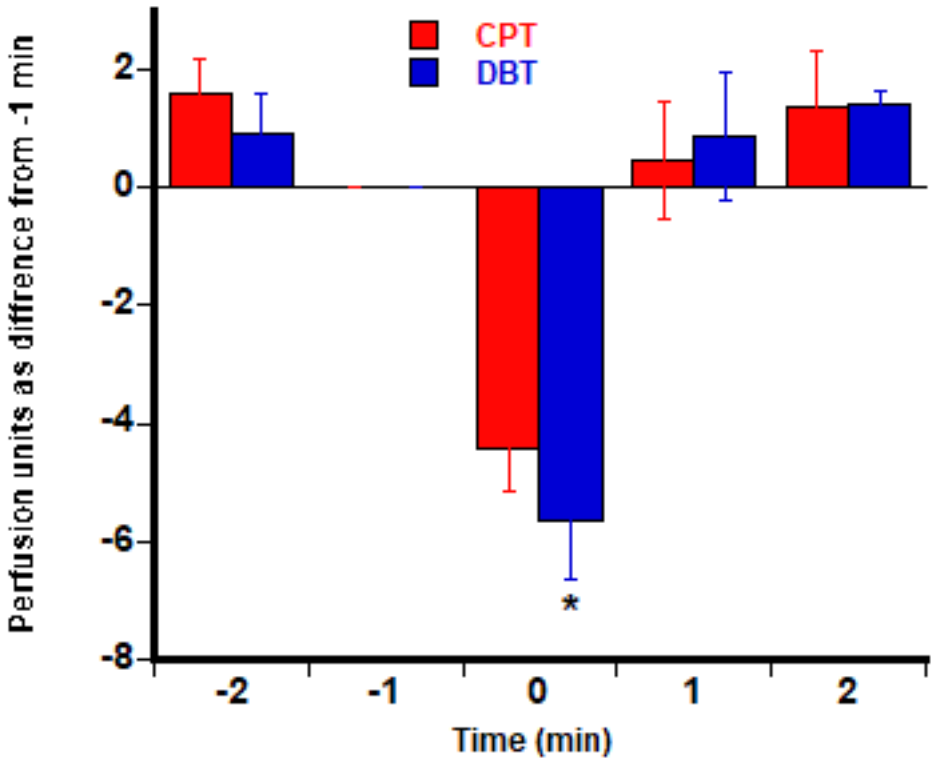

Figure 6-11 The time course for the changes in blood flow expressed as a perfusion unit before and after performing the physiological interventions with CPT and DBT in 9 healthy volunteers. The value was expressed as a percentage to the reading of -1 minute before the intervention and are mean ± SEM. * - Denotes a statistically significant difference ($p < 0.05$) between tests using a paired Student's *t*-test.

6.3.2 Protocol two: The effect of localised heating on microcirculation perfusion in the forearm of a healthy volunteer using different probes

According to protocol two which is previously mentioned in the methodology of this chapter, three sets of experiments were conducted to examine the effect of local heating on the perfusion of microcirculation in the forearm.

The first set of experiments was conducted in the same volunteer. Three measurements of blood flow were performed during three separate visits. As seen in Figure 6.12 (A) the rising the temperature to 42°C increased blood flow in the heated area. A gradual increase in blood perfusion which reached its peak after about 45 minutes. The effect of heating started to decline slightly but remained very high in comparison to the control, in which the response was stable during the 120 minutes procedure.

In the second set of experiments and as seen in Figure 6.12 (B), using a group from four volunteers with a median age 29 resulted in similar changes in the perfusion of the blood to what we have previously mentioned using the same volunteer. Results were expressed as a percentage of peak response to reduce the individual variation in blood flow.

The third set of the experiment involved the use of iontophoretic chamber to fix the heating probe and as mentioned in protocol two in the methodology. A group of eight male volunteers aged between 18 and 35 with median age 28 were recruited for this test. Increasing the temperature to 42°C without adding a solution to the iontophoretic chamber failed to produce any changes in the baseline blood flow in the microcirculation.

Once we added a solution (at t=10) rapid increase in the perfusion rate of blood flow was recorded this increase reached its peak after 30 minutes of the addition and remain stable for a maximum of 20-30 minutes before the start to decline to baseline. Results were expressed as a percentage of peak response to reduce the individual variation in blood flow, see Figure 6.13

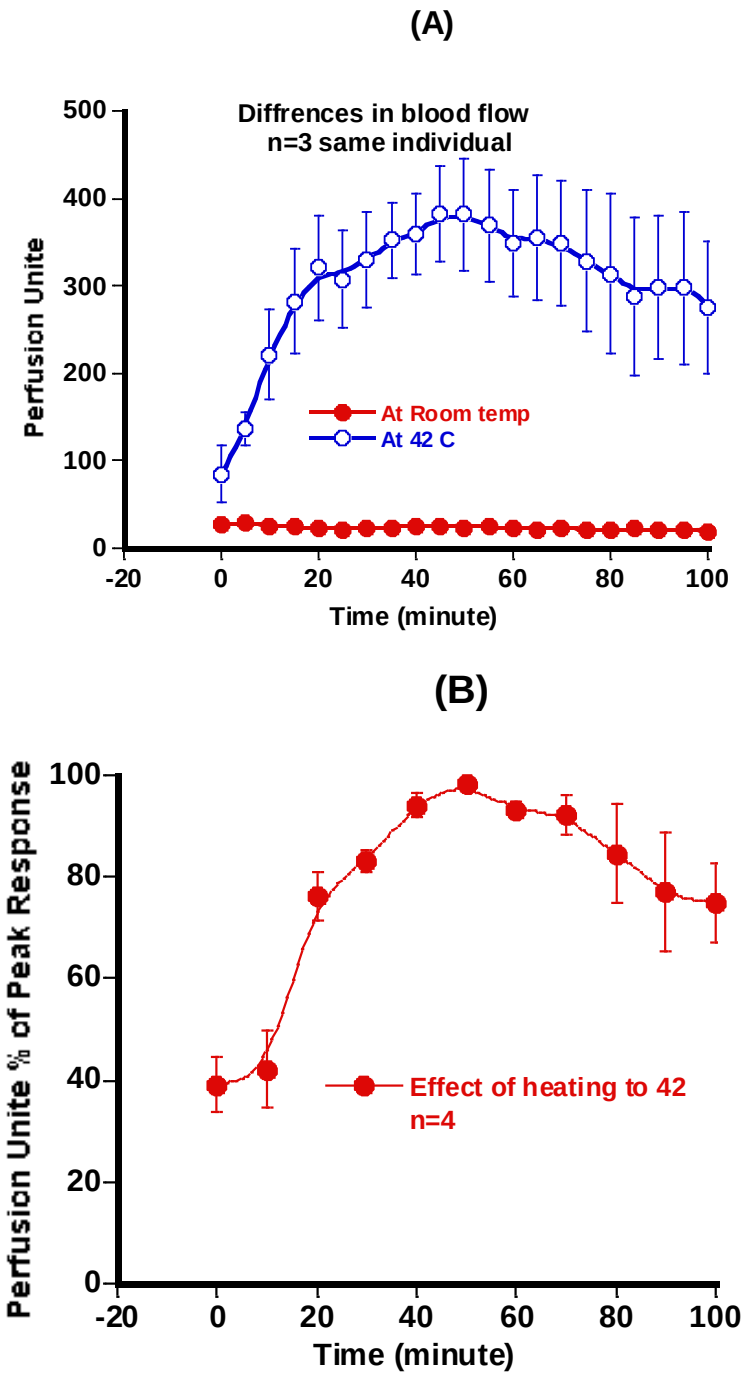

Figure 6-12 The changes in perfusion of blood flow of the forearm of (A) same volunteer or (B) group of 4 volunteers after heating the area to 42°C. The values are mean ± SEM and expressed as (A) a perfusion unit and (B) as a percentage of peak response.

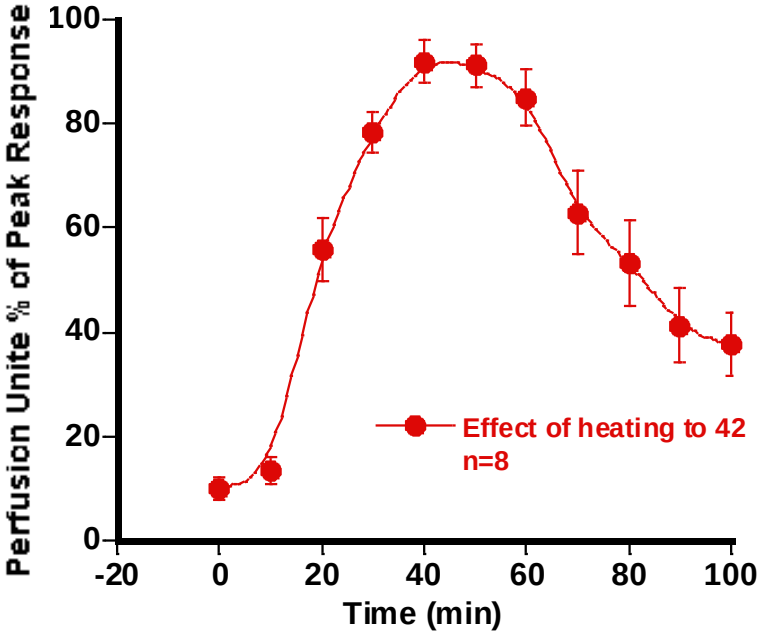

Figure 6-13 The changes in perfusion of blood flow to the forearm of a group of eight volunteers after heating the area to 42°C using the iontophoretic chamber to fix the heating probe. The value is mean ± SEM and expressed as-as percentage of peak response.

6.3.3 Protocol three: The effect of localised heating and iontophoresis of phenylephrine on blood perfusion in the forearm of a healthy volunteer

In this set of experiments, 16 male volunteers were recruited. The exact concentration of phenylephrine to be used was obtained following a screening the response using different dilutions of the stock solution (concentration of the stock solution was 10mg/ml). As can be seen in Table 6.2 all volunteers failed to show response when 0.1mg, 0.3mg were used. The use of 1mg/ml concentration was able to produce vasoconstriction and reduced blood flow in ten volunteers. In the majority of the 16 volunteers responded positively to the vasodilation induced by preheating to 42°C.

We followed the third protocol mentioned in the methodology section to report the changes in the blood perfusion after the iontophoresis of phenylephrine using 50μA current for a 60-second duration. As we reported previously the effect of heating was observed after the solution of the drug being added to the iontophoresis chamber and not before that. Heating process was continued throughout the protocol.

Our results for the iontophoresis of phenylephrine in ten volunteers suggested that the vasoconstriction resulted following the iontophoresis of the drug is irreversible and the circulation needs more than 40 minutes to recover from the effect of the drug and to re-maintain normal perfusion to the area. Figure 6.16

Table 6-3 The responses of 16 volunteers to iontophoresis of different concentrations of phenylephrine and heating to 42˚C, where

Blank= did not try - = no response

+ = positive response (there is more than 30 % change in blood flow)

	Response to different concentration of phenylephrine							**Response to heat 42˚C**	
Volunteer	**10mg/ml**	**5mg/ml**	**2.5mg/ml**	**1.25mg/ml**	**1mg/ml**	**0.3mg/ml**	**0.1mg/ml**		
V1	+	-	-					+	
V2	+	+	+					+	
V3	+	-	-						
V4		+	-	-	+			+	
V5					+			+	
V6					+			+	
V7				+	-	+		+	
V8					+			+	
V9					+			+	
V10					+			+	
V11					-	-	-	+	
V12					+			+	
V13					-		-		
V14					+		-	+	
V15					+	-		+	
V16					+		-	+	

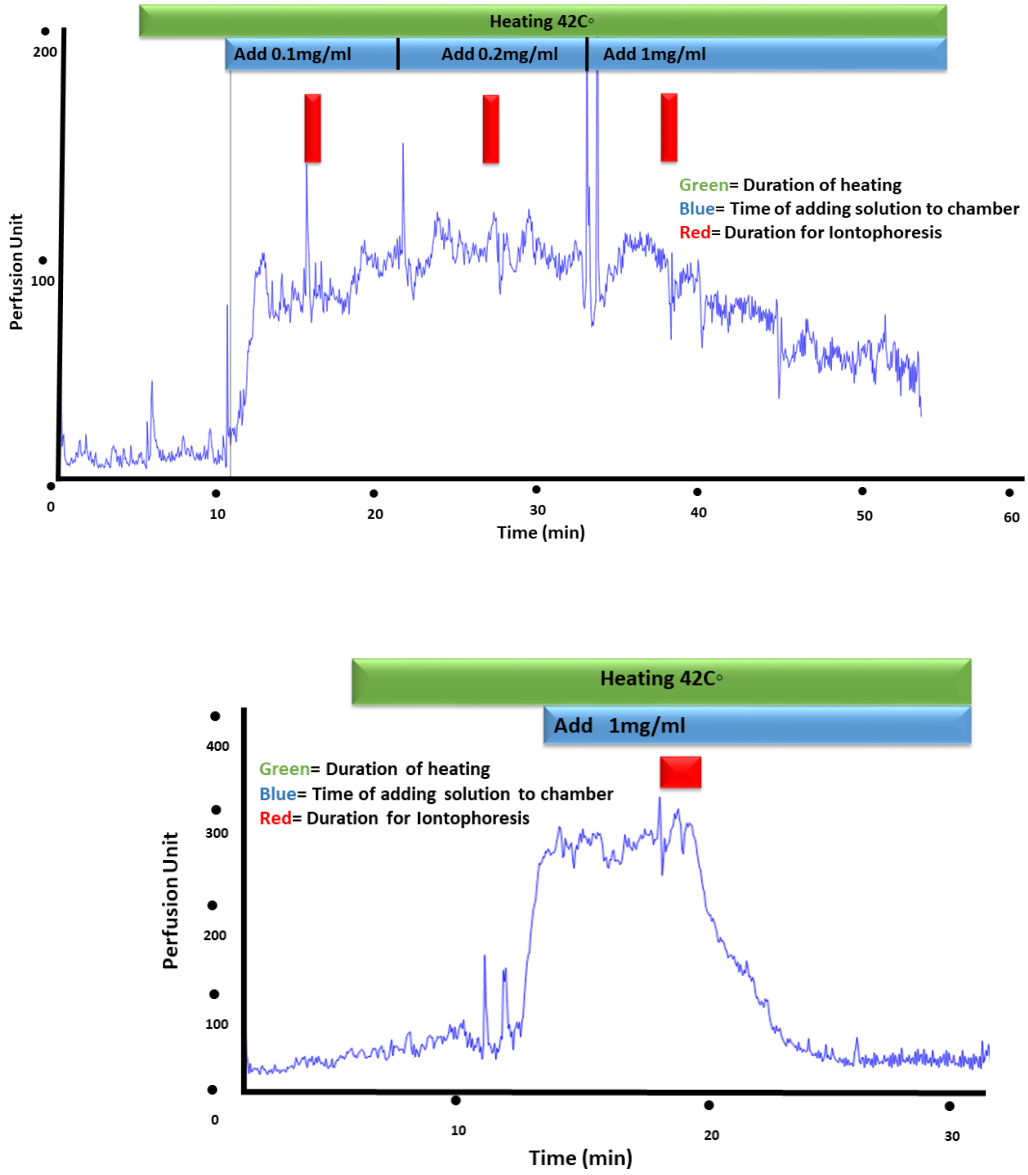

Figure 6-14 The representative traces for the response of two volunteers to the (upper) heating and iontophoresis of three different concentration of phenylephrine and (lower) heating and vasoconstriction response resulted from the iontophoresis of 1mg/ml phenylephrine. The duration of iontophoresis was 60 seconds while the heating process lasts for the entire procedure in both cases.

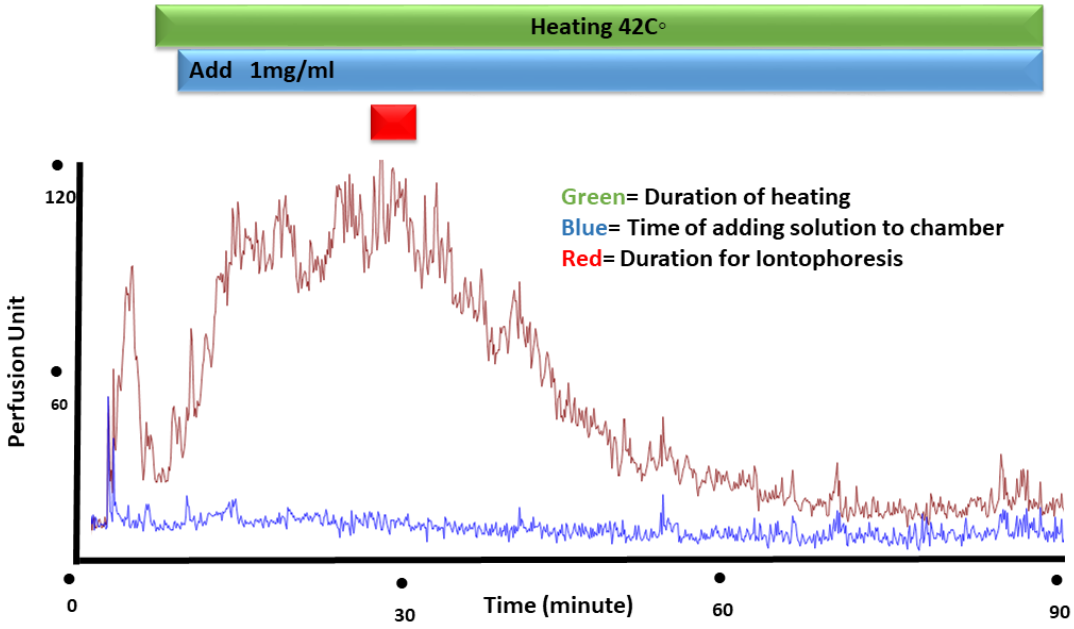

Figure 6-15 The representative traces for the response of heating and vasoconstriction response resulted from the iontophoresis of 1mg/ml phenylephrine. The duration of iontophoresis was 60 second using 50μA current while the heating process lasts for the entire procedure in both cases.

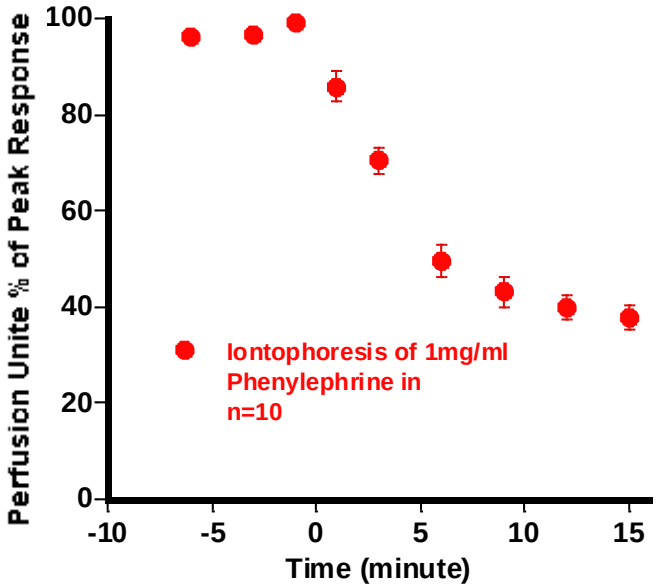

Figure 6-16 The time course for the vasoconstriction effect following the iontophoresis of 1mg/ml of phenylephrine using 50μA current for 60 seconds in 10 volunteers. Data expressed as a percentage of peak response and are mean±SEM.

6.4 Discussion

The main purpose of this study was to investigate the effect of transdermal application of phenylephrine on the microcirculation blood flow in the skin of forearm in healthy men and to compare that response with the time course of the response to phenylephrine if the lithium chloride pre-iontophorised to the same area. Our previous in vitro results using isolated vascular and non-vascular tissue confirmed that LiCl synergistically enhanced the time course of some α_1-adrenoceptors agonists. In order to achieve these goals, we first needed to optimise protocols for measuring the changes in microcirculation blood flow using the LDF after exposure to physiological stimuli such as cold and hypoxia, local heating of the area and the topical application of chemical agents which reduce blood flow using the iontophoresis technique.

Our results show that both CPT and DBT reduced the skin blood flow in the forearm in a reproducible manner. Each intervention last for 60 second and the body recovered from that drop in blood flow after 1-2 minutes. The reduction in blood flow was more significant with the deep breathing comparing to cold pressor. In addition to that, our results show the presence of inter-individual variation in both the basal and the response of blood flow to these physiological interventions was larger than the differences in the reading of repetitive test in the same individual.

Furthermore, localised heating of the area using the SH02 device to 42°C induced vasodilation in the microcirculation and increased blood flow to the area significantly. This was reported with the same individual, group of 4 and a group of 10 using either direct contact between heating probe holder and skin surface or indirect contact using iontophoresis chamber to hold the heating probe. The duration of vasodilatation lasted for more than 100 minutes with the direct contact while it was less stable using the iontophoresis chamber and last for less than 70 minutes before the start to drop. Moreover, the iontophoresis of phenylephrine in a concentration of 1mg/ml using 50mA as a current and for 1-minute duration produced irreversible vasoconstriction in the area, the body failed to recover normal blood flow even after 90 minutes of ceasing the iontophoresis process.

6.4.1 Physiological interventions with CPTs and DBTs

LDF was developed in 1964 to measure the changes in skin blood flow during rest and challenged situations. Different types of physiological interventions were used to induce vasoconstriction which mainly results from the increase of sympathetic nerve activity. Two of the most common, reliable and simple methods to induced vasoconstriction are CPT and DBT.

The CPT has been used for more than 90 years in the prediction of cardiac diseases such as hypertension (Hines and Brown 1936). While deep breathing is well documented to stimulate autonomic activity and induce vasoconstriction (Bolton and Carmichael, 1935). Both tests were commonly used to assess the responsiveness of cardiovascular circulation and they act by stimulation of the sympathetic nervous system and causing an increase in both the peripheral resistance and heart rate. Recently the skin microcirculation has been used as representative vascular bed to examine the potential vascular diseases including renal disease, hypertension, type II diabetes, peripheral vascular disease, ageing, coronary artery disease and heart failure (Carberry *et al.*, 1992; Shamim-Uzzaman *et al.*, 2002; *Rajashekar et al*., 2003; Stewart *et al.*, 2004; Cui *et al.*, 2005; Sokolnicki *et al.*, 2006; Holowatz *et al.*, 2008).

The over-reactivity of skin microcirculation due to some physiological stimulus such as cold and hypoxia can indicate high suitability to develop hypertension or another vascular disease in the future, this fact was reported in a cohort study which lasts for 28 years (Kasagi *et al*., 1995).

The CPT can produce significant changes in blood flow at a different time point before, during and after the test. Zhao and his group in (2012) reported that the reproducibility of CPT can be measured by recording the changes in blood flow at three-time points 1, 0, 2 minutes after the test. In their study which included 568 participants, they showed that during the test which lasts for 1 minute there was a significant drop in blood flow to the forearm and the body recovered this drop after 2-4 minutes. These results are in line with our results associated with CPT and DBT in healthy men which show that the effect of these test can last for a 1-2 minute followed by a rapid recovery of the normal microcirculation in the tested area. Di Marco and his colleagues in (2012) tested the effect of deep breathing in 20 healthy subjects on the changes in systolic and diastolic blood pressure. They reported that deep breathing induced a significant drop in systolic blood pressure, this drop ultimately affected the skin blood flow in the skin microcirculation. In another study conducted by Feger and Braune (2005) using

60 subjects, the effect of CPT and DBT were compared. All subject produced vasoconstriction following DBT while 95% produced vasoconstriction following CPT. Their vasoconstriction responses were inversely proportional with the age while gender differences were only significant with deep breathing but not CPT. This was attributed to the size of men usually larger than women which increase the vital capacity of the lung.

These results are in line with our finding that compares the changes in skin blood flow in healthy subjects following both DBT and CPT. The drop in forearm blood flow was significant following both tests however, it was much more pronounced following DBT.

6.4.2 Effect of local heating and iontophoresis of phenylephrine of skin blood flow

As mentioned in the introduction the reactivity of microcirculation can be impaired and affected by many medical disorders. Hence researchers start using the microvasculature to reflect the state of the macrovascular and provide an accurate tool for assessing, diagnosis, prognosis and even treatment strategy for any medical condition (Holowatz *et al.,* 2008).

The transdermal drug delivery using iontophoresis is performed by a non-invasive application of small electrical current that enhances the penetration of the ionic drug to the epidermis layer, the action of the drug can be detected by the use of LDF system (Beed 2011). The majority of drug applied using iontophoresis were vasodilator drugs such as nitroprusside, bradykinin and acetylcholine with minimal focus on vasoconstrictors. That could be due to the fact that, the low flow rate in the skin microcirculation during the resting condition (Brown *et al*., 2003).

In order to overcome this difficulty in detecting the action of vasoconstrictor agents on skin blood flow using LDF, local heating of the area was introduced (Beed *et al*., 2009). Local heating produces vasodilation or (hyperaemia) in the heated area, partially through inhibition of vasoconstriction induced by adrenergic stimulation; as denervated skin still shows vasodilation response when heated after sympathectomy. In addition to neuronal control of blood flow the local endothelial produced vasoactive substances which also control blood flow; the key role here is for nitric oxide (Lipnicki and Drummond 2001).

The hyperaemia can be defined as the regulation of blood flow according to body needs in health and disease states. There are two main types of hyperaemia, the active hyperaemia which increases blood flow to tissue during an extra activity such as increase blood flow to the muscle during exercise. While the reactive hyperaemia result after tissue deprived of oxygen for different reasons such as applying pressure to the peripheral organs (Bliss 1998).

In this study, we used local heating to 42°C to produce a reproducible hyperaemia. All subjects initiated strong responses which were reported in the same individual, in a group of 4 as well as in a group of 10 subjects. The time course for the vasodilation induced by direct local heating using SHP2 probe decline by 20% after 100 minutes in a group of 1 and 4 subjects.

On the other hand, using the local heating with the iontophoresis chamber produced a peak response after 40 minutes with a plateau for less than 40 minutes before the body starts to adapt the vasodilation and recover the baseline blood flow when NaCl was added to the chamber. Furthermore, the experiments which involve the iontophoresis of phenylephrine, LDF reported irreversible contraction induced by phenylephrine which was expressed as a 50% drop in the blood flow.

Although some of the previous studies investigated the skin response to local heating and described two phases of response with an initial rapid vasodilatation followed by a plateau (Brusselmans *et al.*, 2015), we couldn't specify similar phases in our results which showed a delay in the peak response before reaching the plateau which did not last for less than 30 minutes. This could be because they have used a higher temperature (44, 45) °C to induce local vasodilation while we avoid using such high temperature to avoid complication. In a similar study (Henricson *et al*., 2011) preheated forearm tissue to 44 °C to induced vasodilation before iontophorised phenylephrine. Their results showed that phenylephrine reduced blood perfusion by 25-33%. Another study reported a 50% reduction in blood flow in response to noradrenaline following preheating the area to 40 °C (Wilson *et al.*, 2002).

These results are in agreement with our finding in this study which shows that in 10 subjects the perfusion curve was reduced by 50% following the iontophoresis of 1mg/ml phenylephrine using low current 50mA for the 60-second duration the response reached a plateau and last for more than 20 minutes even after iontophoresis was stopped. This could be attributed to the local action of the drug delivered by iontophoresis as well as the remaining drug which kept in contact with the skin for that

period. The concentration of the phenylephrine used in this study was optimised in the previous investigation to prevent using a too high or too low concentration of the drug. We avoided the use of higher current and duration for iontophoresis protocol that was reported in other studies to prevent the non-specific vasodilation produced by the current itself which can interfere with the analysis of responses (Droog *et al.*, 2004).

6.4.3 Limitations local heating and iontophoresis of phenylephrine of skin blood flow

The skin of the forearm consider as non- glabrous skin, in which the response of the blood vessels to vasoconstriction is less pronounced after exposure to noradrenaline from an external source, this was ascribed to an assumption of increasing the rate of production of nitric oxide rather than the direct effect of temperature per *se (Wingo et al.,* 2009).

In this study we did not test the effect of iontophoresis phenylephrine in non- heated skin, because other researchers reported that the response of blood vessels to vasoconstriction is much lower if iontophoresis was applied to skin in resting state (Fredriksson *et al.*, 2009), comparing to dilated blood vessels with local heating which increased the blood flow by 10-15 times (Carberry *et al.,* 1992).

Other scientists reported that the response to vasoconstriction effect after iontophoresis of adrenalin and phenylephrine was not consistent with no significant effect on transient hyperaemic response in the forearm of twenty subjects. Although they have used higher current and longer duration the experiment was conducted without local heating (Brown *et al., 2003)*. In this procedure, no washing out step for the remaining phenylephrine in the chamber was performed. Some studies suggested that drug removal can affect tissue response to the iontophorised drug *(Henricson et al.*, 2011). Washing the drug can result in a movement that could interfere with the reading of the LDF device.

We could not compare the time course of iontophorised phenylephrine in pre-dilated skin in the presence and absence of lithium solution as we planned in the aim of this chapter, although we have obtained a medical dosage form of LiDCO solution which contains 1.5mM concentration LiCl. The main reason for that is we have noticed that the response to iontophorised phenylephrine was stable and maintained for more than 30 minutes, and it was unethical and difficult to convincing the Ethical Committee to expose the volunteers to LiCl so decided to terminate the research at this point.

In summary, this chapter shows that, firstly, the physiological interventions by CPT and DBT can reduce blood flow in a reproducible manner, and DBT effect was significantly more potent than CPT. Secondly, localised heating of the skin blood vessels to 42° C significantly enhanced blood flow and produced local vasodilation. Thirdly, topical application of phenylephrine by iontophoresis reduced blood perfusion in the microvascular circulation in healthy subjects. This effect lasted for more than 30 minutes which significantly differ from our *in vitro* results that described the time course to this α_1- agonist.

Chapter 7: General Discussion and Summary

7.1 General discussion

The core outcome of the work in this thesis is that the time course of the contraction induced by most of GPCR agonists in smooth muscle *in vitro* is not sustained and start to decline after a short period. This observation is most common with the use of high concentration of the agonist. The incubation of porcine isolated vascular and non-vascular smooth muscle with a therapeutic concentration of lithium ions (1mM) significantly enhances and better maintains the contraction induced by some phenethylamine related α_1-adrenoceptors agonists assessed using the isometric tension recording.

It was important to describe that the contraction elicits by KCl and U46619 are relatively stable for more than 180 minutes in comparison with that induced by other agonists in porcine isolated vascular tissue. Examining noradrenaline-induced contraction in this preparation highlighted the fact that the response fades with time and this decline was due to auto-destruction of the drug in bathing solution rather than by factors related to the tissue itself such as metabolism, uptake or chelating with heavy metals or desensitisation. We also reported that the time course of noradrenaline-induced contraction is better-maintained if the air is used to gas the bath instead of 95% oxygen.

The importance of this interaction comes from the fact that although both groups have been used for more than 70 years, researchers continue discovering new uses and clinical benefits nowadays. Phenylephrine is a selective α_1-adrenoceptors agonist acting by binding to $G_{q/11}$ subunit on the cell membrane this binding results in stimulation of IP_3 pathways that increase the concentration of Ca^{2+} and induce contraction (Docherty 2019). There are many clinical applications for phenylephrine in current medicine. It can be used topically as a nasal decongestant (Eccles 2007) or applied to the eye to induce mydriasis (Ostrin and Glasser 2004), used to reduce pain associated nasogastric intubation in children (Craig *et al*., 2015), use as a suppository to enhance the contraction of the internal anal sphincter in faecal incontinence (Baek *et al.*, 2011) beside its vital action in maintaining the blood pressure during medical conditions associated with hypotension such as different type of shock (Morelli *et al*., 2008), post-caesarean spinal anaesthesia (Mon *et al*., 2017). On the other hand, clinical application for lithium also dramatically increase and besides its uses in the prophylaxis and treatment the of bipolar disorder, it was reported that lithium has a unique anti-suicidal action (Lewitzka *et al.*, 2015).

Furthermore, lithium was used *in vitro* and *in vivo* against different types of bacterial and viral infections (Tsao *et al.*, 2015; Zhou *et al.*, 2015). The importance of these clinical potential uses of lithium arises from the fact that they were reported in different animal models and in more than one species using a concentration which is within the therapeutic level (0.4-0.8mM) to avoid the sign of toxicity that appears at a concentration > 1.2mM.

For drugs to exert a beneficial clinical effect for a patient we need to have the means to deliver the molecule to the site of action or target in an adequate concentration. Thus, drugs are given via infusion, injection, topical application or oral intake. For most patients, the acceptability of a drug will depend on the route of administration and the frequency. Reducing the frequency of the administration will enhance patient compliance and acceptability for a given drug. This can be translated as an increase in the duration of action of the drug. When we investigate the properties of a drug and the interaction at the receptor very rarely do, we investigate responses for longer than 15-60 minutes? Indeed, for smooth muscle contraction, the routine use of cumulative concentration-response curves means that individual concentrations are rarely assessed beyond 15 minutes.

Thus, for drugs that activate specific receptors for clinical condition (e.g. decongestants, blood pressure, bowel control), there is perhaps a need to establish *in vitro* that responses are sustained over the likely interval of administration. My results in this thesis indicated that the contractions induced by stimulation of 5HT receptors, histamine receptors, α_1-adrenoceptors, angiotensin II receptors, vasopressin receptors and muscarinic receptors are not sustained and fade with time even with the continuous presence of a high concentration of the agonist in the bathing solution. This observation was reported using isolated vascular and non-vascular tissue in the pig.

It was widely accepted that the response of each drug is proportional to the amount of the drug in the plasma concentration, *i.e.* the higher plasma concentration of a specific drug can induce a higher clinical response. However, this is not the case in all instances. It was reported that the use of L-erythromethoxamine gel (selective α_1 agonist) produced an enhancement in the mean anal resting pressure (MARP) which can be used clinically to treat faecal incontinence in the elderly. The use of 0.3 % and 1% concentrations produced a stable response for 2 hours duration before it fades and loses more than 60% of its peak response after 4 hours.

In contrast, the plasma concentration for the drug was measured following the intra-anal application of 0.3% and 1% LEM gel. It was found that the plasma concentration declined by only 20% of its initial concentration after the same periods (4 hours). This example shows the differences in the time course between biological response and plasma concentration, see Figure 7.1 (Nisar *et al.*, 2007).

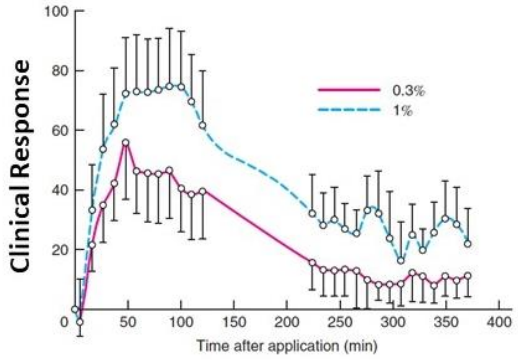

Fig. 2 Mean(s.e.m.) percentage rise in mean anal resting pressure (MARP) from baseline in six patients who received both 0·3 and 1 per cent gels

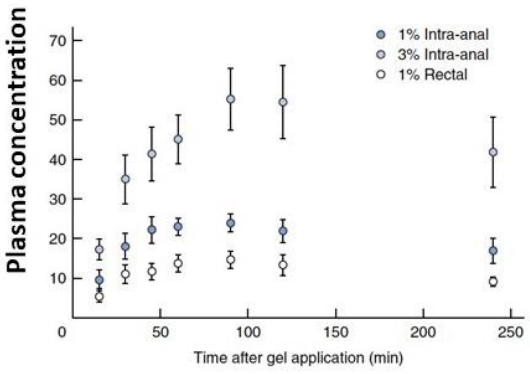

Fig. 3 Plasma concentration of L-erythro methoxamine after intra-anal and rectal application. Values are mean(s.e.m.)

Figure 7-1 Shows the differences between the time course of biological response and plasma concentration of 0.3 % and 1% L-erythromethoxamine gel -figure adapted from (Nisar *et al.*, 2007).

A similar observation was also reported in isolated sheep internal anal sphincter that contract following the addition of L-erythromethoxamine to treat faecal incontinence. Again the response was not maintained and started to decline after less than 15 minutes. In her work, Rayment reported that the use of a low concentration of lithium ions (0.5-3mM) enhanced and better-maintained this response (Rayment *et al .,* 2007).

As we mentioned in the introduction of this thesis, many researchers previously reported the interaction between lithium ions and the response initiated in isolated vascular and non-vascular tissue from different species in both contraction and relaxation studies (see Table 1.3). Our results showed that this interaction seems to be relatively selective to α_1-adrenoceptors agonists and not reported with other agonists which act on different receptors pathways (5HT receptors, histamine receptors, angiotensin II receptors, vasopressin receptors). Even though they all produced their action following the binding with $G_{q/11}$ subunit by stimulating the metabolism of phosphoinositide and enhanced the entrance of Ca^{2+} to the intracellular compartment to initiate and maintained the contraction in this preparation.

Interestingly, my results showed that the synergistic interaction between LiCl and α_1-adrenoceptors agonists only reported with three agents (phenylephrine, metaraminol and L-erythromethoxamine) while it is not happening with cirazoline and A61603. The common feature between these three agonists is they all have phenethylamine structure while both cirazoline and A61603 have an imidazoline structure. Although all the five agents have been reported to elicit a contraction in the vascular smooth muscle through binding with postjunctional α_1-adrenoceptor (Hone *et al.*, 1995; Jones *et al.*, 2003; Mehrotra *et al.*, 2007; Aragão *et al.*, 2014), They are different in term of the time to elicit the contraction, time to reach maximum response and duration of the action following the exposure to maximum or submaximal concentration of the agonist. This difference was easily detected by comparing the traces of the responses to these agonists. This rationalized the uses of traces in the results sections in this thesis besides the concentration-response curves which might hide some aspect of the response.

It is worthy to highlight that the differences between phenethylamine and imidazoline were previously reported by Ruffolo and his group in 1983. Their work involved comparing the potency of noradrenaline isomers, S (+), R (-) and desoxy derivative (dopamine) at α_1-adrenoceptors. Their results showed that the relative order of potency for phenethylamine as follows R (-) >S (+) > desoxy. In contrast, using isomers of 2-(3,4,α-trihydroxybenzyl) imidazoline and its desoxy derivative 2-(3,4,α-dihydroxybenzyl) imidazoline showed a potency at α_1-adrenoceptors as desoxy > R (-) > S (+). These results suggested that both phenethylamine and imidazoline can interact differently with α_1-adrenergic receptors (Ruffolo and Waddell, 1982; Ruffolo *et al.*, 1983).

This difference could be considered a possible explanation for our results regarding the selectivity of the interaction with LiCl especially when we know that the time course for the contractions induced by imidazoline agonist (cirazoline and A61603) were relatively stable in comparison with the contraction induced by phenethylamine derivatives. Further studies using different techniques are required to better understand the mechanisms behind this interaction. In this thesis we excluded several suggested mechanisms including for the action of lithium in the treatment of bipolar disorder such as including inhibition of IMPtase and GSK3 enzymes (Klein and Melton 1996), we provides evidence for the first time that neither receptor trafficking nor desensitisation is responsible for the enhancement in the time course of phenylephrine-induced contraction when preincubated with LiCl. We used concanavalin A which is an internalisation blocker (Perez-Aso *et al.*, 2013). This interaction was

reported with different salts of lithium and in a concentration range 0.3mM-2mM. In contrast, the interaction strongly inhibited in the absence of Ca^{2+}, which suggest an important role for this cation in initiating the contraction. It is noteworthy to mention that using Na^{+}/K^{+} ATPase inhibitor (ouabain) in nano concentration produced a similar enhancement in time course but with much lower enhancement in the magnitude of the contraction induced by phenylephrine. All these mechanisms in addition to other factors which were mentioned in appendix including (the role of NO, the role of Mg^{2+} and role of Rho kinase inhibitors) failed to explain the enhancement in the time course of this adrenergic agonist. However, this is not limiting the possibility of preincubation a low concentration of LiCl with these drug to enhance their duration especially when we know that the FDA approved the clinical uses of lithium in psychiatry in 1970 (Bauer and Gitlin 2016) depending on its clinical value in managing the symptoms without adequate information about how it produces these effect.

After I confirmed such interaction *in vitro* in both isolated vascular and non-vascular smooth muscles of the pig, I decided to examine the time course for the contraction induced by phenylephrine if it applied to the skin of the forearm of healthy volunteers using the iontophoresis method and LDF. Phenylephrine was used to assess the abnormal vascular reactivity associated with many acute (sepsis) and chronic (hypertension) diseases. It was suggested that the skin microcirculation is in relatively vasoconstriction state during the normal resting condition so it is difficult to detect further vasoconstriction that results from the addition of external pressor agent (Brown *et al.*, 2003).

As a solution for this Beed and his colleagues suggested the use of localised heating to induce vasodilation locally (Beed *et al.*, 2009). In this study, I followed the same procedure of preheating the skin to induced vasodilatation before applying phenylephrine and measured the time course of the vasoconstriction induced in skin microcirculation. The heating to 42 produced relatively stable vasodilation that lasts for less than 20 minutes. Surprisingly my results showed that the time course for phenylephrine-induced vasoconstriction following the iontophoresis was stable and did not decline in a similar rate as we reported early using the porcine isolated vascular tissues. An explanation for this could be the concentration that reached the superficial blood vessels in the skin is low and as we have reported in chapter 3 that low concentration of phenylephrine associated with relatively stable contraction in compare with high concentration. Due to this observation, we decided to stop the research at this point because it was unethical to expose the volunteers to the LiCl even if it is

through the iontophoresis after we reported that the vasoconstriction produced by phenylephrine was stable.

As a limitation to my work, It could be suggested that recording the time course for the contraction induced by phenylephrine for a longer period could expose a decline in response, however, the problem associated with this could be the fact that the vasodilation produced by heating is only stable for 20 minutes so it will be difficult to distinguish between the cause of relaxation is it due to decline in the activity of phenylephrine or its due to loss the vasodilation effect of the heating. Thus, another method to induce the vasodilation rather than heating or the use of an alternative method to deliver or monitor the response rather than iontophoresis and LDF could be warranted for future work. Another limitation for this study could be the inability to access to human tissue to test some of the intriguing observations which were noted in the porcine tissue using the isometric tension technique.

7.2 Summary

The results of this thesis highlighted two main areas first, the contractile agents, in general, have been used in an enormous number of vascular researches either to characterise their potency and maximum response in different tissue or to characterise the action of other vasodilator agents. The α_1-adrenoceptor agonists are an example of these vasoconstrictors commonly used in the contraction-relaxation study in the vasculature. Despite the numerous researches conducted in porcine vascular smooth muscle in which α_1-adrenoceptor agonists were used to inducing contraction, only a few studies highlight the fact that contraction induced by these group of drugs was not sustained and decline with time especially when used in high concentration. The endogenous catecholamine can prolong their time course slightly by blocking their metabolism and the uptake mechanism. On the other hand, some other phenethylamine related α_1-adrenoceptor agonists such as phenylephrine, methoxamine and metaraminol significantly enhanced their time course by preincubation the isolated porcine smooth muscle from the vascular and non-vascular organs with a therapeutic concentration (1mM) of lithium ions. The latter is a clinically used drug in the management of bipolar disorder. This sort of synergistic interaction could have a potential clinical application to extend the time course of the response to these agonists because the three agents have a clinical role. The exact mechanism of this interaction is not explained by the suggested mechanisms for the action of lithium and further studies are needed to examine the specific protein involved in this interaction and the exact site to provide a better understanding to this interesting

observation. The second important outcome made by this thesis that not all the *in vitro* observations can be mirrored using *in vivo* models. We confirmed that the contractile response to some α_1-adrenoceptor agonists is not sustained in porcine, but it was more stable in man when we measured the changes in blood flow following the iontophoresis application of this agonist.

It is important to acknowledge that lithium was used as a consumer product over 70 years ago and phenylephrine is now found as a consumer-based orally active decongestant product ..... yet we are learning about these two drugs.

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Appendices

Appendix I Ethical form

University of Nottingham
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Email: FMHS-ResearchEthics@nottingham.ac.uk

Faculty of Medicine &Health Sciences
Research Ethics Committee
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Queen's Medical Centre Campus
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13 August 2018

Mr Saif Yahaya
PhD Student
c/o Dr Vince Wilson
Associate Professor of Autonomic Pharmacology
School of Life Sciences
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Dear Mr Yahaya

Ethics Reference No: 21-1805 – please always quote	
Study Title: The effect of short-term physiological interventions and Iontophoresis on the skin blood flow in healthy male volunteers, as determined by the Laser Doppler Flowmetry.	
Chief Investigator/Supervisor: R P Mahajan, Professor of Anaesthesia and Intensive Care, Clinical Neurosciences, School of Medicine,	
Lead Investigators/student: Saif Yahya, PhD Student, School of Life Sciences	
Other Key Investigators: Dr Vince Wilson, Associate Professor of Autonomic Pharmacology, School of Life Sciences, Dr Martin Beed, Consultant in Intensive Care Medicine & Anaesthesia, Nottingham University Hospitals NHS Trust, Debbie Bush, Research Nurse, Division of GI Surgery, School of Medicine.	
Type of Study: PhD project, physiological intervention	
Proposed Start Date: 01/06/2018	**Proposed End Date:** 31/12/2018 6 mths
No of Subjects: 10-12	**Age:** 18+years

Thank you for your e-mail dated 10 August 2018 responding to the comments made by the Committee and some additional changes as detailed. The following revised documents were received:

- FMHS REC Application form and supporting documents version 2.0: 10.08.2018

These have been reviewed and are satisfactory and the study has been given a favourable opinion.

A favourable opinion has been given on the understanding that:

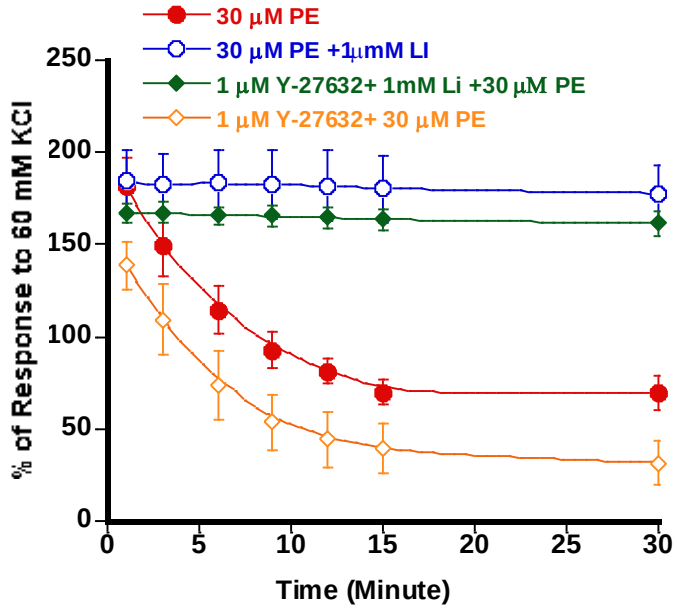
1. The protocol agreed is followed and the Committee is informed of any changes using a notice of amendment form (please request a form).
2. The Chair is informed of any serious or unexpected event.
3. An End of Project Progress Report is completed and returned when the study has finished (Please request a form).

Yours sincerely

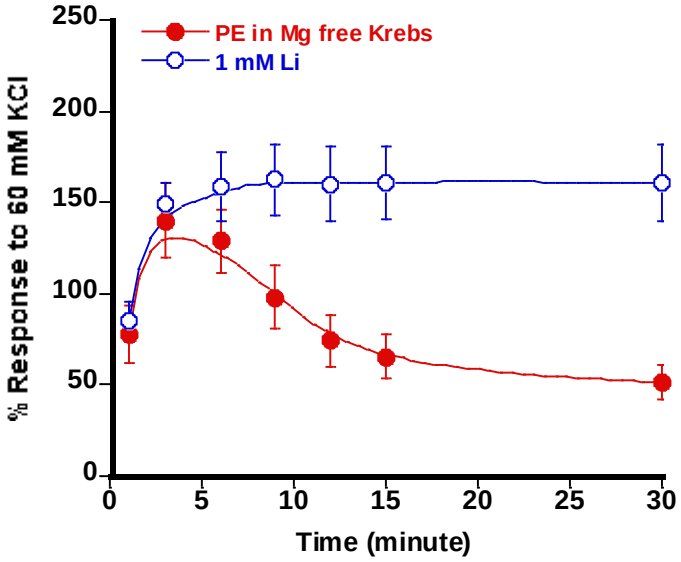
pp

Dr Bethan Phillips
Acting Chair, Faculty of Medicine & Health Sciences Research Ethics Committee

Appendix II Role of Rho kinase inhibitors in the action of LiCl in porcine splenic artery



Appendix III Role of Mg^{2+} in the action of LiCl in porcine splenic artery



Appendix IV Role of NO in the action of LiCl in the porcine splenic artery