EFFECT OF HIGH SALT DIET ON BLOOD VESSEL FUNCTION: ALTERATION IN VASCULAR β-ADRENOCEPTOR SIGNALLING

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Effect of high salt diet on blood vessel function: Alteration in vascular β-adrenoceptor signalling

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ABSTRACT

Consumption of high salt leads to the alteration of vascular β-adrenoceptor response which subsequently modifies the blood vessel responses. This change could be due to an alteration in β-adrenoceptor-mediated signalling in blood vessels. To test this hypothesis, an evaluation of β-adrenoceptor-mediated mechanical and electrical responses was undertaken in low-pressure segment of the circulatory system i.e. main pulmonary arteries, of Sprague-Dawley rats fed either normal or high salt diets for 18-23 days. Blood pressure and heart rate were monitored in anesthetised rats using intra-arterial catheters. Isometric tension was measured in isolated pulmonary artery ring preparations. Glass microelectrodes were used for the recording of membrane potential (E_m). Consumption of high salt resulted in a modest increase in blood pressure but not heart rate. Isoprenaline evoked hyperpolarization in pulmonary arteries of rats fed a normal diet. Hyperpolarization evoked by stimulation of β-adrenoceptor involved multiple processes including the activation of K^+ channels and Na^+/K^+ATPase.

The removal of endothelium produced hyperpolarization of vascular smooth muscle cells in blood vessels obtained from rats fed a normal diet. The hyperpolarisation due to denudation was the result of the activation of K^+ channels and Na^+/K^+ATPase. Moreover, hyperpolarization as a consequence of β-adrenoceptor stimulation appeared to be mutually dependent on the presence of endothelial cells since this event (i.e. hyperpolarization) as evoked by isoprenaline was absent in denuded tissues. Isoprenaline-mediated relaxation was found to be partly dependent on the presence of endothelium.

Isoprenaline produced similar relaxations in intact pulmonary arteries obtained from animals fed a high salt compared to normal diet. However, the E_m was found to be more negative in intact pulmonary arteries of animals fed a high salt diet compared to those fed a normal diet. The isoprenaline evoked hyperpolarization was notably absent in the pulmonary arteries. In addition, in intact versus denuded blood vessels obtained from rats on high salt diet, the E_m was not significantly different.

Taken together, the data suggests that the presence of the endothelium is essential for the propagation of β-adrenoceptor-mediated relaxation. Moreover, the evidence seems to also imply that consumption of high salt leads to the activation of K^+ channels and Na^+/K^+ATPase channels preserving β-adrenoceptor-mediated relaxations in pulmonary arteries.

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AC:	adenylyl cyclase
ATP:	adenosine 5-triphosphate
Ba^{2+}:	barium ions
BP:	blood pressure
BK_{Ca}:	large-conductance Ca^{2+}-activated K^+
Ca^{2+}:	calcium ion
CaM:	Calmodulin
cAMP:	cyclic adenosine 3,5-monophosphate
cGMP:	cyclic guanosine 3.5-monophophate
CGRP:	calcitonin gene-related peptide
Cl^-:	chloride ion
CNP:	C-type natriuretic peptide
CO:	carbon monoxide
Cx	Connexins
DAG:	Diacylglycerol
E_m:	membrane potential
EETs:	cytochrome P450 mono-oxygenases derived epoxyecosatrienoic acids
EDHF:	endothelium-derived hyperpolarizing factor
EDRF::	endothelium-derived relaxing factor
e.j.ps:	excitatory junctional potentials
GC:	guanylyl cyclase
GDP:	guanine nucleotide diphosphate
GPCR:	guanine -protein-coupled receptors
GTP:	guanine nucleotide triphosphate
H_2O_2:	hydrogen peroxide
H_2S:	hydrogen sulphide
HETE:	hydroxyeicosatetraenoic acid
HUVECs:	human umbilical vein endothelial cells
IK_{Ca}:	Intermediate conductance Ca^{2+} activated K^+ channels
IP_3:	inositol 1,4,5-triphosphte
K^+:	potassium ions
K_{ATP}:	ATP-sensitive potassium channels
K_{Ca}:	Ca^{2+}-sensitive potassium channels
$K_{ir2.1}$	inward rectifier potassium channels
K_{2P}:	two pore domain potassium channels
K_V:	voltage-gated K^+ channels
LDVs:	large dense cored vesicles
L-NAME:	N^{ω}-nitro-L-arginine methyl ester
L-NMMA:	NG-monomethyl-L-arginine
MEGJs:	myoendothelial gap junctions
MLCK:	myosin light chain kinase

MLCP:	myosin light chain phosphatase
Na^+:	sodium ion
Na^+/K^+ATPase:	electrogenic pump/sodium-potassium pump
O^{2-}:	superoxide ion
P2X:	Purinoceptors
PA:	phosphatidic acid
PI:	Phosphatidylinositol
PIP_2:	phosphatidylinositol 4, 5-bisphosphate
PKA:	protein kinase A
PKC:	protein kinase C
PKG	protein kinase G
PGI_2	Prostacyclin
PLC:	phospholipase C
RAAS:	renin-angiotensin-aldosterone system
SCVs:	small clear vesicles
SDVs:	small dense cored vesicles
SK_{Ca}:	small conductance Ca^{2+} activated K^+ channels
TASK:	Two-pore domain acid sensitive K^+ channels
TEA:	tetraethyl ammonium
TWIK:	tandem of pore domains in weak inward rectifier K^+ channels
VIP:	vasoactive intestinal peptide
α:	alpha
β:	beta
γ:	gamma
βARK:	β-adrenergic receptor kinase

1.0. INTRODUCTION

1.1. Overview of cardiovascular system

The cardiovascular system is one of the integral components of the body that helps in maintaining cellular homeostasis. It includes the heart and blood vessels (Guyton and Hall, 2000; Tortora and Grabowski, 2010). The heart pumps blood and blood vessels channel and deliver it throughout the body. Arteries carry blood saturated with oxygen and nutrients away from the heart to all parts of the body. Arteries are thick-walled tubes with a circular covering of yellow, elastic fibres, which contain a filling of muscle that absorbs the tremendous pressure wave of a heartbeat and slows the blood down (Guyton and Hall, 2000; Tortora and Grabowski, 2010). This pressure can be felt in the arm and wrist and is referred to as the pulse. Eventually, arteries divide into smaller arterioles and then into even smaller capillaries, the smallest of all blood vessels. One arteriole can serve a hundred capillaries. Here, in every tissue of every organ, capillary exchange takes place where cells take up oxygen and nutrients and send away the waste products. Capillaries join together to form small veins called venules, which flow into larger main veins, and these deliver deoxygenated blood back to the heart. Veins, unlike arteries, have thin, high distensible walls, because the blood has lost the pressure which forced it out of the heart, so the deoxygenated blood which flows through the veins on its way to the lungs moves along very slowly on its way to be reoxygenated. From the right side heart, deoxygenated blood flows to the lung via unique vessels i.e. pulmonary arteries to be oxygenated, and then back to the left side of heart to begin its journey around the body once again. The blood vessels consist of various cells types that communicate with each

other in response to physiological stimuli to maintain adequate blood flow in the circulatory system (Guyton and Hall, 2000; Tortora and Grabowski, 2010).

1.2. Blood vessels

The blood vessels of the cardiovascular system are divided into arteries, arterioles, capillaries, venules and veins. The vascular wall is made up of three layers or tunics of three different cells (Pugsley and Tabrizchi, 2000). Starting from the lumen, the first layer (tunica interna/intima) is composed of an epithelial cell lining also referred as endothelium, middle layer (tunica media) consists of smooth muscle cells and connective tissue, and the outermost layer (tunica externa) is made up of connective tissue and contains nerve supply to the blood vessels. The composition of the blood vessel layers varies in structure in correlation to the difference in function that occurs throughout the cardiovascular system (Guyton and Hall, 2000; Pugsley and Tabrizchi, 2000; Tortora and Grabowski, 2010).

In the cardiovascular system, pulmonary arteries in contrast to systemic arteries have lesser smooth muscle cell layers under normal conditions, consistent with low pressure system (Daly and Hebb, 1966). The pulmonary vascular resistance is offered by the small arteries with an internal diameter of a few hundred μm. The pulmonary capillary bed responds differently from the systemic capillary bed. Pulmonary veins are similar in structure to pulmonary arteries with less layers of smooth muscle cells and may be regulated differently (Daly and Hebb, 1966). The structure of pulmonary arteries may change with an increase in pressure, i.e. pulmonary hypertension. The chronic increase in

pressure leads to structural remodelling of the medial layer (increase in number or size of smooth muscle cells) along with fibrosis (Barer *et al.*, 1989). This change may manifests alteration in various functional mechanisms involved in the regulation of pulmonary artery (Barnes and Liu, 1995).

1.2.1. Sympathetic innervations of blood vessels

The sympathetic nervous system modifies blood flow under physiological conditions and may be involved in the pathophysiology of vascular diseases (Dzau *et al.*, 1994). In blood vessels, smooth muscle cells are innervated by postganglionic sympathetic nerves. The density of innervation varies widely from vessel to vessel. The postganglionic sympathetic nerve terminals form a network of unmyelinated slender processes that widen at regular intervals into varicosities (Thaemert, 1966). In most blood vessels, a plexus of branching varicose axons sits in the adventitial layer of blood vessels. The blood vessels with multiple smooth muscle cell layers have direct contact with axons at the medio-adventitial border. In addition, branching of the axons has also been observed in the two to three outer layers of the tunica media (Burnstock, 1986). These varicosities are arranged in series along the length of axon and are separated by narrow intervaricose regions. The nerve varicosities are irregularly distributed along the axons and the distance between individual varicosities ranges from almost nothing to >7 μm (Luff *et al.*, 1987). Generally, varicosities are fusiform shape with variable sizes ranging from 0.4-3.0 μm in diameter and 0.25-4.0 μm in length (Luff *et al.*, 1987). Other than cell organelles like mitochondria and sarcoplasmic reticulum; sympathetic nerve varicosities also have a

number of vesicles that contains a "cocktail" of neurotransmitters. Further these are classified into three different types that include large dense cored vesicles (LDVs), small dense cored vesicles (SDVs) and small clear vesicles (SCVs) (Stjärne, 1989). Both SDVs and LDVs have been shown to store noradrenaline and adenosine triphosphate (ATP) (LDVs contain 8-20 times more than SDVs), and some LDVs contain neuropeptides (neuropeptide Y, substance P, Calcitonin gene related peptide) (Stjärne, 1989). Following stimulation of sympathetic nerves these transmitters are released in the sympathetic neurovascular junctions, the width of which range from 50-100 nm (Luff *et al.*, 1987) and interact with membrane receptors such as adrenergic (α-, β-adrenoceptors), purinoceptors resulting in vascular contraction and relaxation.

1.2.1.1. Sympathetic nerve stimulation of blood vessels

The sympathetic nerve stimulation has been linked to depolarization of vascular smooth muscle cells of guinea-pig pulmonary artery & vein and mesenteric veins (Suzuki, 1981, 1983; Van Helden, 1988, 1991). The nerve mediated depolarization consists of two components a) transient one, which occurs at the beginning of the response and; b) a sustained phase that appears thereafter. Both of these components are blocked by α-adrenoceptor antagonists. Van Helden (1991) had reported an ongoing discharge in mesenteric vein, referred to as a spontaneous transient depolarization. Following, the nerve stimulation these spontaneous transient depolarizations increase in frequency with a time lag of 1-2 seconds. Moreover, these high frequency spontaneous transient depolarizations summate to generate a slow excitatory junctional potential. Interestingly,

these spontaneous transient depolarizations are not produced due to transmitter released from sympathetic nerve terminala but are generated from the irregular oscillating release of Ca^{2+} from the internal stores that might as well activate contractile proteins in the vascular smooth muscle cells (Van Helden 1991). Collectively, this would suggest that α-adrenoceptors play an important role in vascular contraction by releasing Ca^{2+} from internal stores that might cause depolarization of vascular smooth muscle cells.

The membrane potential changes in response to sympathetic nerve stimulation vary in arteries and veins. A single sympathetic nerve stimulus evokes a membrane depolarization that is referred to as excitatory junctional potential (e.j.p.). Several e.j.ps summate to induce depolarization of a few mV that will activate voltage sensitive Ca^{2+} channels and this then leads to the contraction of smooth muscle cells (Hirst and Edwards, 1989). These rapid nerve responses are resistant to inhibition by α-adrenoceptor antagonists (Angus *et al.*, 1988). In arteriolar smooth muscle cells, e.j.ps are observed that have short latencies of 10-20 ms (Hirst and Neild, 1978; Hirst and Edwards, 1989). The time course of e.j.ps is faster in neuronal or skeletal synapses than those recorded from any other autonomic organ tissue (Hirst and Edwards, 1989). The rapid onset of the response suggests that the sympathetic transmitter is activating a ligand-gated channels and the decay phase reflects a slow rate of closure of such channels. There is ample amount of evidence to support the idea that e.j.ps in arterioles and arteries result from the release of ATP and the activation of purinoceptors (P2X) (Suzuki, 1985; Hirst and Jobling, 1989). In addition, it has been demonstrated that non selective channels activated by ATP in vascular smooth muscle cells also show high selectivity for Ca^{2+} ions (Benham, 1989). Altogether, evidence suggests that sympathetic

nerve stimulation induces e.j.ps and leads to depolarization via the action of the co-transmitter, ATP, on cation permeable ligand gated ion channels (P2X receptors) (North, 2002).

Sympathetic nerve stimulation has been linked to an increase in the pulmonary vascular resistance (Pace *et al.*, 1972; Piene, 1976). Adrenergic innervation of pulmonary vasculature largely varies amongst mammals (Richardson, 1979). Histochemical examination revealed that catecholamine containing nerve fibres are generally absent in intra-pulmonary artery of rat (El-Bermani, 1978; McLean *et al.*, 1985). Whereas, extra-pulmonary arteries are abundant in sympathetic innervation but the extent and density varies between the species (Richardson, 1979). Interestingly, the pulmonary trunk of rat pulmonary artery is well innervated and produces noticeable contraction following stimulation of nerve. However, the nerve stimulated contractions are barely detectable in rat main pulmonary arteries, which suggest that the sympathetic innervation become scarce further along the pulmonary vascular tree (Duggan *et al.*, 2011). Stimulation of sympathetic nervous system to the pulmonary artery seems to cause stiffening of the pulmonary vascular bed. This in turn can aid in the propulsion of blood into lungs, especially in a state of low cardiac output. This hypothesis has been supported by *in vivo* investigations conducted in dog and cat pulmonary artery (Pace *et al.*, 1972; Piene 1976).

1.3. Vascular contraction

In order to modulate blood flow, numerous endogenous agents act on blood vessels in response to physiological and pathophysiological stimuli. In blood vessels, the maintenance of resting ionic gradient is a fundamental property of the vascular cells that

facilitates propagation and transmission of messages by electrical conduction (Hirst and Edward, 1989). Modulation of the electrical properties of smooth muscle cells leads to generation of force, referred to as electromechanical coupling. Although spike generation is involved in triggering contraction, tension may be regulated by depolarization of quiescent smooth muscle that does not generate action potentials (Su *et al.*, 1964). It is also clear that contraction can occur by processes that are independent of cell depolarization and that have been referred to as pharmacomechanical coupling (Su *et al.*, 1964; Somlyo and Somlyo, 1968; Casteels, 1980; Baron *et al.*, 1984). Pharmacomechanical responses are elicited by agonist-induced changes in the concentration of second messenger molecules such as intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$), inositol 1,4,5-triphosphate (IP_3), 1,2 diacylglycerol (DAG). In vascular smooth muscle cells, neurotransmitter or autacoids produce contraction through elevation of $[Ca^{2+}]_i$ via Ca^{2+}influx and release of Ca^{2+} from intracellular stores (such as sarcoplasmic reticulum). An increase in $[Ca^{2+}]_i$ is known to cause smooth muscle contraction. The neurotransmitters released by sympathetic nerve stimulation can increase $[Ca^{2+}]_i$ in smooth muscle cells by activating post-junctional membrane receptors such as α-adrenoceptors or purinoceptors. The α-adrenoceptors are guanine-protein-coupled receptors (GPCR) that increase Ca^{2+} by releasing Ca^{2+} from internal stores via the $G_{\alpha q}$/ phospholipase C (PLC)/ inositol phosphates (IP)/IP3 pathway (Hirst and Edward, 1989; Van Helden, 1991). The purinoceptors are linked to non selective cation channels which cause depolarization of the membrane resulting in Ca^{2+} influx via voltage-gated Ca^{2+} channels (Figure 1) (Sneddon and Burnstock, 1984; Hirst and Edward, 1989).

1.3.1. Vascular smooth muscle cells

The vascular smooth muscle cells are uni-nucleated, non striated muscle cells. They appear thickest in the middle and taper at each end (Tortora and Grabowski, 2010). Like striated muscle, smooth muscle also contains actin and myosin filaments that play an integral role in vascular contraction and relaxation. The absence of regular alignment of filaments into a sarcomere gives the muscle tissue a smooth appearance under the microscope (Somlyo and Somlyo, 1968). In various blood vessels, smooth muscle cells communicate with each other and endothelial cells via hemi-channels known as gap junctions (Figueroa and Duling, 2009; Dora, 2010).

The diverse cellular functions of vascular smooth muscle are mediated by complex interactions between membrane proteins (receptors, ion-channels, ion exchangers) and intracellular components/systems (sarcoplasmic reticulum, second messengers, contractile proteins) (Somlyo and Somlyo, 1970).

1.3.1.1. Receptors

The site of interaction of endogenous and/or exogenous chemical mediators with the surface of the cell leading to a response is known as a receptor (Rang and Dale, 2007). The binding of chemical mediators to receptors initiates the intracellular chemical cascade. The cellular responses to receptor activation vary considerably according to the structure and nature of the receptor. There are four different types of receptor activated signal transduction. These include guanine-nucleotide binding protein (G-protein) coupled receptors, nuclear receptors, tyrosine-linked kinase receptor system and receptor coupled to ion channels (Rang and Dale, 2007)

GPCRs are the largest class of cell surface receptor super-family. The GPCRs were named for their ability to associate with heterotrimeric G-proteins ($G_{\alpha\beta\gamma}$) (Gilman, 1987). These G-proteins function as intermediaries between extracellular receptors and intracellular effectors proteins for a variety of neurotransmitters and hormones, thus controlling intracellular concentrations of signal molecules such as cyclic nucleotides, Ca^{2+} and IP (Gilman, 1987; Casey and Gilman, 1988). The GPCR is a bundle of seven-transmembrane α-helices alternatively connected through intracellular and extracellular loops. The amino terminal (N-terminal) part of the receptor is located on the extracellular side of the cytoplasmic membrane, whereas its carboxyl terminal (C-terminal) faces the cytoplasm. Heterotrimeric G proteins are composed of α, β and γ-subunits. The β- and γ-subunit are considered to be a single functional complex ($G_{\beta\gamma}$), because they do not dissociate in non-denaturing conditions. The α-subunit can bind and hydrolyze guanosine triphosphate (GTP). They are called heterotrimeric because of the three different subunits

and G-proteins because they display highly selective binding affinity for guanine nucleotides. In the basal state, an α-subunit is bound to guanosine diphosphate (GDP) and is associated with the βγ complex. Following receptor-ligand binding, the GPCR undergoes a conformational change such that it promotes the exchange of GDP for GTP on the α-subunit. In the GTP-bound state, the α-subunit dissociates from the βγ-complex, and both the α-subunit and the βγ-complex can interact with and regulate downstream effectors to evoke physiological responses. However, the hydrolysis of GTP associated with the α-subunit results in its re-association with the βγ complex, leading to the dissipation of the intracellular response (Neer and Clapham, 1988; Neer, 1994). There are several other levels of regulation for this system, such as regulators of G-protein signalling (RGS), activators of G-protein signalling (AGS), and G-protein receptor kinases (GRKs) (Vögler *et al.*, 2008). The RGS proteins are known to increase the intrinsic GTPase activity of the α-subunit; AGS proteins activate G-proteins independent of GPCR-mediated signalling, and GRKs phosphorylate key residues on the GPCR, leading to desensitization and/or endocytosis of the receptor (Ferguson, 2001; Sato *et al.*, 2006). There are approximately twenty G_{α}-subunits known to date. They are divided into four major G_{α} subfamilies such as $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q/11}$and $G_{\alpha 12/13}$ (Hubbard and Hepler, 2006; Plagge *et al.*, 2008). Stimulatory $G_{\alpha s}$ and inhibitory $G_{\alpha i}$ exist as distinct entities, with $G_{\alpha s}$ being responsible for activating the adenylyl cyclase (AC) and $G_{\alpha i}$ inhibiting the enzyme and stimulating potassium channels (Gilman, 1987; Brown and Birnbaumer, 1988). The $G_{\alpha q/11}$ is coupled to the activation of phospholipase C-β (PLC-β). Further, activation of PLC-β leads to the hydrolysis of phosphatidylinositol 4, 5-bisphosphate (PIP_2) and the production of IP_3 and DAG. The signalling via this pathway evokes

responses that are mediated through Ca^{2+} and protein kinase C (PKC) (Lapetina, 1987; Hubbard and Hepler, 2006). These mechanisms for receptor-effector coupling are applicable to a gamut of vascular smooth muscle receptor systems such as adrenergic (α, β), muscarinic (M), purinergic (P_2) and prostaglandins (PGI_2 and PGE_2).

The nuclear receptors are intracellular receptors that transduce signals from glucocorticoids, mineralocorticoids, the sex steroids (estrogen, progesterone, and androgen), thyroid hormones, and vitamin D3. They are characterized by a central DNA-binding domain, which targets the receptor to specific DNA sequences known as response elements (Berg, 1989; Zilliacus *et al.*, 1995). Glucocorticoids, mineralocorticoids, estrogen and peroxisome proliferator-activated receptor gamma have been shown to play an important role in cardiovascular and metabolic diseases by regulating vascular smooth muscle migration, proliferation, perivascular inflammation and ultimately contractility to endogenous mediators such noradrenaline (Walker, 2007; Ketsawatsomkron *et al.*, 2010). Tyrosine-linked kinase receptors are characterised by ligand-stimulated autophosphorylation of tyrosine kinase that is intrinsic to the receptor (Hunter and Cooper, 1985). Unlike G-protein receptors, tyrosine-linked kinase receptors consist of a single polypeptide containing one hydrophobic region that forms a single transmembrane domain. The extracellular surface of the receptor encompasses an agonist recognition site and the intracellular surface incorporates a tyrosine kinase enzyme. The ligands for these receptors include: platelet-derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factor (TGF), fibroblastic growth factor (FGF), insulin like growth factor I (IGF) and atrial natriuretic peptide (ANP). PDGF promotes mitogenesis and is a potent vasoconstrictor. Mechanical stress (myogenic constriction) and vascular

remodelling due to hypertension has also been linked to activation of tyrosine phosphorylation pathways (Zou *et al.*, 1998; Murphy *et al.*, 2002). Activation of Angiotensin II type I receptors induce growth promoting factors via EGF. This cross-talk between growth factors by G-protein couple receptors is known as trans-activation mechanism (Eguchi and Inagami, 2000; Kalmes *et al.*, 2001).

The group of transmembrane ion channels that are regulated by the binding of a ligand referred to as ligand gated ion channels or receptor-coupled ion channels. These complexes are all pentameric, comprised of five similar subunits, which form a barrel-like structure with a channel pore running through the middle of the complex (Large, 2002). The receptor-coupled to ion channels upon interaction with ligand results in opening of transmembrane ion channels allowing the flux of cations or anions down their concentration gradient. This transduction mechanism may or may not be dependent on intracellular second messengers and the response to agonist binding is faster than that of biochemical transduction. These ionotropic receptors play a major role in vascular tone (Gibson *et al.*, 1998; Large, 2002). Members for this family are Na^+ channels and cholinergic nicotinic receptors.

1.3.2. Electrical properties of vascular cells

The smooth muscle cells of blood vessels maintain large ionic gradients across their plasma membranes (Bolton, 1979; Hirst and Edwards, 1989). The asymmetric distribution of ions across the membrane of vascular smooth muscle cells generates the potential difference known as the resting membrane potential. The ability of vascular cells to respond to changes in their ionic environment and maintain a relatively constant internal milieu is one of their most fundamental properties. In response to external perturbations, cells will activate specific membrane bound ion transport systems in an effort to maintain overall homeostasis (Bolton, 1979; Hirst and Edwards, 1989).

The concentration gradients for different ions (K^+, Cl^-, Ca^{2+} and Na^+) have been estimated for arterial smooth muscle cells and are listed in Table 1 (Hirst and Edwards, 1989). The electrical properties of vascular smooth muscle have been studied extensively in various tissues amongst various vertebrates. Various studies suggest that the membranes of vascular smooth muscle are more permeable to K^+ ions compared to other ions (Bolton, 1979; Kuriyama *et al.*, 1982; Hirst and Edwards, 1989). This higher permeability to K^+ ions results in a resting membrane potential of vascular smooth muscle cells between -55 to -75 mV (Hirst and Edward, 1989; Nelson *et al.*, 1990). Ionic differences are tightly regulated by membrane proteins such as K^+ channels, Ca^{2+} channels, Cl^- channels, electrogenic pumps and various ion-exchangers.

The membrane potential (E_m) as given by the Goldman-Hodgkin-Katz equation (Goldman, 1943; Hodgkin and Katz, 1949):

$$E_m = RT/F \ln (P_K [K]_o + P_{Na} [Na]_o + P_{Cl} [Cl]_o) / (P_K [K]_i + P_{Na} [Na]_i + P_{Cl} [Cl]_i)$$

Where E_m is the membrane potential as determined by the Nernst potential (E_K, E_{Na} and E_{Cl}) and the relative conductances of the ions. Individual E_P calculated by the Nernst equation mentioned are in Table 1. For example:

$E_K = RT/F \ln [K]_o/[K]_i$ at 37°C.

Table 1.

Intracellular $[X]_i$ and extracellular $[X]_o$ ion concentration; and equilibrium Nernst potential (E_p) of vascular smooth muscle cells (Hirst and Edwards, 1989)

Ions	**$[X]_i$ (mM)**	**$[X]_o$ (mM)**	**E_p (mV)**
Sodium (Na^+)	10 – 20	~ 150	+50
Potassium (K^+)	130 – 160	3 – 5	-90
Chloride (Cl^-)	40 – 70	~ 140	-20
Calcium (Ca^{2+})	10^{-6} - 10^{-8} M	~ 2	> +150

1.3.2.1. The electrogenic pump (Na^+/K^+ATPase)

The Na^+/K^+ATPase is a membrane bound protein that establishes and maintains high internal K^+ and low internal Na^+ concentrations in the smooth muscle cells (Skou, 2004). It is an oligomer composed of α-, β- and γ-subunits (Collins *et al.*, 1982; Mercer *et al.*, 1993). The α-subunit is responsible for the catalytic and ion transport properties of the enzyme. The β-subunit contains the binding sites for the cations, ATP, and the inhibitor, ouabain. The γ-subunit is a regulatory subunit and responsible for the normal activity of the enzyme. The latter subunit appears to be involved in the occlusion of K^+ by modulating K^+ and Na^+ affinity of the enzyme. During each active cycle three Na^+ ions are pumped out of the cell in exchange for two inflowing K^+ ions for each ATP molecule consumed, which implies that the enzyme complex contains multiple binding sites for Na^+ and K^+ (Sweadner, 1989). This exchange of cations plays a critical role in regulating the Na^+ electrochemical gradient across the membrane. In various cells, the Na^+ electrochemical gradient regulates various cellular ion counter transporters (Na^+/Ca^{2+}, $Na^+/H^+/Cl^-/HCO_3^-$, Na^+/H^+) and co-transporters (Na^+/sugar/amino acids, Na^+/Cl^-, $Na^+/K^+/Cl^-$) (Mobasheri *et al.*, 2000). Inhibition of the electrogenic pump with ouabain increases the intracellular concentration of Na^+, thus decreasing its electrochemical gradient that leads to an increase in cytosolic Ca^{2+} concentration (Juhaszova and Blaustein, 1997). The increase in intracellular Ca^{2+} in vascular smooth muscle could initiate different signal transduction mechanisms that affect vascular myogenic responses of the circulatory system (Davis and Hill, 1999; Blaustein *et al.*, 2009).

1.3.2.2. K^+ Channels

The membranous spanning proteins that selectively allow K^+ ions to pass through their pores are known as K^+ channels. The K^+ channels are the most widely distributed ionic channels in the body. In the vascular system, these proteins contribute to regulating the resting membrane potential, hence vascular tone of the circulatory system. Efflux of K^+ ions due to the opening of K^+ channels results in membrane hyperpolarization in vascular smooth muscle. This hyperpolarization leads to the closure of voltage-dependent Ca^{2+} channels, and subsequently reduction in Ca^{2+} entry, thus vasodilation (Nelson and Quayle, 1995). On the other hand, inhibition of K^+ channels leads to membrane depolarization and vasoconstriction (Nelson and Quayle, 1995). Four major classes of potassium channels have been identified in vascular smooth muscle; these include voltage-gated potassium channels (K_v), inwardly rectifying potassium channels (K_{ir}), calcium-activated potassium channels (K_{Ca}) and ATP-sensitive K^+ channels (K_{ATP}) (Nelson and Quayle, 1995; Standen and Quayle, 1998; Ko *et al.*, 2008). Molecular biology along with electrophysiological techniques introduced another class of K^+ channels, i.e. the two pore domain potassium channel (K_{2P}) (Lotshaw, 2007; Enyedi and Czirják G, 2010).

Voltage-gated potassium channels (K_v channels): K_v are ubiquitously expressed in vascular smooth muscle cells (Nelson and Quayle, 1995). The K_v has six transmembrane spanning domains made up of hydrophobic amino acids (Sl-S6), linked by sequences of hydrophilic amino acids. Each subunit also has a cytoplasmic amino- and carboxy-terminal domain. One of the membrane-spanning domains in each subunit (the S4 region)

is charged, having a basic amino acid (lysine or arginine) every third residue that acts as a voltage sensor to regulate the function of the channel (Korovkina and England, 2002). Each channel has two subunits, α and β. Structurally, α-subunits in K_v have cytoplasmic N- and C-termini and contain a pore. Further, each α-subunit is associated with ancillary β-subunits, which influences the characteristics of the channel (Bahring *et al.*, 2001; Korovkina and England, 2002). The opening of K_v in response to a change in voltage, i.e. depolarization, results in repolarization and thus return to the resting membrane potential. The small-scale depolarization in vascular smooth muscle cells leads to an influx of Ca^{2+} via L-type Ca^{2+} channels and activation of the contractile machinery. Taken together, this suggests that K_v function as a buffer and limit membrane depolarization, thus maintain resting vascular tone (Nelson and Quayle, 1995; Korovkina and England, 2002; Ko *et al.*, 2008). Prolonged depolarization leads to the inactivation of the K_v channel. Compared to the process of activation, K_v channel inactivation is relatively slow and involves an initial peak in the K_v current due to voltage-dependent activation followed by a drop in the current due to voltage dependent inactivation (Nelson and Quayle, 1995). The compound 4-aminopyridine (4-AP) has been used in many studies of vascular smooth muscle as a K_v channel blocker (Smirnov and Aaronson, 1992; Nelson and Quayle, 1995). In addition, tetraethylammonium ion (TEA) also inhibits K_v, although at higher concentrations than needed to inhibit K_{Ca} channels (Robertson and Nelson, 1994).

Inwardly rectifying potassium channel (K_{ir}): An ion channel that passes K^+ (positive charge) more easily into the cell (inward direction) is known as inward rectifying. The inward rectifing K^+ channels contain tetrameric subunits, each of which consist of only two transmembrane domains and are encoded by a member of the K_{ir} gene family

(Standen and Quayle, 1998; Kubo *et al.*, 2005). In blood vessels, K_{ir2} channels exhibit strong inward rectification (Park *et al.*, 2008). Bradley *et al.* (1998) have reported mRNA and protein expression of $K_{ir2.1}$, but not $K_{ir2.2}$ or $K_{ir2.3}$, in coronary, mesentery and cerebral (basilar) arteries of Sprague-Dawley rats. There is a general agreement that K_{ir} channels are abundant in the smooth muscle of small-diameter resistance vessels (Knot *et al.*, 1996; Park *et al.*, 2008; Ko *et al.*, 2008). However, Tennant and colleagues (2006) have also identified $K_{ir2.1}$ in cultured human pulmonary artery smooth muscle cells. The vascular smooth muscle K_{ir} channels mediate inward currents at membrane potentials that are negative relative to the E_K and small outward currents at membrane potentials that are positive relative to the E_K (Park *et al.*, 2008; Ko *et al.*, 2008). Ba^{2+} ions block $K_{ir2.1}$ in arterial smooth muscle cells. The inhibition by Ba^{2+} ions is amplified at more negative membrane potentials (Robertson *et al.*, 1996; Kubo *et al.*, 2005; Park *et al.*, 2008; Ko *et al.*, 2008). The $K_{ir2.1}$ channels play an integral role in K^+ induced vasodilation. A local increase in extracellular K^+ in the range of 6-15 mM has been shown to produce vasodilation and pronounced hyperpolarization of rat portal vein, mesenteric, coronary and cerebral arteries in the absence or presence of endothelium (Robertson *et al.*, 1996; Knot *et al.*, 1996; Edwards *et al.*, 1998; Weston *et al.*, 2002). The hyperpolarization induced by high extracellular K^+ ions involved $K_{ir2.1}$ and electrogenic pump (Na^+/K^+ATPase).

Ca^{2+}-activated potassium channels (K_{Ca}): On the basis of their conductance, K_{Ca} ramified into three subtypes that include large (BK_{Ca}), intermediate (IK_{Ca}) and small conductance (SK_{Ca}) (Nelson and Quayle, 1995). The BK_{Ca} channels seemed to be preferentially expressed in vascular smooth muscle cells, while IK_{Ca} and SK_{Ca} are

preferentially located in endothelial cells (Ghatta *et al.*, 2006; Félétou, 2009; Grgic *et al.*, 2009). As the name suggests, these K^+ channels are regulated by the cytoplasmic concentration of Ca^{2+}. Like K_v channels, BK_{Ca} also buffers pressure- or chemical-induced depolarization and vasoconstriction by hyperpolarizing the membrane potential (Tanaka *et al.*, 2004; Ledoux *et al.*, 2006). The BK_{Ca} channels are comprised of a pore-forming α-subunit and regulatory β-subunit. The pore forming α-subunits contain six transmembrane-spanning domains (S1–S6) that include a voltage sensor (S4) (Nelson and Quayle, 1995, Ledoux *et al.*, 2006). An additional seventh transmembrane region (S0) at the exoplasmic NH_2 terminus of BK_{Ca} has also been reported. The β-subunits enhance the Ca^{2+} sensitivity of the channel (Nelson and Quayle, 1995; Ko *et al.*, 2008). The BK_{Ca} induces vasodilatory responses through the cAMP/PKA or NO/cGMP/PKG pathway. Treatment with TEA, iberiotoxin, and charybdotoxin blocks the vascular BK_{Ca} channels (Nelson and Quayle, 1995). Of these blockers, iberiotoxin is the most selective blocker of the BK_{Ca}. Charybdotoxin has also been used as a selective BK_{Ca} channel blocker; however, it also affects IK_{Ca} (Ko *et al.*, 2008). Potential activators of BK_{Ca} are NS1619, NS1608 and estrogen (Ledoux *et al.*, 2006).

ATP-sensitive potassium channels (K_{ATP} channels): A low intracellular concentration of ATP leads to efflux of K^+ ions thus limiting myogenic depolarization and control of myogenic tone. Standen *et al.* (1989) first reported the role of K_{ATP} channels in arterial dilation in rats. The inhibition of K_{ATP} channels induce vasoconstriction and membrane depolarization in various vascular smooth muscles (Nelson *et al.*, 1990; Nakashima and Vanhoutte, 1995, Quayle *et al.*, 1997; Teramoto, 2006). Several endogenous agonists such as calcitonin gene-related peptide (CGRP) and adenosine activate K_{ATP} leading to

hyperpolarization and relaxation, a response that is mimicked by treatment with K_{ATP} openers. In contrast, various neurotransmitters (noradrenaline, 5-hydroxytryptamine (5-HT), neuropeptide Y and vasoconstrictors angiotensin II, endothelin-1) inhibit K_{ATP} leading to depolarization and contraction (Quayle *et al.*, 1997). Pathophysiological involvement of K_{ATP} is reported in systemic arterial dilation during hypoxia, reactive hyperaemia in coronary and cerebral circulation, and acidosis- and endotoxic shock-induced vasodilation (Brayden, 2002, Teramoto, 2006; Ko *et al.*, 2008). Moreover, the inhibition of K_{ATP} leads to impaired coronary and cerebral autoregulation (Nelson and Quayle, 1995). The K_{ATP} are hetero-octameric complexes containing four pore-forming, inwardly rectifying channel subunits ($K_{ir6.1}$ or $K_{ir6.2}$), together with four sulphonylurea receptors (SURs), which are an ATP-binding cassette (ABC) family of proteins (Teramoto, 2006; Ko *et al.*, 2008). In vascular smooth muscle cells, the K_{ATP} are composed of Kir6.X ($K_{ir6.1}$ or $K_{ir6.2}$)/SUR2B (Standen and Quayle, 1998; Teramoto, 2006; Ko *et al.*, 2008). Exogenously, the K_{ATP} in smooth muscle are known to be inhibited by glibenclamide and tolbutamide (anti-diabetic sulphonylurea drugs) (Nelson and Quayle, 1995; Quayle *et al.*, 1997; Standen and Quayle, 1998; Teramoto, 2006; Ko *et al.*, 2008). Various agents that open K_{ATP} are cromakalim, levcromakalim, nicorandil, pinacidil, minoxidil, diazoxide, and BRL-55834. The vasodilation and hyperpolarization induced by these agents can be blocked by glibenclamide (an antagonist of K_{ATP} channels) (Quayle *et al.*, 1997).

Two-pore domain potassium channel (K_{2P}): The membrane proteins with four trans-membrane domains (TMD) and two pores (P) domains that selectivity allow K^+ ions efflux are referred as two-pore domain potassium channels. This unique feature is at the

origin of their name, 2P domain K^+ channels or K_{2P} channels (Lotshaw, 2007). The K_{2P} channels are composed of four potential transmembrane segments (M1-M4), the 2P domains (P1 and P2), short NH_2-terminal and long COOH-terminal cytoplasmic parts, and an extended extracellular loop between M1 and P1. The connecting loops between M domains are generally short, although the sequence connecting M1 and M2 is typically longest and includes the first pore domain (P1). The M1-M2 extracellular linker domain usually contains one or more asparagines which may function in N-linked glycosylation and one or more cysteines which may form disulfide bridges between subunits (Lesage and Lazdunski, 2000). Isoform-specific amino acids have also been identified within this domain that contributes to important pharmacological characteristics (Enyedi and Czirják, 2010). The second pore domain (P2) is located between the M3 and M4 domains. The protonation of histidine 98 (His98) in the first pore (P1) is partly responsible for inhibition of the Tandem of P domains in Weak Inward rectifier K^+ channel (TWIK)-related acid sensitive K^+ channels (TASK) (Duprat *et al.*, 2007). The cytosolic C-terminal domain has also been shown to play several important roles in modulation of channel gating. Other than pore domains, these subunits do not share significant sequence homologies with the 6TMS/1P and 2TMS/1P subunits (Lesage and Lazdunski, 2000; Lotshaw, 2007; Enyedi and Czirják, 2010). However, based on gene sequence similarities, K_{2P} channels are divided into six subfamilies that includes Tandem of P domains in Weak Inward rectifier K^+ channel (TWIK), TWIK-Related K^+ channel (TREK) and TWIK-Related Arachidonic Acid stimulated K^+ channel (TRAAK), TWIK-related Acid Sensitive K^+ channel (TASK), TWIK-related Alkaline pH activated K^+ channel (TALK), Tandem-pore domain Halothane Inhibited K^+ channel (THIK)

(Lotshaw, 2007; Gurney and Manoury, 2009, Enyedi and Czirják G, 2010). The latter mentioned proteins are expressed in various tissues and extensively studied in the central nervous system (Talley *et al.,* 2001; Lesage, 2003; Lotshaw, 2007). The K_{2P} channels expressed in the systemic and pulmonary circulations are regulated by various chemical and physiological stimuli such as pH, oxygen, phospholipids, neurotransmitters, GPCRs and volatile anaesthetics. Moreover, stretch regulates the functions of K_{2P} channels under a range of physiological and pathological situations (Lesage and Lazdunski 2000; Mathie 2007). This sensitivity to a wide range of physiological signals (polyunsaturated fatty-acids, pH, O_2) that are important in regulating blood flow makes them important candidates as regulators of vascular tone, especially in small resistance vessels of the systemic circulation, such as mesenteric and cerebral arteries (Blondeau *et al.,* 2007; Garry *et al.,* 2007), and the pulmonary circulation (Gardener *et al.,* 2004; Gurney *et al.,* 2003). The arachidonic acid sensitive K_{2P} channels expressed in the cerebral artery, rat mesenteric and femoral arteries are TREK-1, TREK-2 and THIK (Lesage and Lazdunski, 2000). The TRAAK was reported in mesenteric but not femoral artery (Gardener *et al.*, 2004; Gurney and Manoury, 2009). The carotid arteries and pulmonary arteries lack TREK-1 and TRAAK. The pulmonary artery contains TASK-1, TASK-2, TREK-2, TWIK-2 and THIK 1 (Gardener *et al.*, 2004; Blondeau *et al.*, 2007; Gurney and Manoury, 2009). Functional and electrophysiological studies performed on mice deficient in TREK-1 suggest that it has a role in endothelial cells to regulate cerebral vascular tone (Blondeau *et al.*, 2007). The TASK-1 and TASK-2 channels expressed in pulmonary artery of various animals have shown to regulate arterial tone in response to various physiological stimuli (pH, O_2). Treatment with acidic buffer (pH 6.5), Zn^{2+}, or

anandamide depolarize the resting membrane potential by blocking the background channel, i.e. TASK (Maingret *et al.*, 2001). The participation of TASK in control of vascular tone seems to be more important in pulmonary vasculature as compared to systemic arteries (Gardener *et al.*, 2004).

1.3.2.3. Calcium channels

An increase in intracellular Ca^{2+} levels mediated by voltage-gated channels triggers a range of cellular responses such as the regulation of Ca^{2+}-dependent second messengers, Ca^{2+} release from cytoplasmic stores, gene expression and smooth muscle contraction (Xiong and Sperelakis, 1995). Based both on pharmacological and biophysical profiles, voltage-dependent Ca^{2+} channels have been classified into T-, L-, N-, P/Q-, and R-types (Bean, 1989; Pietrobon, 2005). Out of five different subtypes of voltage-dependent Ca^{2+} channels, L-type (long lasting/kinetically slow) and T-type (transient/ kinetically fast) have been described in various vascular smooth muscle cells (Sturek and Hermsmeyer, 1986; Bolton and Pacaud, 1992). The kinetics of activation or inactivation is slow for L-type Ca^{2+} compared to T-Type channels with different operational voltage range. The L-type Ca^{2+} channels operate at high threshold (activation voltage of -45 to -35 mV) with single channel conductance of 18-26 pS; and T-type Ca^{2+} channels operate at low threshold (activation voltage of -60 to -50 mV) with single channel conductance of 8-12 pS (Xiong and Sperelakis, 1995; Cribbs, 2006). Whereas L-type channels function primarily to regulate Ca^{2+} entry for contraction, it is generally accepted that T-type Ca^{2+} channels do not contribute significantly to arterial vasoconstriction, with the possible

exception of the renal microcirculation. The L-type channels are regulated by cyclic nucleotides and phosphorylation while T-types are not (Cribbs, 2006). Finally, L-type Ca^{2+} channels are inhibited by verapamil, diltiazem and nifedipine, and activated by Bay K8644 (Ca^{2+} channel agonist). The T-type channels are blocked by tetramethrine, R (-)-efonidipine and NNC 55-0396 (Jensen and Holstein-Rathlou, 2009).

1.3.2.4. Chloride Channels (Cl^-):

The chloride channels are abundantly distributed in smooth muscle membranes (Kitamura and Yamazaki; 2001). Vascular smooth muscle cell mainly includes two types of Cl^- currents, i.e. calcium dependent chloride currents and volume regulated currents (Chipperfield and Harper 2000; Kitamura and Yamazaki; 2001). Agents (such as noradrenaline, caffeine and the Ca^{2+} ionophore A-23187) that increase intracellular concentration of Ca^{2+} have been reported to activate Cl^- currents in the smooth muscle cells (Byrne and Large, 1987a,b). This change in chloride conductance produces membrane depolarization, which leads to the opening of voltage dependent Ca^{2+} channels thus leading to an influx of Ca^{2+} and hence contraction (Baron *et al.,* 1991; Criddle *et al.,* 1997). A second type of Cl^- currents has been reported to be activated by mechanical stress that occurs during vascular distention caused by high systolic pressure. The mechanical stress induced elevations in Cl^- conductance leads to the vascular contraction, thus reduces the arterial diameter (Nelson *et al.*, 1997). Criddle *et al.* (1997) reported that niflumic acid selectively inhibited the agonist-induced contraction without altering the high K^+-evoked contraction in mesenteric artery of rat. In parallel, He and Tabrizchi

(1997) demonstrated that niflumic acid antagonizes the α_1-adrenoceptor-induced contractions in isolated perfused mesenteric blood vessels. In addition, Cl^- channels play a role in maintenance of E_m of vascular smooth muscle cells by altering the transmembrane chloride concentration. This is affirmed by the use of various Cl^- channel agonists and antagonists (niflumic acid, indanyloxyacetic acid 94 and diphenylamine-2-carboxylic acid) (Large and Wang, 1996; Criddle *et al.,* 1997; Kitamura and Yamazaki; 2001).

1.3.3. Endothelial cell layer (Endothelium)

Until the late 1900s, endothelial cells were considered a homogeneous population of cells merely forming an inert barrier to separate the vascular space from the interstitium. Ground-breaking investigations by different research groups recognized the fundamental importance of endothelial cells in the regulation of vascular smooth muscle tone. Further, recognizing the role of endothelium in coagulation stressed the fact that it's not a merely passive barrier (Moncada *et al.* 1977; Furchgott and Zawadzki, 1980; Furchgott, 1983; Palmer *et al.*, 1987, 1988). The landmark report by Furchgott and Zawadzki (1980) unravelled the obligatory role of endothelial cells in the acetylcholine-evoked relaxations in isolated arteries of the rabbit. Since then, this pivotal observation has since been extended to a wide variety of blood vessels from different species, including humans (Félétou and Vanhoutte, 2009). Later in 1987, Ignarro *et al.* proposed that the endothelial mediator involved in endothelium-dependent relaxation is nitric oxide (NO), which stimulates soluble guanylate cyclase in vascular smooth muscle cells leading to increased

levels of cyclic guanosine monophosphate (cyclic GMP), and thus relaxation. The latter ground-breaking observation was further substantiated by reports published by Palmer and coworkers (1987, 1988). Evidently, there is a general agreement in the scientific community that NO is indeed a major endothelium-derived relaxing factor (EDRF). The discovery of various nitric oxide synthase (NOS) inhibitors aided in exploring the physiological role of NO in the vascular walls (Rees *et al.,* 1989). These pharmacological tools (NOS inhibitors) also made evident that NO is not a sole mediator of EDRF (Rees *et al.,* 1989). In certain blood-vessels, prostacyclin released from endothelial cells evoked noticeable vasorelaxation by activating the production of cyclic adenosine monophosphate (cyclic AMP) (Moncada and Vane, 1979; Furchgott and Vanhoutte, 1989). The endothelium dependent relaxation was observed in various blood vessels in the presence of NOS and a prostacyclin inhibitor. These endothelium-dependent relaxations were attributed to hyperpolarization of vascular smooth muscle triggered by the endothelial cells, and led to the postulation of the existence of endothelium-derived hyperpolarizing factor (EDHF) (Félétou and Vanhoutte, 1988; Nakashima *et al.*, 1993).

1.3.3.1. Nitric oxide (NO)

Nitric oxide generated by the vascular endothelium is a major regulator of vascular homeostasis, and changes in its bioavailability are now known to play a role in the development of a number of diseases (hypertension, atherosclerosis, stroke etc.) (Vanhoutte *et al.*, 2009). The impaired vascular function is common to the latter clinical conditions (Vanhoutte *et al.*, 2009). Palmer *et al.* (1987, 1988) reported the release of NO

and enzymatic formation of NO in the vascular endothelium. NO is a by-product from the conversion of the semi-essential amino acid L-arginine to L-citrulline, a reaction that is catalyzed by eNOS (Palmer *et al.*, 1988). NO is a free radical species that carries unpaired electrons on its outer shell. It is highly reactive with a half life of 3-5 seconds (Moncada *et al.*, 1989). NO reacts with superoxide (O^{2-}) and other reactive oxygen species to produce a highly cytotoxic reactive nitrogen species known as peroxynitrite. In addition, NO exhibits low water solubility that aids the gaseous molecule to diffuse rapidly across physiological membranes into the adjacent smooth muscle cells (Andrews *et al.*, 2002).

The endothelial nitric oxide synthase (eNOS or NOS III) is one of three isoforms of the enzyme that is mainly localized in caveolae in the membrane of endothelial cells. The other two isoforms are neuronal nitric oxide synthase (nNOS or NOS I) and inducible nitric oxide synthase (iNOS or NOS II) (Moncada and Higgs, 2006). The eNOS is constitutively active in the endothelial cells. An enzyme contains various domains; the C-terminal of eNOS is referred as reductase domain that provides a binding site for nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), and flavin adenine mononucleotide (FMN) (Palmer *et al.*, 1987; Moncada and Higgs, 1995). The N-terminal provides an oxygenation site (heme protein) for L-arginine and is known as the oxygenation domain. This domain also has a site for a co-factor, tetrahydrobiopterin (BH_4). The commissure where both reductase and oxygenase domains join is a binding site for calmodulin and acts as a switch to regulate the flow of electrons between the two domains (Moncada and Higgs, 2006).

The generation of NO by agonists (bradykinin, substance P and acetylcholine) was originally considered to be regulated by $[Ca^{2+}]_i$ ions. This was confirmed by chelation of extracellular Ca^{2+} or with the presence of an antagonist of calmodulin that abolishes NO production in response to these agonists (Luckhoff *et al.,* 1988; Busse and Mulsch 1990). The mechanism of Ca^{2+} dependent activation has been extensively studied and it is recognized that the various agonists activate phospholipase C, leading to increases in cytoplasmic Ca^{2+} and diacylglycerol. The increases in $[Ca^{2+}]_i$ cause displacement of the eNOS, thus allowing calmodulin access to its binding site on the eNOS. The binding of Ca^{2+}/calmodulin to the domains connecting region of eNOS results in an NADPH-dependent flow of electrons from the reductase region of one monomer of eNOS to the heme iron in the oxygenase region of the other monomer initiating the synthesis of NO (Moncada and Higgs, 2006). However, it is also now evident that eNOS could be activated by certain stimuli without a sustained increase in $[Ca^{2+}]_i$ being necessary. For example, shear stress, a stimulus generated by blood flowing on the endothelial cell surface enhances the production of NO by two to fourfold over basal values that is maintained as long as the stimulus is applied (Hoyer *et al.*, 1998). Initially, the generation of NO in response to shear stress due to the activation of eNOS was thought to be Ca^{2+}-independent. However further, investigation reveals that there is an initial transient increase in $[Ca^{2+}]_i$ when shear stress is applied (Hoyer *et al.*, 1998). This was also confirmed by inhibition of shear stress-induced activation of eNOS by chelation of intracellular Ca^{2+}. This has led to the view that activation of eNOS by shear stress actually requires Ca^{2+}; and the enzyme could be phosphorylated at resting $[Ca^{2+}]_i$ levels to generate NO (Dimmeler *et al.*, 1999). The mechanism by which shear stress generates

NO involves the phosphatidylinositol 3-kinase (PI3K) pathway, leading to the activation of serine kinase Akt-1which phosphorylates eNOS on Ser^{1177} (Dimmeler *et al.*, 1999; Fulton *et al.*, 1999). In endothelial cells, eNOS has also been reported to be activated via G-protein-coupled receptors (Schini-Kerth and Vanhoutte, 1995).

In vascular smooth muscle cells, the main target for NO is the enzyme, soluble guanylyl cyclase (sGC), that catalyses the conversion of GTP into the second messenger molecule (cyclic guanosine monophosphate; *cGMP*) that ultimately causes relaxation. The activation of sGC arises when NO interacts with its heme moiety to form a complex that ultimately induces conformational changes in the enzyme which exposes the catalytic site to GTP (Hobbs, 1997). The cGMP induces relaxation by decreasing Ca^{2+} flux by inhibiting the flow through voltage-gated Ca^{2+} channels (Lincoln, 1989). In addition, cGMP also activates cGMP-dependent protein kinases (Schlossmann and Hofmann 2005) protein kinase G-I (PKG I). Further, PKG I phosphorylates proteins in the sarcoplasmic reticulum, including the Ca^{2+}-activated K^+ channels (Archer, 1994), the IP3 receptor-associated cGMP kinase substrate (Hirata *et al.*, 1990) and phospholamban (Cornwell *et al.*, 1991). The phosphorylation of these proteins leads to the sequestration of Ca^{2+} in the sarcoplasmic reticulum, reduction of cytosolic Ca^{2+} and vascular relaxation.

Damage to the endothelium perturbs vascular homeostasis. The impairment in NO production or bioavailability is an important risk factor for hypertension and other cardiovascular diseases; and is referred to as endothelial dysfunction. The endothelial dysfunction is measured as a decrease in endothelium-dependent vasodilation induced either by appropriate agonists (Schachinger *et al.*, 2000) or by shear stress due to flow

(Neunteufl *et al.*, 2000). This has been demonstrated in both animal and human hypertensive populations (Vanhoutte *et al.*, 2009). In an animal model, Ca^{2+} dependent NOS activity was reported comparable between aortas of hypertensive and normotensive rats (Nava *et al.*, 1996). However, NO production was normal in spontaneously hypertensive rats, but O^{2-} production was elevated and led to increased oxidation of NO, resulting in a decreased vasodilatation response (Heitzer *et al.*, 2000). The superoxide ion could arise from a variety of sources, including NADPH oxidases and cyclo-oxygenase and these enzymes are reported to be up-regulated in hypertension (Vanhoutte *et al.*, 2009). Taken together, evidence seems to suggest that essential hypertension is associated with reduced endothelium-dependent relaxation, where the functional role of NO is regulated through its destruction or modification by another radical, namely superoxide.

1.3.3.2. Endothelium dependent Hyperpolarizing factors (EDHF)

The EDHF is not a single factor that is synthesized in endothelial cells diffusing to adjacent vascular smooth muscle cells to elicit hyperpolarization. The EDHFs represent a pool of various mediators that are released with EDRF from endothelial cells by various endothelium-dependent vasodilators (Félétou and Vanhoutte, 2009). Along with NO, numerous other factors such as arachidonic metabolites (cyclo-oxygenase derived prostacyclin, PGI_2; cytochrome P450 mono-oxygenase derived epoxyecosatrienoic acids, EETs; lipoxygenase derived 12 -(*S*)- hydroxyeicosatetraenoic acid, HETE), gaseous molecules (carbon monoxide, CO; hydrogen sulphide, H_2S), reactive oxygen species

(hydrogen peroxide, H_2O_2), vasoactive peptides (C-type natriuretic peptide, CNP; vasoactive intestinal peptide, VIP; calcitonin gene-related peptide, CGRP and adrenomedullin) and adenosine, mediate their effects by activating K^+ channels and/or gap junctions causing hyperpolarization of underlying smooth muscle cells (Félétou and Vanhoutte, 2009).

The EDHF-mediated responses are characterized as a non-NO/non-PGI_2 mediated endothelium-dependent vasorelaxation response that is accompanied by hyperpolarization of vascular smooth muscle. The EETs (11, 12 EET; 14, 15 EET), generated in the endothelial cell by cytochrome P450, diffuse into adjacent smooth muscle cells and activate BK_{Ca} channels causing hyperpolarization (Félétou and Vanhoutte, 2009; Campbell and Fleming, 2010). In blood vessels, bradykinin, pulsatile stretch and shear stress have been reported to release EETs that hyperpolarize underlying vascular smooth muscle cells by activating large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels (Huang *et al.*, 2005; Weston *et al.*, 2005). Alternatively, Earley *et al.* (2005) reported that EETs activate vanilloid transient receptor potential channels type 4(TRPV4). The Ca^{2+} influx through TRPV4 increases the frequency of unitary Ca^{2+} release events (Ca^{2+} sparks) via ryanodine receptors located on the sarcoplasmic reticulum of cerebral artery smooth muscle cells. Further, EET-induced Ca^{2+} sparks activate nearby sarcolemmal BK_{Ca} channels. Like EETs another arachidonic metabolite, lipoxygenase derived 12-*S*-HETE, was reported to be released by endothelial cells of porcine coronary artery and rabbit aorta and activate sarcolemmal BK_{Ca} channels, thus causing hyperpolarization of smooth muscle cells (Pfister *et al.*, 1987; Zink *et al.*, 2001). The endothelium-dependent relaxations evoked by lipoxygenase derivatives are restricted to few blood vessels

(Félétou and Vanhoutte, 2009). Other mediators that recently joined the EDHF club are CO, H_2S and H_2O_2. Carbon monoxide is a by-product derived from degradation of heme by heme-oxygenase. Like 12-*S*-HETE, CO vasodilator action is restricted to few blood vessels. CO is a diffusible gaseous molecule that induces cGMP-mediated activation of BK_{Ca} and direct activation of K_{ATP} and voltage-gated K^+ channels (K_V) in the smooth muscle cells (Wu and Wang, 2005). In contrast, CO is also recognized as a NOS inhibitor via binding to prosthetic heme site of the latter enzyme and could contribute to endothelial dysfunction (Baragatti *et al.*, 2007). Although, the H_2S molecule has been shown to hyperpolarize blood vessels, it is mostly expressed in vascular smooth muscle cells and virtually absent in endothelial cells (Wang, 2002). The last member of gaseous EDHFs is H_2O_2 which is derived by the reaction of superoxide dismutases with superoxide ion. The H_2O_2 molecule acts as an important antioxidant defense in nearly all cells exposed to oxygen. The H_2O_2 elicits endothelium-dependent relaxations by various pathways such as by activation of cGMP and various K^+ channels (BK_{Ca}, K_{ATP}, K_V and K_{ir}) (Ellis and Triggle, 2003; Shimokawa and Matoba, 2004). However, in certain vessels H_2O_2 mediated relaxation is not always associated with hyperpolarization of smooth muscle cells (Chaytor *et al.,* 2003). Amongst the vasoactive peptides, C-type natriuretic peptide (CNP) has been investigated in mesenteric and carotid arteries and various veins (Ahluwalia and Hobbs, 2005; Félétou and Vanhoutte, 2009). As with other EDHF candidates, CNP evoked hyperpolarization is due to stimulation of cGMP that activates the BK_{Ca} channels (Wei *et al.*, 1994). In addition, CNP has also been reported to activate G-protein regulated inward-rectifier K^+ channels (GIRK) independent of stimulation of cGMP (Villar *et al.*, 2007).

Common to all EDHF induced hyperpolarization is activation of the K^+ channels present on vascular smooth muscle cells. This underscores the importance of K^+ ions as front runner in the race for the EDHF crown. The contribution of two calcium activated K^+ channels that have been rigoursly investigated for EDHF are the intermediate and small conductance Ca^{2+} activated K^+ channels (IK_{Ca}, SK_{Ca}). These channels are expressed in the plasma membrane of vascular endothelial cells. The endothelium-dependent relaxant agonists elevate the intracellular level of Ca^{2+} and activate IK_{Ca} and SK_{Ca} channels that further cause efflux of K^+ into the extracellular space. The accumulation of K^+ in the extracellular space can provoke relaxation and/or hyperpolarization of vascular smooth muscle cells by activating K_{ir} and/or the Na^+/K^+ATPase pump (Edward *et al.*, 1998). Similar hyperpolarization was observed by raising the extracellular K^+ concentration (5-15 mM) in various blood vessels (Hendrickx and Casteels, 1974; Nelson and Quayle, 1995; Prior *et al.*, 1998; Edward *et al.*, 1998). In most blood vessels, pharmacological tools used to study these two channels are charybdotoxin and TRAM-34 (for blocking IK_{Ca}); and apamin, scyllatoxin and UCL1684 (for inhibiting SK_{Ca}) (Neylon *et al.*, 1999). Following activation of IK_{Ca} and SK_{Ca} channels, the efflux of K^+ from intracellular space to extracellular space in the endothelium in intact blood vessels supposedly occurs through hetero-cellular hemi-channels known as myoendothelial gap junctions (MEGJ). The MEGJ play an integral role in the EDHF-mediated responses. Inhibition of these junctions by gap junction inhibitors (peptides, GAP-27; α-glycyrrhetinic acid derivatives; carbenoxolone and heptanol) attenuates the EDHF-mediated and endothelium-dependent relaxant responses in various blood vessels (Yamamoto *et al.*, 1999; Sandow and Hill, 2000; Sandow *et al.*, 2002; Dora *et al.*, 2003; Haddock *et al.*, 2006). The gap junctions

are hemi-channels (connexins) that aid electrical coupling between same cells (smooth muscle cells-smooth muscle cells; endothelial cells-endothelial cells) referred to as homo-cellular gap junctions; and between two different cells (smooth muscle cells-endothelial cells) referred to as hetero-cellular gap junctions (Schmidt *et al.*, 2008). In blood vessels, the gap junctions are made of connexins (Cx) 37, 40 and 43 (Haddock *et al.*, 2006; Schmidt *et al.*, 2008). Sandow and Hill (2000) reported that the number of MEGJs increase with the reduction in the size of the artery. In small arteries such as mesentery artery, SK_{Ca} co-localizes with Cx37, Cx40, and Cx43 and is adjacent to endothelial cell gap junctions, whereas IK_{Ca} co-localize with Cx37 and Cx40 in myoendothelial gap junctions (Sandow *et al.*, 2006). This co-localization of channels in MEGJs aids in the transmission of endothelial hyperpolarization to the underlying smooth muscle which plays an important role in EDHF-mediated responses in many blood vessels. In addition, with the aid of MEGJs vascular electrical responses are conducted through a low-resistance pathway provided by the endothelial cell layer (Dora, 2010). Various studies with IK_{Ca}/ SK_{Ca}/ Cx knock-out mice have shown the importance of these components in the cardiovascular system (Félétou and Vanhoutte, 2009). In genetically modified mice (eNOS knockout mice), EDHF mediated responses play a compensatory role for the absence of eNOS (Waldron *et al.*, 1999; Brandes *et al.*, 2000). Deletion of IK_{Ca} and SK_{Ca} expression in the endothelial cells of transgenic mice showed elevated blood pressure with reduced hyperpolarization noted in blood vessels in response to acetylcholine (Taylor *et al.*, 2003; Si *et al.*, 2006). The endothelium-mediated relaxant responses have been shown to attenuate in Cx 40 knockout mice. Further, theses connexins are suggested to play a role in the development of hypertension and irregular arteriolar vasomotion (de

Wit *et al.*, 2003). Taken together, alteration of the EDHF pathway can also contribute to these endothelial dysfunctions. The EDHF has been suggested to play a compensatory role in the loss of NO bioavailability (Félétou and Vanhoutte, 2009). An anomaly in the function or expression of various proteins expressed in vascular smooth muscle and endothelial cells could affect the dilator function of blood vessels that is critical for optimal tissue perfusion.

1.3.4. Signalling pathways involved in vascular contraction

The interaction of chemical compounds (agonists) with cell surface receptors results in the activation of various intracellular signalling pathways and generation of numerous second messengers. The second messengers target various intracellular targets to generate vascular smooth muscle responses.

The phosphophatidylinositol 4, 5, diphosphate (PIP_2) is an important constituent of all plasma membranes (Hirasawa and Nishizuka, 1985). Its phosphodiesteric cleavage to inositol 1, 4, 5 triphosphate (IP_3) and 1, 2, diacylglycerol (DAG) has been linked to various agonists such as acetylcholine (Van Breemen *et al.*, 1984), histamine (Tsuru, 1984), angiotensin (Smith, 1986), noradrenaline (Bulbring and Tomita, 1987), serotonin (Lapetina, 1987), thromboxane A_2 (Lapetina, 1987), ATP (North, 2002), and vasopressin (Crooke *et al.*, 1988). The phosphatidylinositol (PI) undergoes two-stage phosphorylation in the inner plasma membrane to form PIP_2, which is the precursor for receptor activation by external stimuli (Hirasawa and Nishizuka, 1985). Receptor occupation stimulates G-protein regulated phospholipase C that hydrolyse PIP_2 to

produce IP_3 and DAG (Hirasawa and Nishizuka, 1985). DAG is immediately phosphorylated to phosphatidic acid (PA) by kinase in presence of ATP (Hirasawa and Nishizuka, 1985). IP_3 is the intracellular mediator of Ca^{2+} release from the endoplasmic

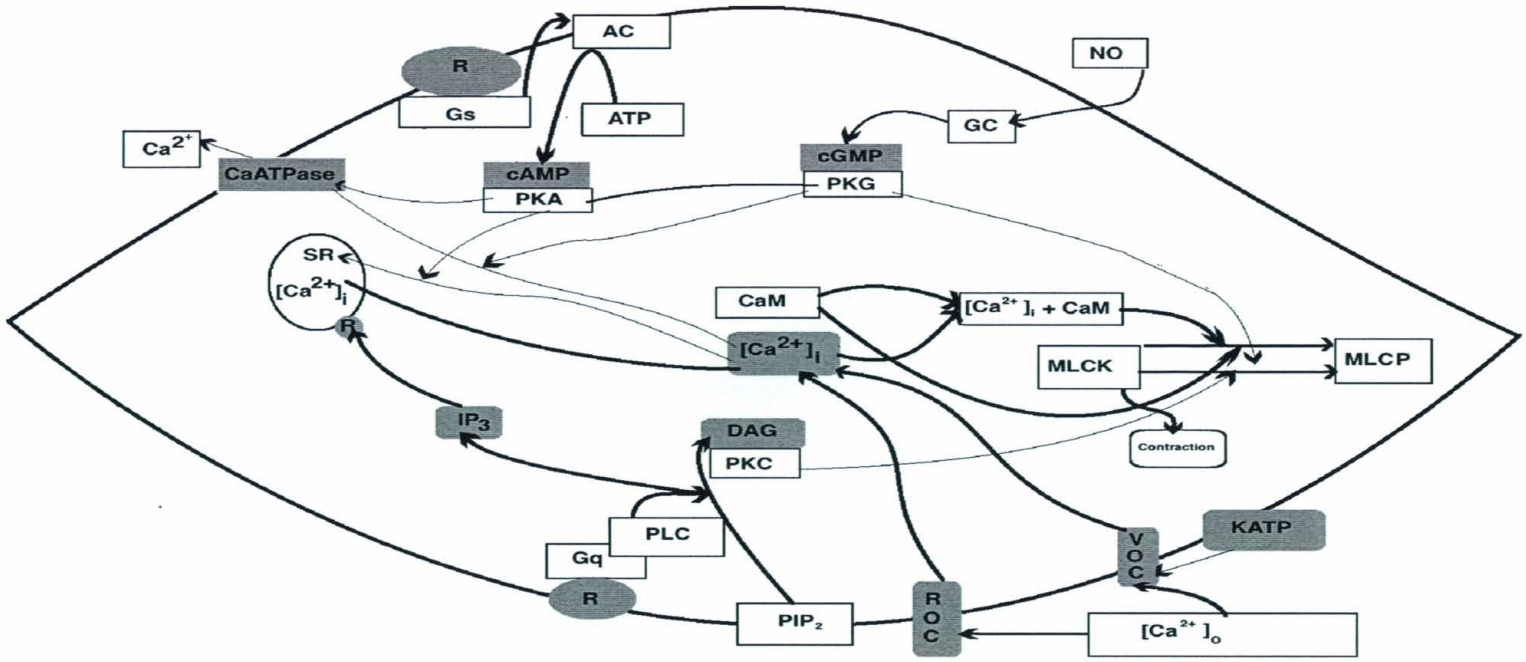

Figure 1. A schematic summarizing signalling involved in vascular smooth muscle cell for the process of contraction and relaxation. Adenylyl cyclase, (AC); adenosine 5-triphosphate, (ATP); ATP-sensitive potassium channel, (K_{ATP}); calcium, [Ca^{2+}]; intracellular concentration of Ca^{2+}, ([Ca^{2+}]$_i$); extracellular concentration of Ca^{2+}, ([Ca^{2+}]$_o$); calmodulin, (CaM); calcium ATPase enzyme, (CaATPase); 3'-5'-cyclic adenosine monophosphate, (cAMP); cyclic guanosine monophosphate, (cGMP);

guanylyl cyclase, (GC); G-protein, (G_q); stimulatory G-protein (G_s); inositol 1,4,5-triphosphate, (IP_3); 1,2 diacylglycerol, (DAG); myosin light chain kinase, (MLCK); myosin light chain phosphatase, (MLCP);phospholipase C, (PLC); nitric oxide, (NO); protein kinase A, (PKA); protein kinase G, (PKG); phosphophatidylinositol 4, 5, diphosphate, (PIP_2); receptor operated Ca channel, (ROC); sarcoplasmic reticulum, (SR); receptor, (R); voltage operated Ca channel, (VOC); . Solid dark lines represent pathways mediating contraction and solid light lines represent pathways mediating relaxation

reticulum via IP_3 receptors (Streb *et al.*, 1984). Elevation of $[Ca^{2+}]_i$ leads to binding of Ca^{2+} to calmodulin (CaM) to form Ca^{2+}-CaM complex. This complex binds and activates myosin light chain kinase (MLCK) (Dabrowska *et al.*, 1978). Further, active kinase complex (Ca^{2+}-CaM-MLCK) phosphorylates the two 20-kDa light chain subunit of myosin at serine[19] and induces the cross-bridge cycling to generate force (Walsh, 1994). The phosphorylated myosin is dephosphorylated by myosin light chain phosphatase (MLCP), thus relaxing smooth muscle (Walsh, 1994). DAG is the membrane-associated activator of protein kinase C (PKC) (Hirasawa and Nishizuka, 1985). PKC has been shown to play an important role in sensitization of the smooth muscle leading to contraction. This is referred to as sensitization of contractile elements that evoke enhanced contraction at a given intracellular Ca^{2+} concentration (Somlyo and Somlyo, 2003). In addition, PKC targets nucleotide exchange factor on small molecular weight G-protein (Rho subfamily) that further phosphorylates MLCP and results in sustained contraction (Somlyo and Somlyo, 2003). PKC-induced sensitization could be also caused by direct or indirect phosphorylation of MLCK by CaM kinase II and subsequently decrease the affinity of the enzyme for Ca^{2+}-CaM complex (Tansey *et al.*, 1994; Somlyo and Somlyo, 2003).

1.4. β-adrenoceptors in cardiovascular system

Catecholamines such as noradrenaline and adrenaline regulate physiological processes via their interactions with a variety of membrane bound receptors. A landmark study by Ahlquist (1948) divided these surface receptors into α- and β-adrenergic receptors on the

basis of the relative potency of various agonists in generating cardiovascular responses. Studies revealed that β-adrenoceptors can be sub-divided into β_1, β_2, β_3, atypical or β_4 (Lands *et al.*, 1967; Emorine *et al.*, 1989; Kaumann *et al.*, 1997). It has also been suggested that β_4 might be the low affinity state of the β_1 receptor (Kaumann *et al.*, 2001). The β-adrenoceptors are expressed in the cardiovascular system and play an integral role in the regulation of the same. β_1- and β_2-adrenoreceptors are expressed in the pump of the circulatory system, i.e. the heart. Under normal physiologic conditions contractility and/or heart rate is regulated by cardiac β_1-adrenoceptors but it has been suggested that in situations of stress, when large amounts of adrenaline is released from the adrenal medulla, stimulation of cardiac β_2-adrenoceptors could contribute to additional increases in contractility and heart rate (Motomura *et al.*, 1990; Brodde and Michel, 1999). Moreover, the existence of β_3-adrenoreceptors has been suggested to mediate negative inotropic effects in myocardium (Gauthier *et al.,* 2000), but their role remains unclear (Heubach *et al.,* 2000). The modifications in β-adrenoceptors seem to play an important role in heart disease such as heart failure. The pronounced activation of the sympathetic system in patients with heart failure seems to be inversely correlated with survival. The cardiac β-adrenoceptors, in particular the β_1 subtype, are down-regulated in heart failure (Brodde, 1993), and the remaining receptors are uncoupled from $G_{\alpha s}$, presumably via increased activity of G-protein receptor kinases (GRK2 and/or GRK5) (Ungerer *et al.*, 1993). In addition, an increase in $G_{\alpha i}$ proteins in heart failure and congestive cardiomyopathy direct its antagonizing effect on β-adrenoceptor-mediated cardiac responses (Neumann *et al.*, 1988; Bohm *et al.,* 1990). Furthermore, the increased sympathetic activity could aggravate the disease process via increased renin secretion and

sodium retention, both of which could increase peripheral resistance and further increase the work load for the heart. Increased renin secretion in part occurs by the activation of β_1-adrenoceptors in kidneys. The β_1-adrenoceptors are present in high density on the renin-containing juxtaglomerular cells in the afferent arterioles (Johns, 1981). Stimulation of these receptors results in increased renin secretion rate causing no or minimal changes in renal hemodynamics (Kopp *et al.*, 1980). In addition, β_2-adrenoceptors have been found to localize in the collecting ducts, and activation of these receptors may alter the transport properties of various tubular segments (DiBona and Kopp, 1997). The increased production of renin leads to formation of angiotensin II via the renin-angiotensin-aldosterone system (RAAS). Further, angiotensin II causes blood vessels to constrict, resulting in increased blood pressure, and it also stimulates the secretion of the hormone aldosterone from the adrenal cortex. Aldosterone causes the tubules of the kidneys to increase the reabsorption of sodium and hence water into the blood. This increases the volume of fluid in the body, which also increases blood pressure (Paul *et al.*, 2006). Lastly, β-adrenoceptors are also expressed in the blood transporting vessels of the circulatory system. The β-adrenoceptor-mediated vasodilation plays an important physiological role in the regulation of vascular tone (Guimarães and Moura, 2001). Stimulation of peripheral β-adrenoceptors leads to relaxation of the vascular smooth muscle and can regulate peripheral vascular resistance resulting in the distribution of blood to the different organs. So far, three subtypes of β-adrenoceptors have been identified in the mammalian blood vessels, i.e. β_1, β_2 and β_3 (O'Donnell and Wanstall, 1984; Shen *et al.*, 1996; MacDonald *et al.*, 1999; Chruscinski *et al.*, 2001; Briones *et al.*, 2005). Stimulation of β_1 and β_2 were suggested to play role in the

relaxation of both arteries and veins (Taira *et al.*, 1977; Vatner *et al.*, 1985). The maximal relaxant response mediated via β-adrenoceptors varies amongst vascular bed and species, for example: in some arteries such as cerebral and coronary arteries, the β_1-subtype plays an important role in relaxation (Edvinsson and Owman, 1974; O'Donnell and Wanstall, 1985) whereas β_2 evoked relaxation responses is larger in veins (Molenaar *et al.*, 1988). In addition, the relaxant responses mainly depend on the level of tone of the tissues (Begonha *et al.*, 1995). In the 1990s, the existence of a subtype other than classical β_1- and β_2-adrenoceptor involved in relaxation was reported in rat carotid, mesentery and pulmonary arteries; rat aorta and canine pulmonary artery (Gray and Marshall, 1992; Sooch and Marshall, 1995; Tamaoki *et al.*, 1998; MacDonald *et al.*, 1999). With the discovery of β_3-agonists (BRL-37344, CL-326243, CGP-12177 and ZD-2079) and antagonists (SR-59230), β_3-signalling has been studies in experimental models (both *in vivo* and i*n vitro*). Although the β_3-adrenoceptor has been shown to play a role in regulating vascular tone, its role varies amongst species. Stimulation of β_3-adrenoceptor by BRL-37344 and CL-326243 in mice, rats and dogs led to reduction of blood pressure and total peripheral resistance (Tavernier *et al.*, 1992; Shen *et al.*, 1996; Rohrer *et al.*, 1999). In contrast, Shen *et al.* (1996) reported that β_3-adrenoceptor agonists were not able to evoke any cardiovascular effect in conscious primates like monkey and baboon. Further, a propranolol (non-selective β-antagonist) resistant relaxant component in rat aorta is not antagonized by selective inhibitors of β_1, β_2 and β_3 receptors (atenolol, β_1-antagonist; *ICI* 11855, β_2-antagonist; SR-59230) (Brawley *et al.*, 2000). The later suggests the presence of a fourth or atypical (β) subtype that participates in the relaxation of blood vessels. Emerging literature on the atypical β-adrenoceptors suggests that it is a

low affinity form of β_1-stubtype that has a typical interaction with CGP-12177 (β_3-agonist) (Konkar *et al.*, 2000). The physiological importance of β-adrenoceptor has been elucidated in transgenic mice. In β_1-knockout mice, high variability in blood pressure and significantly low heart rate in response to isoprenaline were observed (Rohrer *et al.*, 1999). In β_2-knockout mice, heart rate and blood pressure appear normal at rest but these parameters were found elevated during exercise (Chruscinski *et al.*, 1999). In addition, the hypotensive response to isoprenaline is significantly attenuated in β_2-knockout mice (Chruscinski *et al.*, 1999). Interestingly, in double knockout mice (β_1 and β_2) the upregulation of β_3-adrenoceptor compensated for unaltered cardiovascular responses. This was shown by significantly enhanced cardiovascular responses (hypotension) to β_3-agonist CL-316243(Rohrer *et al.*, 1999). Taken together, β-adrenoceptors play major roles in blood flow distribution to various organs (depending on the metabolic need) and regulation of vascular tone.

1.4.1. β-adrenoceptor signalling

The β-adrenoceptor is a GPCR and has α, β, γ subunits as part of its infrastructure. The β-adrenoceptor is comprised of 413 amino acid residues of approximately 46,500 Daltons. Like all GPCRs, the β-adrenoceptor has seven transmembrane-spanning α-helices with three extracellular loops, with one being the amino terminus and three intracellular loops with a carboxy-terminus (Emorine *et al.*, 1991). The overall amino acid sequence identity between the three receptors is about 50% and may reach up to 90% in transmembrane regions that participate in catecholamine binding (Marullo *et al.*, 1990).

The residues involved in the binding of ligands to β-adrenoceptors are associated with the seven transmembrane regions, arranged in such a way that they form a pocket, and a part of the extracellular hydrophilic loops joining the transmembrane regions (Fraser, 1989). Following binding of the ligand to the receptor, the conformational changes activate $G_{\alpha s}$ in vascular smooth muscle cells (Scheid *et al.*, 1979) or $G_{\alpha i}$ in endothelial cells (Zheng *et al.*, 2005) that stimulates second messenger systems or activates ion channels. The interaction between β-adrenoceptors and the $G_{\alpha s}$ protein depends on residues from the intra-cytoplasmic loops and the C-terminal region (O'Dowd *et al.*, 1988). Furthermore, coupling of $G_{\alpha s}$ to adenylyl cyclase (AC) results in the activation of the enzyme and catalyses the conversion of ATP to cAMP. The cAMP activates protein kinase A (PKA) by binding to its regulatory subunit, causing PKA to dissociate from the catalytic subunit, thereby rendering it active. PKA is a serine/threonine protein kinase that targets a number of intracellular proteins to produce vascular relaxation, involving a) PKA-induced phosphorylation of MLCK that result in decreasing the affinity of MLCK for calmodulin thus relaxation of smooth muscle cells (Somlyo and Somlyo 1994; Walsh 1994); b) activation of large-conductance, Ca^{2+} activated K_{Ca} channels; c) activation of K^+ channels resulting in hyperpolarization and reduced Ca^{2+} influx (Chen and Rembold, 1992) and d) sequestration of Ca^{2+} into internal stores (Tawada *et al.*, 1988). In addition, the eNOS inhibitor N^G-monomethyl-L-arginine (L-NMMA) attenuated the relaxation response to β_2-adrenoceptors stimulation or to the cAMP analogue dibutyryl cAMP, indicating that the NO-dependent component of the β-adrenoceptor relaxation response is mediated largely through the elevation of cAMP. The levels of eNOS serine phosphorylation were also found elevated on stimulation of β-adrenoceptors in human

umbilical vein endothelial cells (HUVECs) (Yao *et al.*, 2003). This was confirmed by pre-treatment of HUVECs with the PKA inhibitor H89 that resulted in inhibition of β-adrenoceptor-mediated serine phosphorylation of eNOS (Ferro *et al.*, 2003). Taken together, it seems that β-adrenoceptor-mediated vasorelaxation has both cAMP-dependent and cAMP-independent components.

1.4.1.1. cAMP-independent pathways

Endothelial/Nitric oxide/cGMP pathway: The participation of endothelial cells/NO in β-adrenoceptor-mediated vascular relaxation is controversial. On one hand, it has been suggested that the removal of the endothelium or inhibitors of NOS was found to have no influence on isoprenaline evoked relaxations in rat aorta (Moncada *et al.*, 1991), canine coronary arteries (White *et al.*, 1986), rat carotid artery (Oriowo, 1994), or human internal mammary artery (Molenaar *et al.*, 1988); and on other hand, many authors showed that removal of endothelium reduces the relaxations caused by β-adrenoceptor agonists in several isolated vessels that include rat thoracic aorta (Brawley *et al.*, 2000), canine coronary arteries (Rubanyi and Vanhoutte, 1985), mouse aorta (Akimoto *et al.*, 2002) and rat pulmonary artery (Bieger *et al.*, 2006). Surprisingly, two different investigators Moncada *et al.* (1991) and Brawley *et al.* (2000) showed opposite results in the same tissue (rat thoracic aorta). It is plausible that different endothelial β-adrenoceptor (β_3) subtype mediate isoprenaline-evoked hyperpolarisation in rat thoracic aorta. This hypothesis was investigated by Trochu *et al.* (1999) and the evidence suggested that β_3-adrenoceptors are mainly located on endothelial cells, and act in conjunction with β_1- and

β_2-adrenoceptors to mediate relaxation through activation of an NOS pathway and a subsequent increase in cGMP levels. It was also found that β_2-adrenoceptor-mediated eNOS activation occurs independently of Ca^{2+} and might be mediated via the phosphoinositide 3-kinase/Akt (serine/threonine protein kinase) pathway. This was confirmed in rat thoracic aorta by treating the tissue with wortmannin (inhibitor of phosphoinositide 3-kinases) and an Akt inhibitor resulting in the inhibition of β-adrenoceptor mediated eNOS activation (Ferro *et al.*, 2004). Taken together, the role of the endothelium in β-adrenoceptor mediated vasodilation is still unclear.

Role of K^+ channels in β-adrenoceptors signalling: The stimulation of β-adrenoceptors is also associated with hyperpolarization of smooth muscle cells (Prehn *et al.,* 1983; Nakashima and Vanhoutte, 1995; Ming *et al.*, 1997; Fujii *et al.*, 1999; Goto *et al.*, 2001; Bieger *et al.*, 2006; Garland *et al.*, 2011). This change in membrane potential has been linked to the opening of K^+ channels that vary amongst the vascular beds and species. For example, Nakashima and Vanhoutte (1995) demonstrated that stimulation of β-adrenoceptors in canine saphenous vein involves the opening of K_{ATP} channels and venodilation. Similarly, the activation of β-adrenoceptors in rat mesenteric arteries has been found to result in the opening of K_{ATP} channels leading to the hyperpolarization of vascular muscle (Fujii *et al.*, 1999; Goto *et* al., 2001; Garland *et al.*, 2011). Moreover, Ming and colleagues (1997) reported that in conscious dogs, β_2-adrenoceptor-mediated dilation of resistance coronary arteries involved the opening of K_{ATP} channels as well as the generation of NO. Bieger *et al.* (2006) demonstrated that isoprenaline induced hyperpolarization in the rat main pulmonary artery was associated with the opening of two-pore acid sensitive K^+ channels (TASK), but not the K_{ATP} channels. The activation

of TASK following stimulation of β-adrenoceptors did not appear to be mediated via cAMP/PKA activation (Bieger *et al.*, 2006).

The β-adrenoceptor-activated AC/cAMP/PKA phosphorylates serine/threonine residue on β-adrenergic receptor kinase (βARK) that further phosphorylates serine/threonine residues on the β-adrenoceptor, thus facilitating binding of β-arrestins to the receptor (Pitcher *et al.*, 1992; Pippig *et al.*, 1993). Binding of arrestin to the cytoplasmic loop of the receptor occludes the binding site for the heterotrimeric G-protein ($G_{\alpha s}$) thus preventing its activation (desensitization). Alternatively, arrestins cause the internalization of the receptor via clathrin coated pits and subsequent transport to an internal compartment, called an endosome. Subsequently, the receptor could be either targeted to degradation compartments (lysosomes) or recycled back to the plasma membrane where it can signal again (Lefkowitz and Shenoy, 2005). The binding of arrestin prevents the activation of β-adrenoceptor by agonists. In conclusion, βARK acts as a negative feedback enzyme which will prevent over-stimulation of the β-adrenoceptor.

The β-adrenoceptor-mediated responses follow cAMP-dependent and independent pathways. Interestingly, involvement of K^+ channels in β-adrenoceptor signalling varies vastly with vascular tissues. What this diversity in β-adrenoceptor signalling signifies is still unclear. Definitely much remains to be learnt about the mechanisms of β-adrenoceptor signalling in various tissues.

1.5. High salt diet and cardiovascular diseases

Dietary salt is composed of sodium (Na^+) and chloride (Cl^-) ions. The sodium ions are the most abundant ions in extracellular fluid and they play a pivotal role in fluid and electrolyte balance and in generation and conduction of membrane potentials in neurons and muscle fibres (Tortora and Grabowski, 2010). Evidently, the daily increase of Na^+ intake in most countries worldwide is largely due to excess dietary salt. This elevated consumption of dietary salt cause a rise in blood pressure (hypertension) that further lead to increases in cardiovascular (strokes, heart attacks and heart diseases) and renal diseases (Intersalt study, 1988; Meneton *et al.*, 2005; He and MacGregor, 2010). Hypertension has been reported as the biggest cause of death and the second biggest cause of disability after malnutrition in children worldwide (Lopez *et al.*, 2001). Consequences of high blood pressure are mainly due to its effects on the cardiovascular system that cause a high mortality rate worldwide. In the company of other factors such as high cholesterol and smoking, hypertension accounts for over 80% of cardiovascular diseases (Emberson *et al.*, 2003). However, high blood pressure is the single most important cause, responsible for 62% of strokes and 49% of coronary heart disease (World Health Report, 2002). Over the years, assiduous work of many researchers across the globe on salt-induced hypertension and cardiovascular diseases has brought out the concept that in addition to raising blood pressure dietary salt has direct harmful effects. Some of these cardiovascular effects occur independently of pressure change (He and MacGregor, 2010). Tobian (1991) was the first to show that in various forms of experimental hypertension in the rat, a high salt intake induces structural alterations in cerebral and

renal vessels independent of the change in blood pressure (Tobian and Hanlon, 1990; Tobian, 1991).

1.5.1. Evidence linking high salt diet with cardiovascular diseases

A higher number of deaths due to stroke were reported from Japan (in the late 1950s) and interestingly, these incidences had a regional distribution that paralleled high salt consumption as part of the diet (Sasaki, 1964). The salt intake in northern Japan on average was 27 g/day, and 70% of the population (age of 50-60 years) had a high blood pressure (systolic and/or diastolic over 150/90 mmHg) resulting in the highest prevalence of cerebral haemorrhage (Sasaki, 1964). However, the southern region had a much lower prevalence of cerebral haemorrhage due to lower salt intake, an average of 14 g/day; only 10% of the population (same age range) had high blood pressure. A reduction in dietary salt intake in these populations lowers arterial blood pressure and leads to an 80% reduction in mortality due to stroke. Thus overall the reduction in salt intake appeared to be associated with a large decrease in deaths from stroke (Sasaki, 1979; Intersalt study, 1988). Further, a large international study on salt and blood pressure (INTERSALT) was conducted using a standardized method for monitoring blood pressure and 24-h urinary sodium. Fifty-two communities with a wide range of salt intake from 0.5 to 25 g/day were enrolled. However, the majority of recruited communities had salt intake of 6 and 12 g/day and only four had a low salt intake (3 g/day or less). In the latter study, it was demonstrated that a significant positive relationship between salt intake, 24 hour urinary sodium and blood pressures exists. There was also a highly significant positive

relationship between salt intake and the increase in blood pressure with age. It was estimated that an increase of 6 g/day salt intake over 30 years would lead to an increase in systolic BP by 9 mmHg (Intersalt study, 1988). A commendable joint effort by the food industry and Government of Finland in order to reduce salt in food products and to raise the general awareness among consumers of the harmful effects of high salt consumption on health has led to reduction of salt intake by one-third (Karppanen and Mervaala, 2006). This results in a decrease of approximately 10 mmHg in both systolic and diastolic blood pressures. Moreover, a pronounced reduction of 75-80% in mortality associated with stroke and coronary heart diseases and an increase of 5-6 years in life expectancy were also recorded (Laatikainen *et al.*, 2006). The two large randomized trials, the Trial of Hypertension Prevention (TOHP) I and II have demonstrated the long-term effects of salt reduction on cardiovascular diseases (Cook *et al.*, 2007). More than 3000 individuals with an average baseline blood pressure of 127/85 mmHg participated in the studies and were randomized to a reduced-salt group (for 18 months in TOHP I and 36-48 months in TOHP II) or to a control group. The individuals in the intervention group were advised to reduce their salt intake (average of 10 g/day) by 25–30%. These reductions in salt intake resulted in a fall in blood pressure of 1.7/0.9mmHg at 18 months (TOHP I) and 1.2/0.7mmHg at 36 months (TOHP II). The dietary advice was stopped after the completion of the initial studies. The participants were followed for subsequent development of cardiovascular diseases up to 15 years after the end of THOP I and 10 years after the end of THOP II. The intervention group with reduced-salt intake had a 25% lower incidence of cardiovascular events (Cook *et al.*, 2007). Furthermore, epidemiological studies in humans have shown that a high salt diet may have a direct

effect in causing stroke, independent of, and additive to its effect on blood pressure. Ferrara *et al.* (1984) reported that the increase in left ventricular mass associated with essential hypertension can be reduced by lowering the intake of salt. The Treatment of Mild Hypertension Study Research Group showed a significant correlation between the reduction in salt intake and left ventricular mass. In another study group lowering salt intake was also the only factor which was significantly correlated with a reduction in left ventricular mass (Liebson *et al.*, 1995). Lastly, consumption of low salt diet has been shown to improve distensibility of central aorta and large peripheral arteries in normotensive and elderly hypertensive (stage I) patients (Avolio *et al.*, 1986; Gates *et al.*, 2004). This should have beneficial effects on the cardiovascular system.

1.5.2. Evidence from animal studies

The influence of a high salt diet on the cardiovascular system has been widely studied in animal models. Numerous studies in rats, mice, dogs, chickens, rabbits, baboons, pigs and chimpanzees have all shown that high salt intake plays a role in regulating blood pressure (Penner *et al.*, 2007; Elliott *et al.*, 2007). Denton *et al.* (1995) conducted a study in chimpanzees (98.8% genetic homology with man) and demonstrated that a gradual increase of salt intake from 0.5g/day to 10g/day over a period of 20 months, results in progressive elevation in blood pressure. After stopping the salt supplementation, the blood pressure declined to that of control group over a period of 3-6 months. A study conducted in pigs in which one group was fed a diet containing either 0.5% salt or 3% salt for 8 months after weaning with free access to pure water. The average diastolic and

systolic blood pressures became progressively elevated from the second to the eighth month in the group of pigs with the high salt intake (Corbett *et al.*, 1979). Among all the mammalians, by far the most extensive investigations on the effect of high salt diet and the underlying mechanisms have been conducted in rats. A number of studies reported that Dahl salt sensitive and Spontaneous-Hypertensive rats when fed a high salt diet (4 - 8% NaCl) for 4-10 weeks develop hypertension and cause pressure induced adaptive vascular hypertrophy (Folkow *et al.,* 1971; Hampton *et al.*, 1989; Simon *et al.*, 2003), ventricular hypertrophy (Kihara *et al.,*1985), hypertrophied kidneys with glomerular and interstitial fibrosis (Blizard *et al.*, 1991; Vaskonen *et al.*, 1997), stroke (Tobian and Hanlon , 1990; MacLeod *et al.*, 1997), and cardiac failure (Frolich, 1999). The normotensive (Dahl salt-resistant, Wistar-Kyoto) rats fed a high salt diet (2 - 8% NaCl) maintains a normal blood pressure but other effect of excess Na^+ on the cardiovascular system were observed such as increased left ventricular mass (Frolich, 1993), vascular hypertrophy (Tobian and Hanlon , 1990; Tobian, 1991), increased weight and size of kidney (McCormick *et al.,* 1989), stroke (Tobian and Hanlon, 1990) and cardiac failure (Frolich, 1999). Thus, these observations suggested a link between high consumption of dietary Na^+ and cardiovascular diseases by both blood pressure-dependent and blood pressure-independent mechanisms.

The underlying mechanisms that result in salt induced vascular dysfunction have been widely investigated. Numerous studies in rats and mice have suggested that salt-induced vascular dysfunction may be linked to a defective L-arginine/eNOS/ NO pathway. Chen and Sanders (1991) demonstrated that the formation of NO plays an integral role in resting arteriolar tone in Dahl salt-resistant and Sprague-Dawley rats. The N^G-

monomethyl-L-arginine (L-NMMA; eNOS inhibitor) increased blood pressure by abolishing endothelium-derived vasodilation. It has also been reported that basal cyclic GMP levels were significantly higher (7.8-fold) in blood vessels of normotensive rats compared to hypertensive rats (Bieger *et al.,* 2004). These results suggest that during salt loading, salt-resistant rats are able to increase NO production, preventing the enhanced blood pressure response. In contrast, salt-sensitive rats showed a defect in their ability to increase NO production in response to high salt intake. It is plausible to state that Dahl salt-sensitive rats exhibit a marked down-regulation of vascular expression of eNOS. A high salt diet could induce production of superoxide ions which reduce the "bioavailability" of NO in the cardiovascular system. Furthermore, blood vessels from hypertensive animals have been reported to show more sensitivity to vasoconstrictor agents such as noradrenaline, serotonin, ouabain, Bay K8644 (L-type calcium channel agonist), phorbol ester (a protein kinase C activator), and endothelin (Bohr and Webb, 1988; Dominiczak and Bohr, 1989; Storm *et al.*, 1990). The resting E_m recorded from vascular smooth cells of rats fed a high salt diet varies from vessel to vessel. Abel *et al.* (1981) recorded resting membrane potential of similar magnitude in caudal arteries from Dahl salt-sensitive (-50.2 ± 1.1 mV) and Dahl salt-resistant (-51.4 ± 0.9 mV) rats fed a high salt diet (8% NaCl). Similarly, Parai and Tabrizchi (2005) reported that the resting E_m of smooth muscle cells of mesentery arteries of Dahl salt-resistant (-68.0 ± 4.2 mV) and Dahl salt-sensitive (-67.2 ± 4.8 mV) rats fed a high salt diet (4% NaCl) were not different. In contrast, Fujii *et al.* (1997) reported different E_m in superior mesenteric arteries from Dahl salt-sensitive (-41.4 ± 0.5 mV) rats that were significantly depolarized compared to Dahl salt-resistant (-47.0 ± 0.7 mV) rats. A similar observation was made by

Wellman *et al.* (2001) in cerebral arteries form Dahl salt-sensitive and Dahl salt-resistant rats. The resting E_m of tail artery of spontaneously hypertensive rats (-56.1 ± 1.1 mV) was significantly depolarized compared to their aged matched normotensive controls, the Wistar-Kyotos (-63.7 ± 1.5 mV) (Cheung, 1984). Interestingly, Bieger *et al.* (2004) noted hyperpolarization of resting E_m in smooth muscle cells from pulmonary artery of Dahl salt-sensitive compared to Dahl salt-resistant rats fed a high salt diet (4% NaCl). This lack of consistency between measured E_m could be attributed to the regional difference among the blood vessels. Collectively, evidence suggests that the consumption of high salt could lead to an altered E_m of smooth muscle cells and changes in signal transduction within the vascular system that further affects the functional responses of blood vessels to various physiological mediators.

1.6. Objectives of the study

Since Furchgott and Zawadski (1980) first reported that acetylcholine-mediated relaxation of vascular smooth muscle requires the presence of an intact endothelium, the role of the endothelium in the cardiovascular system has been extensively studied in normal and disease states. Endothelium dysfunction has been found a common factor in the gamut of cardiovascular diseases (Vanhoutte *et al.*, 2009). The β-adrenoceptor-mediated vasorelaxation has long been considered as a cAMP dependent phenomenon that take place in the vascular smooth muscle cells (Scheid *et al.*, 1979). The original observation for the involvement of endothelial cells in β-adrenoceptor-mediated vasorelaxation was made by Rubanyi and Vanhoutte (1985), who demonstrated that the

removal of the endothelium reduced the relaxation of canine coronary arteries caused by β-adrenoceptor agonists. Since then various researchers have investigated the role of endothelium in different vascular tissues but its role still remains unclear. For example; isoprenaline-induced relaxation in canine coronary arteries (White *et al.*, 1986), human internal mammary artery (Molenaar *et al.*, 1988) or rat carotid artery (Oriowo, 1994), were found to be endothelium-independent. Other reports demonstrated that removal of endothelium reduces the relaxations caused by β-adrenoceptor agonist in canine coronary arteries (Rubanyi and Vanhoutte 1985), mouse aorta (Akimoto et al. 2002) and rat pulmonary artery (Bieger *et al.*, 2006). Interestingly, two different researchers published contradictory reports in the same tissue i.e. rat aorta, one group showed that β-adrenoceptor-mediated relaxations were endothelium-independent (Moncada *et al.*, 1991) and the other reported that this was the case (Brawley *et al.*,2000). This variance in the latter observations could be due to differences in experimental design i.e. different level of tone placed on tissues. In addition, the impact of the presence of endothelium in β-adrenoceptor-mediated electrical responses has remained unresolved.

Hypertension has been linked with reduced endothelium dependent relaxations in isolated arteries from different animals (Lee and Triggle, 1986; Lee, 1987; Luscher *et al.*, 1987). However, consumption of high salt diet has been shown to increase the formation of NO in Sprague-Dawley rat fed a high salt diet (4% NaCl). This was demonstrated by enhanced excretion of NO metabolites (Tolins and Shultz, 1994). In parallel, Bieger *et al.* (2004) suggested that blood pressure of Dahl-salt resistant rats fed a high salt diet (4% NaCl) remained unchanged due to elevation of the vascular cGMP levels.

Dietary salt intake has been shown to influence β-adrenoceptor-mediated vascular response. High salt intake has been suggested to impair β-adrenoceptor-mediated response (Feldman, 1990a). The reduction of salt intake results in normalized β-adrenoceptor-mediated venodilation in borderline hypertensive patients or elderly normotensive individuals (Feldman, 1990a; Feldman, 1992). In animal studies, high salt intake has variable effects on normalized β-adrenoceptor-mediated responses. The isoprenaline-mediated vascular dilation was found to be significantly attenuated in aortic rings from Dahl salt-sensitive rats fed a high salt diet (Soltis and Katovich, 1991). In contrast, in low pressure segment of the circulatory system (i.e. pulmonary artery), β-adrenoceptor-mediated relaxant responses of Dahl salt-sensitive hypertensive rats were not affected by consumption of a high salt diet (Ford *et al.*, 2011). Furthermore, the noradrenaline-mediated vasodilation was attenuated in six and twelve weeks old spontaneous hypertensive rats (Arribas *et al.*, 1994). Altered β-adrenoceptor-induced hyperpolarization has been reported in vascular tissues of hypertensive rats (Stekiel *et al.*, 1993; Goto *et al.*, 2001). Taken together, the evidence suggests that elevation in blood pressure might interfere with β-adrenoceptor-mediated relaxation responses.

The consumption of a high salt diet has also been shown to modestly elevate systemic blood pressure in Sprague-Dawley rats which are considered to be normotensive (Sofola *et al.*, 2002). Bieger *et al.*(2004) reported that blood pressure of Dahl-salt sensitive rats following consumption of high salt diet (4% NaCl) for the period of 7 weeks was significantly elevated without inducing any change in mean pulmonary pressures. The absence of an elevated mean pulmonary pressure in the circulation may allow for the β-adrenoceptor-mediated functional and electrical response to remain intact in a state of

systemic hypertension. Considering the above evidence, we studied β-adrenoceptor-mediated responses in the low pressure system i.e. pulmonary artery of Sprague-Dawley rats. In addition, consumption of high salt also been found to elevate plasma sodium by 1 to 3 mmol/L in patients with essential hypertension and in the spontaneously hypertensive rat (SHR). The increase in sodium concentration greater than 2 mmol/L has been shown to induce changes in vascular response independent of an increase in blood pressure (de Wardener *et al.*, 2004).

In order to study the effect of high salt diet on β-adrenoceptors, we first need to have an understanding of the β-adrenoceptor-mediated response independent of changes in blood pressure. Earlier evidence from this laboratory supported the view that isoprenaline induces hyperpolarization of pulmonary artery (Bieger *at al.*, 2006). This hyperpolarization was sensitive to inhibition by acidification of buffer and seems to be due to the opening of the two-pore domain potassium channel (TASK) (Bieger *et al.*, 2006). Studies from this and another laboratory suggested that isoprenaline-induced relaxant responses in rat pulmonary artery were also in part endothelium dependent (Priest *et al.*, 1997; Bieger *at al.*, 2006).

Therefore, the two main objectives of the present study were to:

A) Investigate the role of endothelium in β-adrenoceptor-mediated electrical responses in the rat pulmonary artery.
B) Examine the effect of high salt diet on isoprenaline-induced electrical and mechanical responses in the rat pulmonary artery.

1.7. Hypotheses

It is possible that a high salt diet alters β-adrenoceptor-mediated signalling independent of changes in systemic blood pressure. In the present project a number of hypotheses were tested in relation to β-adrenoceptor signalling in isolated blood vessels (main pulmonary artery) from the low-pressure segment of the circulatory system. The first hypothesis being that endothelium played an integral role in β-adrenoceptor-mediated vascular response i.e. electrical and mechanical. The second hypothesis being that consumption of a high salt diet would alter β-adrenoceptor-mediated signalling in the blood vessel independent of a change in intravascular pressure.

2.0. METHODS

2.1. Study I. Role of endothelium in vascular β-adrenoceptor signalling

2.1.1. Preparation of animals

Six- seven weeks old male Sprague-Dawley rats (270 - 330 g) were housed (two per cage) with 12h light/dark cycles and given access to normal food (LabDiet 5P00 ProLab, PMI Nutrition International, Brentwood, MO, U.S.A) and tap water *ad libitum.*

2.1.2. Tissue isolation

Each Sprague-Dawley rat was anaesthetized with halothane (5% in 100% oxygen) and exsanguinated. The chest was cut open to access the thoracic cavity to remove cardio-pulmonary organs. Lungs and pulmonary vasculature along with the heart were transferred into a dissecting dish containing physiological buffer with the following composition (in mM): NaCl, 130; KCl, 4.0; glucose, 11; $MgCl_2$, 1.2; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $NaHCO_3$, 12.5; EDTA, 0.1. The pH of the buffer following saturation with a 95% O_2: 5% CO_2 gas mixture was 7.4 at 36 ± 1 °C. The main left and right pulmonary arteries were isolated, dissected free of connective tissue and separated from the pulmonary trunk. The endothelium was removed in some blood vessels by gentle rubbing with a string made of cotton wool.

2.1.3. Experimental protocol

2.1.3.1. Mechanical force measurements

Ring preparations from the right and left main pulmonary arteries (~2 mm in length) were mounted in 20 ml organ baths at 36 - 37°C under a force of 9.8 mN and gassed continuously with a mixture of 95% O_2: 5% CO_2 for mechanical studies. The tissues were equilibrated for 60 min (washed twice at 30 min with physiological buffer), and isometric tension was measured using force displacement transducers (Model FT03, Grass Instruments Co., MA, U.S.A.) connected to a polygraph (Model 7PCPB, Grass Instruments Co., MA, U.S.A.). Tissues were initially contracted with a single concentration of phenylephrine (1.0 μM), and the integrity of endothelial cells was ascertained by the addition of methacholine (1.0 μM). Tissues were then washed with physiological buffer and left for an additional 60 min (washed once at 30 min with physiological buffer) before they were contracted with phenylephrine (0.3 - 1.0 μM) and a control cumulative concentration-response curve to isoprenaline (1.0 nM - 3.0 μM) was constructed (Bieger *et al.*, 2006). Following a wash with regular physiological buffer and/or acidic buffer (pH 6.4; TASK-1 antagonist) each tissue was incubated 20 min with one of the following: vehicle (twice-distilled water, 200 μL), $BaCl_2$ (100 μM; K_{ir} inhibitor), ouabain (100 μM; Na^+/K^+ATPase inhibitor), tetraethylammonium (TEA, 3.0 mM; voltage gated K^+ channel blocker) or combination of these interventions i.e. buffer pH 6.4 plus $BaCl_2$ (100 μM), buffer pH 6.4 plus ouabain (100 μM), pH 6.4 plus TEA (3.0 mM), $BaCl_2$ (100 μM) plus ouabain (100 μM), buffer pH 6.4 plus $BaCl_2$ (100 μM) plus

ouabain (100 μM), $BaCl_2$ (100 μM) plus TEA (3.0 mM), buffer pH 6.4 plus $BaCl_2$ (100 μM) plus TEA (3.0 mM), $BaCl_2$ (100 μM) plus ouabain (100 μM) plus TEA (3.0 mM), pH 6.4 plus $BaCl_2$ (100 μM) plus ouabain (100 μM) plus TEA (3.0 mM). Subsequently, tissues were contracted with phenylephrine (0.3 - 1.0 μM) and a second cumulative concentration-response curve to isoprenaline was constructed (1.0 nM - 30.0 μM). Parallel experiments were carried out in denuded blood vessels.

2.1.3.2. Membrane potential measurements

Pulmonary artery rings were held under a tension of 9.8 mN in a 5 ml sylgard lined tissue chamber perfused with physiological salt solution pregassed with a mixture of 95% O_2: 5% CO_2 delivered at a rate of 3 - 4 ml per min and warmed to 34 - 35°C. Blood vessels were allowed to equilibrate for 60 min, and the E_m was recorded with borosilicate capillary microelectrodes filled with KCl (3.0 M) with a tip resistance of 10 - 20 MΩ. The Ag/AgCl reference half-cell containing KCl (3.0 M) was connected to the bath via an agar salt bridge containing 150 mM NaCl. Impalements were made by means of a Narishige x-y-z micropositioner, typically from the intimal side at depths >50 μm below the surface. Criteria for impalement and measurement of smooth muscle cell E_m were an abrupt drop in voltage upon penetration of the cell membrane, a stable E_m for at least 1 min, and a sharp return of the E_m to zero upon withdrawal of the electrode from the cell (Bieger *et al.*, 2006).

Voltage signals were recorded by means of Axoclamp-2A (Axon Instruments Inc., CA, USA), the output of which was fed into a Digi-Data 1200 Series Interface (Axon Instruments Inc.). Data was acquired and displayed on AxoScope (Version 1.1) and stored on a microcomputer. The E_m from 3 - 6 cells was initially recorded prior to exposure of the blood vessels to drugs. Subsequently, tissues were exposed to solutions containing isoprenaline (1.0 μM) for 5 - 10 min, and the E_m of the cells was sampled. In cases where inhibitors were used, following exposure to isoprenaline, tissues were perfused with buffer containing no drugs for 10 - 15 min and then exposed to buffer containing either buffer pH 6.4, $BaCl_2$ (100 μM), ouabain (100 μM), TEA (3.0 mM) and combined interventions i.e. buffer pH 6.4 plus $BaCl_2$ (100 μM), buffer pH 6.4 plus ouabain (100 μM), pH 6.4 plus TEA (3.0 mM), $BaCl_2$ (100 μM) plus ouabain (100 μM), buffer pH 6.4 plus $BaCl_2$ (100 μM) plus ouabain (100 μM), $BaCl_2$ (100 μM) plus TEA (3.0 mM), buffer pH 6.4 plus $BaCl_2$ (100 μM) plus TEA (3.0 mM), $BaCl_2$ (100 μM) plus ouabain (100 μM) plus TEA (3.0 mM), pH 6.4 plus $BaCl_2$ (100 μM) plus ouabain (100 μM) plus TEA (3.0 mM) . E_m recordings were carried out by sampling 3 - 4 cells individually in each tissue over a period of at least 30 min after the addition of drugs or a change in buffer (pH 6.4) before and during continuous perfusion with isoprenaline (1.0 μM). Parallel experiments were carried out in denuded tissues. The integrity of the endothelial cell was assessed with methacholine as outlined in our mechanical force investigation in a portion of the denuded blood vessel. Inclusion of phenylephrine (1.0 μM) in pilot experiments revealed 1-2 mV of depolarization in rat main pulmonary artery.

2.2. Study II. Influence of high salt diet on β-adrenoceptor signalling

2.2.1. Preparation of animals

At four weeks of age male Sprague Dawley rats were housed (two per cage) with 12h light/dark cycles and given access to either normal diet (0.26% Na, 0.44% Cl; LabDiet 5P00 ProLab, PMI Nutrition International, Brentwood, MO, U.S.A) or high salt diet (1.52% Na, 2.41% Cl; Modified LabDiet 5001; PMI Nutrition International, Brentwood, MO, U.S.A) along with tap water *ad libitum* for 18 - 23 days.

2.2.2. Blood pressure and heart rate measurements

Following 18 - 23 days on either diet, each rat was anaesthetised with halothane (5% in 100% oxygen for induction and 1.25% in 100% oxygen for maintenance) and a catheter (polyethylene tubing ID 0.28 mm, OD 0.61mm) was inserted into the left distal femoral artery for the measurement of blood pressure and heart rate. The catheter was filled with heparinised saline (25 i.u./ml in 0.9% NaCl), and the blood pressure and heart rate were measured for 10-15 min continuously. Arterial blood pressure was recorded with a pressure transducer (Gould Statham, U.S.A.; Model PD23B). The pressure transducer was connected to an amplifier (DA 100A) that in turn was linked to a universal interface module (UIM 100) which then interfaced with an acquisition unit (MP 100) (Bieger *et al.*, 2004).

2.2.3. Electrolyte measurements

Before sacrificing each animal, a 1.0 ml arterial blood sample was taken for the measurement of electrolytes. Each blood sample was centrifuged in an Eppendorf centrifuge, Model 5415C (14000 rpm) for 6 min to separate serum from cells. Approximately of 500 µl of serum was collected and electrolytes were measured using a Beckman Coulter LX20 Pro.

2.2.4. Tissue isolation

The study was performed using only intact blood vessels from rats fed either a regular or high salt diet. The procedure described in 2.1.2. was followed, briefly cardiopulmonary organs were transferred into a dissecting dish containing physiological buffer (pH 7.4) saturated with a 95% O_2: 5% CO_2 gas mixture at 36 ± 1 °C. The main left and right pulmonary arteries were isolated, dissected free of connective tissue and separated from the pulmonary trunk. The right ventricle and left ventricle plus septum were separated, and the wet weights of the hearts were recorded. The ventricles were then placed in an oven, heated (50° C) for 6 h, and the dry weight was also recorded.

2.2.5. Experimental protocol

2.2.5.1. Mechanical force measurements

The protocol described in 2.1.3.1. was followed, briefly pulmonary artery rings were mounted in 20 ml organ baths under a force of 9.8 mN. The artery rings were contracted with phenylephrine (1.0 μM) and integrity of endothelial cells was ascertained by relaxing them with methacholine (1.0 μM). Tissues were washed with physiological buffer. Following equilibration for an additional 60 minutes, tissues were contracted with phenylephrine (0.3 μM - 1 μM) in order to construct cumulative response curve to isoprenaline (1.0 nM - 30 μM) in the absence and presence of vehicle, acidic buffer, N^{ω}-nitro-L-arginine-methyl ester (10 μM; nitric oxide synthase inhibitor, L-NAME), barium chloride (100 μM), ouabain (100 μM), adenosine- 3', 5'- cyclic monophosphorothioate, Rp- isomer (30 μM; cAMP antagonist, Rp-cAMP), tetraethylammonium (3.0 mM; TEA) or combination of these interventions i.e. buffer pH 6.4 plus $BaCl_2$ (100 μM), buffer pH 6.4 plus L-NAME (10 μM), buffer pH 6.4 plus Rp-cAMP (30 μM), $BaCl_2$ (100 μM) plus ouabain (100 μM), buffer pH 6.4 plus $BaCl_2$ (100 μM) plus ouabain (100 μM), $BaCl_2$ (100 μM) plus ouabain (100 μM) plus TEA (3.0 mM), pH 6.4 plus $BaCl_2$ (100 μM) plus ouabain (100 μM) plus TEA (3.0 mM).

2.2.5.2. Membrane potential measurements

The protocol described in 2.1.3.2. was followed, briefly pulmonary artery rings were held under the tension of 9.8 mN in a 5 ml sylgard lined tissue chamber perfused with physiological salt solution pregassed with a mixture of 95% O_2 : 5% CO_2 delivered at a rate of 3-4 ml per min and warmed to 34-35°C. The E_m was recorded with borosilicate capillary microelectrodes filled with KCl (3.0 M) with a tip resistance of 10-20 MΩ. Initially, the E_m from 3-6 cells was recorded prior to exposure of the blood vessels to acidic buffer, N^{ω}-nitro-L-arginine-methyl ester (10 μM), $BaCl_2$ (100 μM), ouabain (100 μM), tetraethylammonium (TEA; 3.0 mM) or combination of these interventions i.e. buffer pH 6.4 plus $BaCl_2$ (100 μM), buffer pH 6.4 plus L-NAME (10 μM), $BaCl_2$ (100 μM) plus ouabain (100 μM), buffer pH 6.4 plus $BaCl_2$ (100 μM) plus ouabain (100 μM), $BaCl_2$ (100 μM) plus ouabain (100 μM) plus TEA (3.0 mM), pH 6.4 plus $BaCl_2$ (100 μM) plus ouabain (100 μM) plus TEA (3.0 mM). Subsequently, tissues were exposed to solutions containing isoprenaline (1.0 μM) for 5-10 min, and the E_m of the cells was sampled.

2.3. Study III. Effect of denudation of endothelium on vascular β-adrenoceptor signalling in rats fed a high salt diet.

2.3.1. Preparation of animals

Male Sprague Dawley rats, four weeks of aged were housed (two per cage) with 12h light/dark cycles and given access high salt diet (1.52% Na, 2.41% Cl; Modified LabDiet 5001; PMI Nutrition International, Brentwood, MO, U.S.A) and tap water *ad libitum* for 18 - 23 days.

2.3.2. Blood pressure and heart rate measurements

The procedure detailed in 2.2.3. was followed.

2.3.3. Tissue isolation

The protocol described in 2.1.2. was followed.

2.3.4. Experimental protocol

2.3.4.1. Mechanical force measurements

The protocol mentioned in 2.1.3.1. was followed excluding experiments involving TEA alone and in combination.

2.3.4.2. Membrane potential measurements

The protocol mentioned in 2.1.3.2. was followed excluding experiments involving TEA alone and in combination.

2.4. Data and Statistical Analysis

Results from the relaxation studies were calculated as a percentage of the maximum relaxation induced by isoprenaline following contraction with phenylephrine. Percent maximal response (E_{max}) and pEC_{50} values were calculated for individual curves with help of statistical software, Sigma Plot 8.0. An analysis of variance was used for statistical analysis of the data. The Student Newman-Keuls multiple range test was used for multiple comparisons between means. Student's *t-test* was also used to compare means. For all cases, a probability of error of less than 0.05 was selected as the criterion for statistical significance.

2.5. Chemicals

Stock solutions of all drugs were made in twice-distilled water. All drugs were purchased from Sigma Life Science (Oakville, Ontario, Canada).

3.0. RESULTS

3.1. Study I. Role of endothelium in vascular β-adrenoceptor signalling

3.1.1. Mechanical Function

3.1.1.1. Intact tissues

Isoprenaline produced concentration-dependent relaxations with E_{max} and pEC_{50} of 82.83 ± 1.00% and 7.26 ± 0.02, respectively (mean ± s.e.m.; n = 87). The relaxant responses to isoprenaline were significantly attenuated in acidic buffer and Ba^{2+} alone, where E_{max} and pEC_{50} were reduced (Table 2; Figure 2B and 3A). The presence of TEA (3.0 mM) or ouabain (100 μM) alone elicited modest but statistically significantly inhibition of relaxant responses to isoprenaline (Table 2; Figures 4A and 5A). The inhibition of the isoprenaline-evoked relaxant responses produced by TEA or ouabain alone was significantly lower compared to acidic buffer and Ba^{2+} alone (Table 2). Inclusion of Ba^{2+}, TEA or ouabain alone in acidic buffer did not produce further inhibition of isoprenaline relaxant responses compared to acidic buffer or Ba^{2+} alone (Table 2; Figure 3B, 4B and 5B). Presence of TEA and Ba^{2+} combined in acidic or regular buffer did not cause further inhibition compared to acidic buffer alone (Table 2; Figure 6A and B). Inhibition of isoprenaline mediated maximal responses, produced by Ba^{2+} and ouabain combined in the absence or presence of TEA was of similar magnitude (Table 2; Figure 7A and 8A). However, in the presence of acidic buffer, Ba^{2+} and ouabain combined or Ba^{2+}, ouabain and TEA combined caused further inhibition of relaxant responses produced by isoprenaline (Table 2; Figure 7B and 8B).

Table 2. Percent maximal response (E_{max}) and pEC_{50} determined from individual concentration–response curves for isoprenaline in rat main pulmonary artery ring preparations with (+E) and without (-E) endothelium.

	Regular diet (+E)		Regular diet (-E)	
	pEC_{50}	E_{max}	pEC_{50}	E_{max}
Control	7.24 ± 0.11	77.00 ± 3.81	7.16 ± 0.08	61.00 ± 4.29
Acidic buffer	6.91 ± 0.08[a,d]	54.33 ± 4.42[a]	6.83 ± 0.11[a,e]	54.00 ± 6.30
Control	7.26 ± 0.05	80.33 ± 5.76	7.01 ± 0.09	62.17 ± 4.77
$BaCl_2$	6.87 ± 0.09[a,b]	42.00 ± 4.14[a,b]	7.00 ± 0.09	50.33 ± 5.41[a]
Control	7.15 ± 0.14	85.50 ± 1.91	7.17 ± 0.10	63.17 ±6.53
Acidic buffer + $BaCl_2$	6.69 ± 0.12[a]	58.00 ± 4.12[a,d]	6.78 ± 0.22	56.67 ± 2.73
Control	7.30 ± 0.07	78.33 ± 1.82	7.10 ± 0.05	63.00 ± 4.52
TEA	7.10 ± 0.04[a]	68.00 ± 2.88[a,b,d]	6.69 ± 0.31	51.17 ± 9.54[a]
Control	7.28 ± 0.09	77.83 ± 6.29	7.05 ± 0.13	68.00 ± 3.56
Acidic buffer + TEA	6.78 ± 0.19	49.83 ± 4.89[a]	6.52 ± 0.49[e]	55.33 ± 4.17[a]
Control	7.29 ± 0.08	86.83 ± 1.70	6.91 ± 0.07	69.83 ± 6.49
Ouabain	6.84 ± 0.10[a]	72.83 ± 3.34[a,b,d]	6.54 ± 0.28	58.67 ± 5.57

Control	7.24 ± 0.12	82.83 ± 2.15	7.18 ± 0.08	54.17 ± 5.69
Acidic buffer + ouabain	6.77 ± 0.07[a]	60.67 ± 7.36[a]	6.85 ± 0.07[a]	49.50 ± 3.20
Control	7.33 ± 0.09	80.83 ± 3.90	7.18 ± 0.12	65.17 ± 5.56
TEA + $BaCl_2$	6.96 ± 0.11	51.17 ± 8.06[a]	6.99 ± 0.03	57.50 ± 5.39
Control	7.39 ± 0.08	86.50 ± 4.86	7.14 ± 0.25	69.00 ± 7.89
Acidic buffer + TEA + $BaCl_2$	6.94 ± 0.19	52.17 ± 7.38[a]	6.87 ± 0.12	46.83 ± 8.12[a]
Control	7.19 ± 0.07	85.38 ± 3.66	6.98 ± 0.06	67.33 ± 3.99
$BaCl_2$ + Ouabain	6.77 ± 0.11[a]	44.00 ± 5.01[a]	6.80 ± 0.07	37.33 ± 10.40[a]
Control	7.19 ± 0.08	80.50 ± 2.95	7.19 ± 0.05	67.17 ± 7.71
Acidic buffer + $BaCl_2$ + Ouabain	6.62 ± 0.08[a]	33.00 ± 5.39[a,b]	6.65 ± 0.19[a]	48.17 ± 7.28[a]
Control	7.35 ± 0.06	87.33 ± 2.94	6.69 ± 0.22	58.33 ± 3.49
$BaCl_2$ + Ouabain + TEA	6.72 ± 0.17[a]	43.83 ± 8.87[a]	6.48 ± 0.26	24.67 ± 7.20[a,c,e]
Control	7.29 ± 0.06	84.17 ± 3.89	6.72 ± 0.26	60.33 ± 6.41
Acidic buffer + $BaCl_2$ + Ouabain + TEA	6.32 ± 0.17[a,d]	26.00 ± 5.03[a,b,d]	6.49 ± 0.03[e]	21.17 ± 4.08[a,c,e]

Acidic buffer (pH 6.4 ± 0.2) $BaCl_2$ (100 μM); Ouabain (100 μM); Tetraethylammonium (TEA, 3.0 mM)

Each value is the mean ± s.e.m., n= 6-8

[a]Significantly different from respective control; $p<0.05$

[b]Significantly different from acidic buffer (intact); $p<0.05$

[c]Significantly different from acidic buffer (denuded); $p<0.05$

[d]Significantly different from $BaCl_2$ (intact); $p<0.05$

[e]Significantly different from $BaCl_2$ (denuded); $p<0.05$

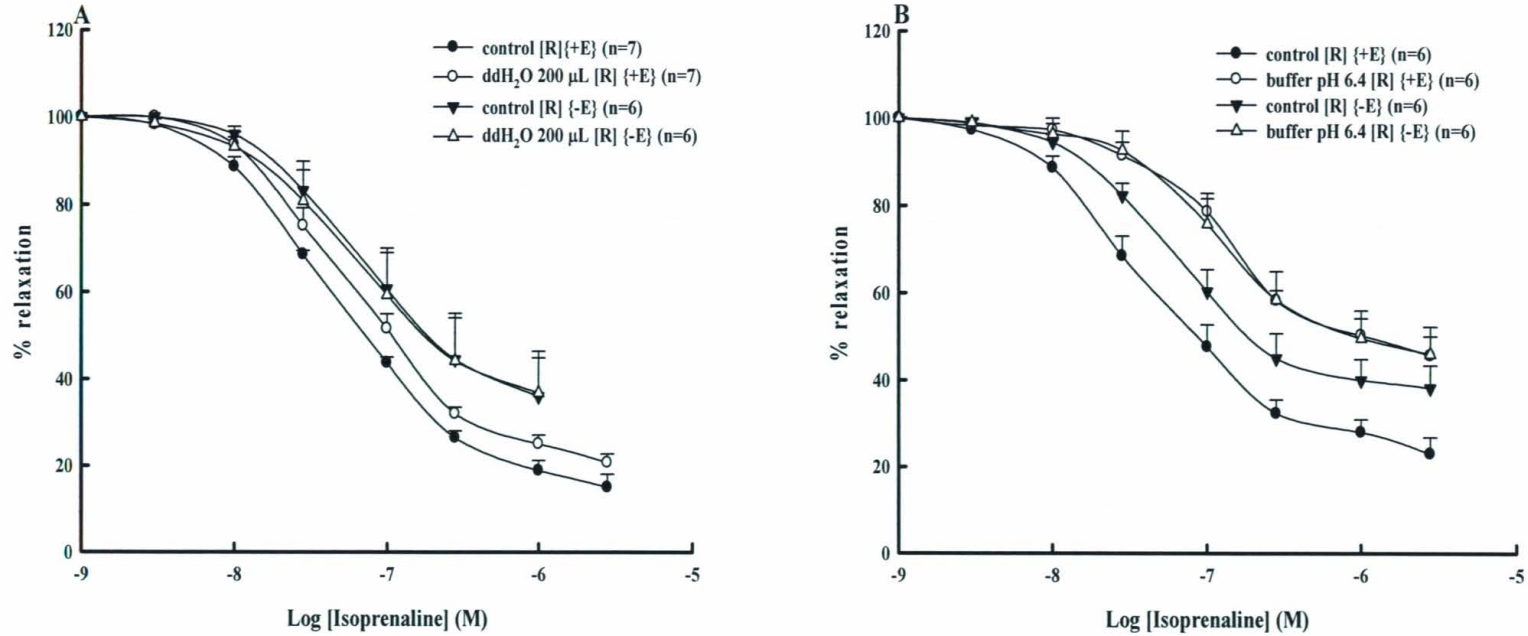

Figure 2. Concentration-response curve for isoprenaline (% relaxation remaining from phenylephrine induced sub-maximal contractions) in intact {+E} and denuded {-E} main pulmonary artery ring preparations from rats fed a regular diet [R] in **A)** regular buffer (control) and twice-distilled water and; **B)** regular buffer (control) and acidic buffer. Each point represents a mean ± s.e.m. of six to seven experiments.

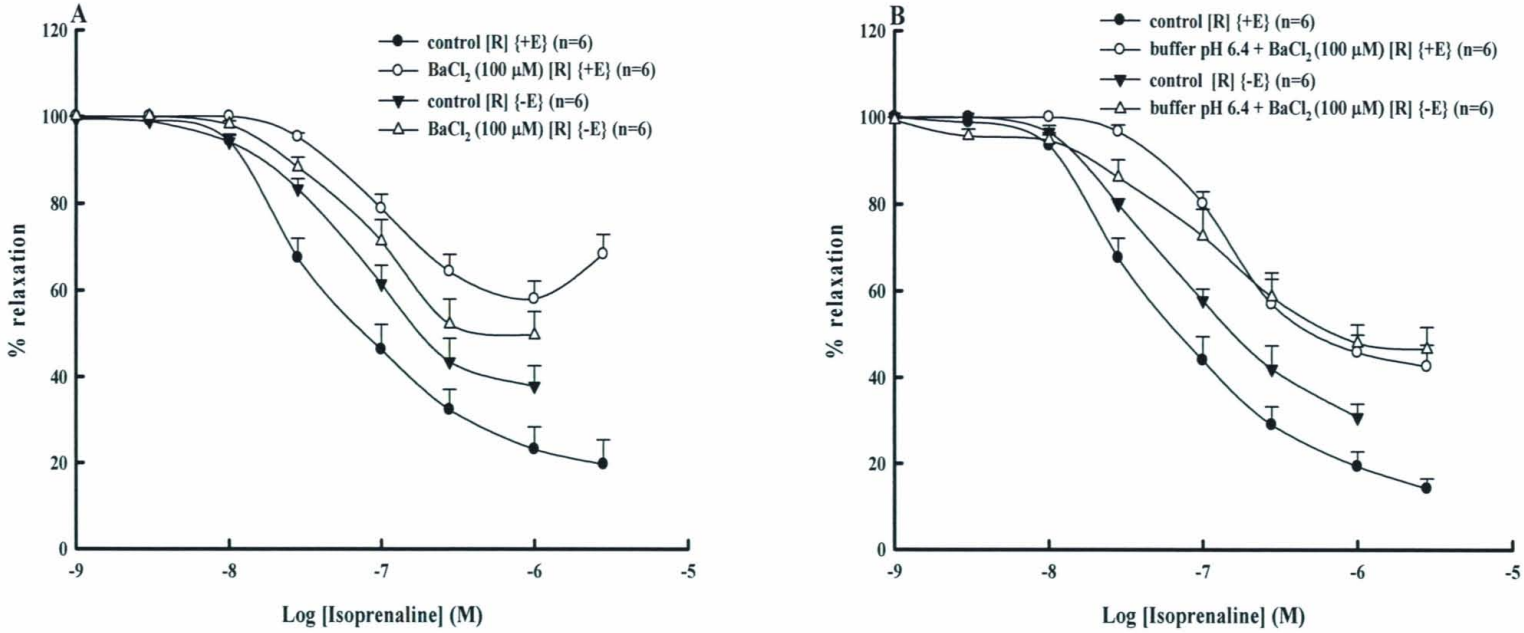

Figure 3. Concentration-response curve for isoprenaline (% relaxation remaining from phenylephrine induced sub-maximal contractions) in intact {+E} and denuded {-E} main pulmonary artery ring preparations from rats fed a regular diet [R] in **A)** regular buffer absence (control) or presence of $BaCl_2$ and; **B)** regular buffer (control) or acidic buffer plus $BaCl_2$. Each point represents a mean ± s.e.m. of six experiments.

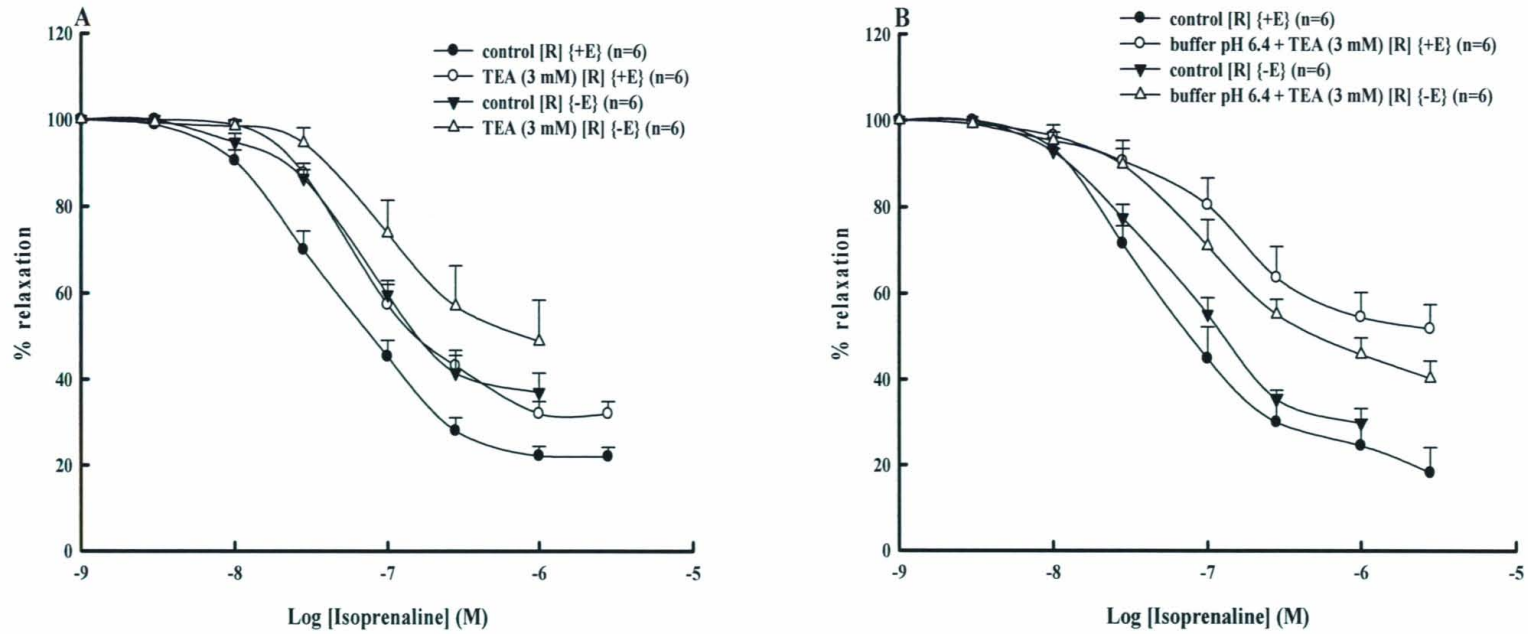

Figure 4. Concentration-response curve for isoprenaline (% relaxation remaining from phenylephrine induced sub-maximal contractions) in intact {+E} and denuded {-E} main pulmonary artery ring preparations from rats fed a regular diet [R] in **A)** regular buffer absence (control) or presence of TEA and; **B)** regular buffer (control) or acidic buffer plus TEA. Each point represents a mean ± s.e.m. of six experiments.

3.1.1.2. Denuded tissues

Isoprenaline produced concentration-dependent relaxations with E_{max} and pEC_{50} of 63.76 ± 1.57% and 7.03 ± 0.04, respectively, (mean ± s.e.m.; n = 84) which were significantly smaller when compared to intact blood vessels. Denudation effectively attenuated the relaxant responses to isoprenaline (Table 2; Figure 2A). Acidic buffer did not cause inhibition of relaxation to isoprenaline (Table 2; Figure 2B). TEA, Ba^{2+} or ouabain alone also did not cause inhibition of relaxant responses to isoprenaline (Table 2; Figure 3A, 4A and 5A). Inclusion of Ba^{2+}, TEA or ouabain alone in acidic buffer did not cause further inhibition of relaxation to isoprenaline when compared to their respective control or acidic buffer (Table 2; Figure 3B, 4B and 5B). The combination of Ba^{2+} and TEA also did not result in further inhibition compared to TEA and ouabain alone (Table 2; Figure 6A). In contrast, Ba^{2+} and ouabain combined produced significant inhibition of the relaxant responses to isoprenaline compared to Ba^{2+} or ouabain alone. The inhibitory effect of Ba^{2+} and ouabain combined in denuded tissue was similar to intact tissues (Figure 7A). The presence of TEA and Ba^{2+} combined, or Ba^{2+} and ouabain combined, in acidic buffer did not cause further inhibition compared to acidic buffer alone (Table 2; Figure 6B and 7B). While the combination of Ba^{2+}, TEA and ouabain in regular or acidic buffer produced additive inhibition compared to acidic buffer and Ba^{2+} alone, the inhibition produced by the former intervention was not different from the effect of Ba^{2+}, TEA and ouabain combined in the regular buffer (Table 2; Figure 8A and 8B).

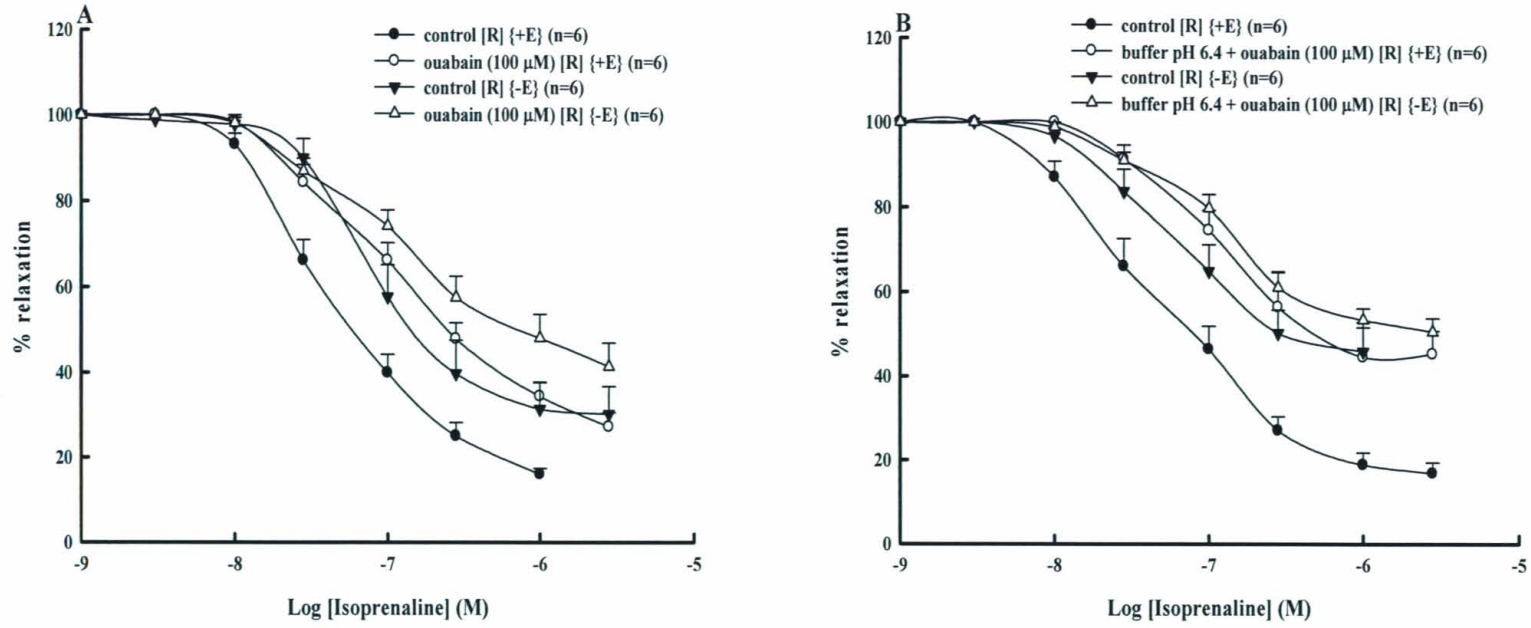

Figure 5. Concentration-response curve for isoprenaline (% relaxation remaining from phenylephrine induced sub-maximal contractions) in intact {+E} and denuded {-E} main pulmonary artery ring preparations from rats fed a regular diet [R] in **A)** regular buffer absence (control) or presence of ouabain and; **B)** regular buffer (control) or acidic buffer plus ouabain. Each point represents a mean ± s.e.m. of six experiments.

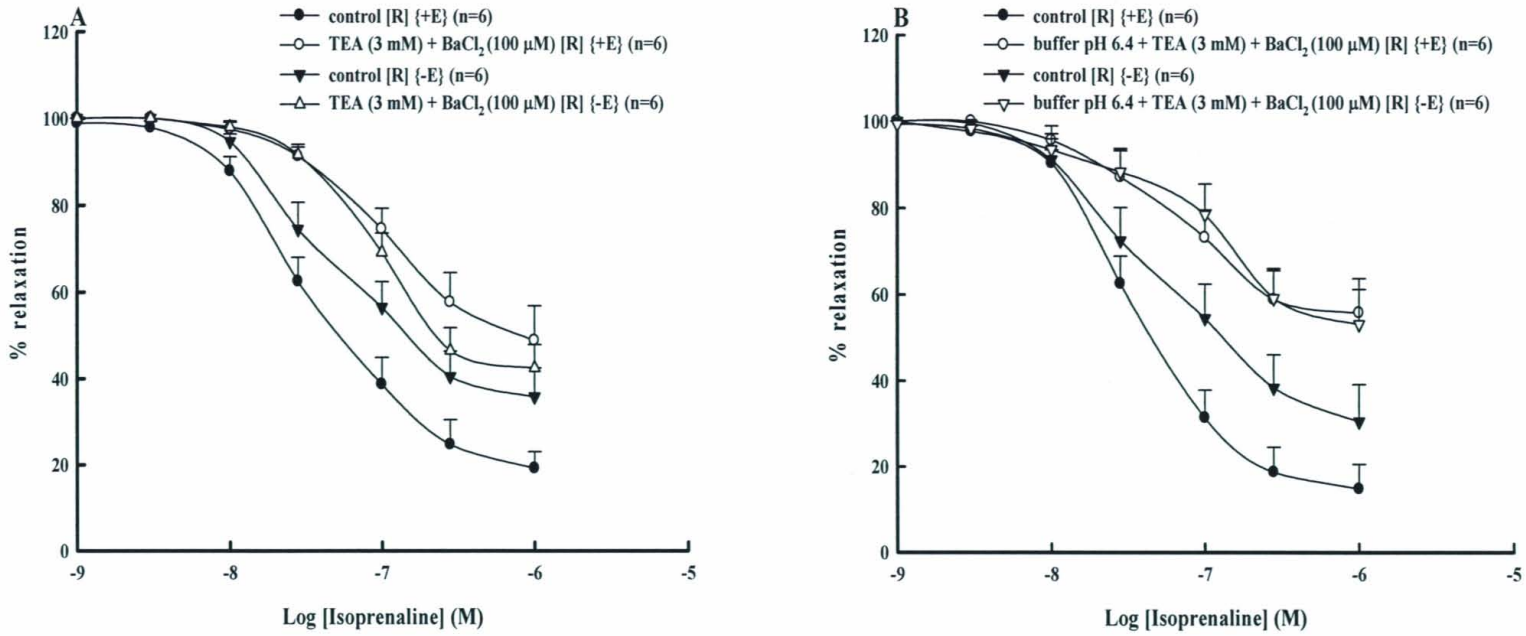

Figure 6. Concentration-response curve for isoprenaline (% relaxation remaining from phenylephrine induced sub-maximal contractions) in intact {+E} and denuded {-E} main pulmonary artery ring preparations from rats fed a regular diet [R] in **A)** regular buffer absence (control) or presence of TEA and $BaCl_2$ together and; **B)** regular buffer (control) or acidic buffer plus TEA and $BaCl_2$ together. Each point represents a mean ± s.e.m. of six experiments.

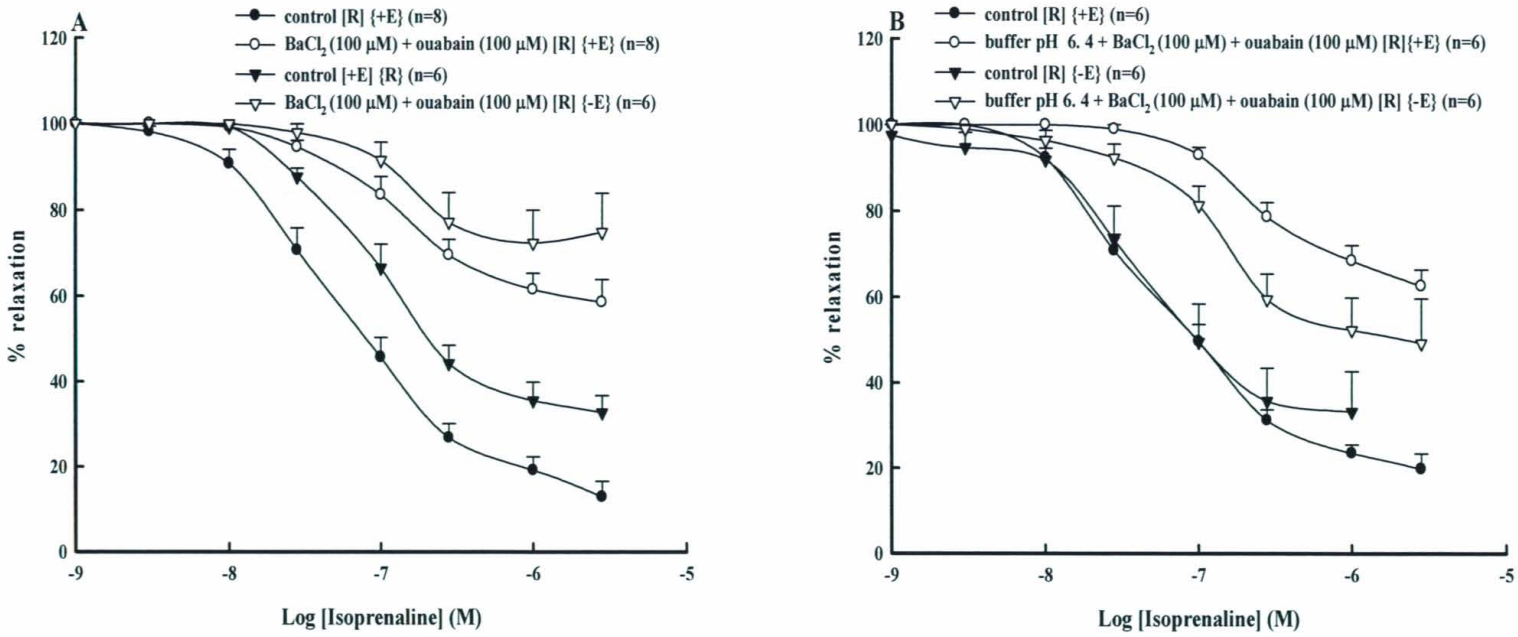

Figure 7. Concentration-response curve for isoprenaline (% relaxation remaining from phenylephrine induced sub-maximal contractions) in intact {+E} and denuded {-E} main pulmonary artery ring preparations from rats fed a regular diet [R] in **A)** regular buffer absence (control) or presence of $BaCl_2$ and ouabain together and; **B)** regular buffer (control) or acidic buffer plus $BaCl_2$ and ouabain together. Each point represents a mean ± s.e.m. of six to eight experiments.

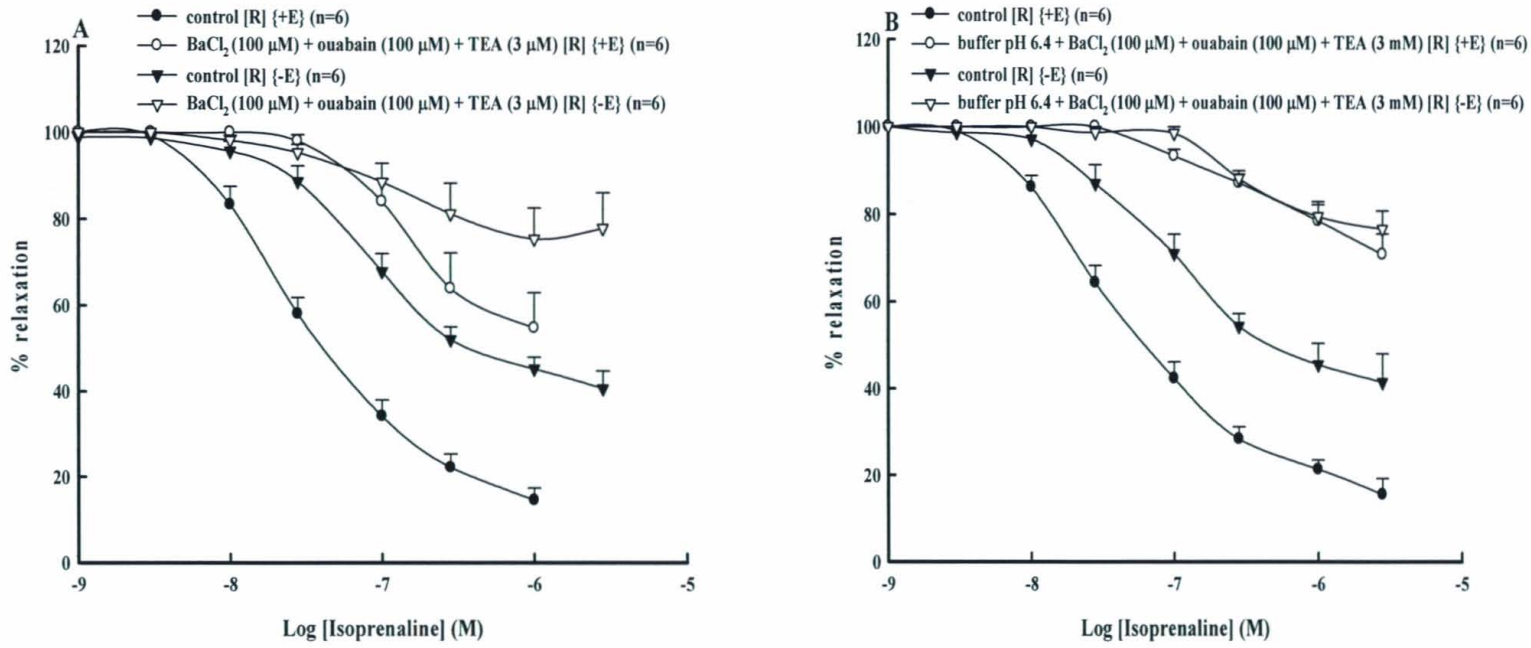

Figure 8. Concentration-response curve for isoprenaline (% relaxation remaining from phenylephrine induced sub-maximal contractions) in intact {+E} and denuded {-E} main pulmonary artery ring preparations from rats fed a regular diet [R] in **A)** regular buffer, absence (control) or presence of $BaCl_2$, ouabain and TEA together; **B)** regular buffer (control) or acidic buffer plus $BaCl_2$ ouabain and TEA together. Each point represents a mean ± s.e.m. of six experiments.

3.1.2. Membrane potential

3.1.2.1. Intact tissue

Acidic buffer, TEA or ouabain alone did not cause significant changes in the resting E_m of the smooth muscle cells (-61.3 ± 1.24, -60.5 ± 1.32, -58.7 ± 0.53 mVrespectively; Table 3). However, Ba^{2+} in the absence or presence of acidic buffer caused modest depolarisation of the vascular smooth muscle cells (-57.7 ± 0.38, -57.5 ± 0.26 respectively mV; Table 3). Ba^{2+} and ouabain combined, but not Ba^{2+} and TEA combined, depolarized pulmonary artery smooth muscle cells (-58.7 ± 0.9, -60.5 ± 0.85 mV respectively; Table 3). Addition of the latter two agents to acidic buffer (-58.2 ± 0.35, -57.6 ± 0.94 mV) resulted in modest but significant depolarization compared to acidic buffer alone (-61.3 ± 1.24 mV) but not more than Ba^{2+} alone (-57.7 ± 0.38 mV) (Table 3). Similarly, the presence of Ba^{2+}, TEA and ouabain in acidic buffer (-56.4 ± 0.54 mV) or in regular buffer (-55.9 ± 0.86 mV) resulted in further depolarization compared to acidic buffer alone (-61.3 ± 1.24 mV) (Table 3). However, the addition of acidic buffer failed to increase the inhibitory effect of combined interventions (Ba^{2+}, TEA and ouabain). Exposure of isoprenaline to blood vessels resulted in hyperpolarisation of approximately 8.0 mV (Table 3). The hyperpolarisation produced by isoprenaline was significantly diminished in acidic buffer (-61.3 ± 1.24 vs -64.2 ± 1.29 mV, respectively; Table 3). TEA modestly but not significantly inhibited isoprenaline induced hyperpolarization (-60.5 ± 1.32 vs -64.2 ± 1.37 mV, respectively; Table 3). Further, inclusion of acidic buffer with TEA caused additive inhibition (-58.3 ± 1.12 vs -59.5 ± 1.38 mV, respectively; Table 3). Presence of Ba^{2+} or ouabain alone in regular buffer did not affect isoprenaline-evoked

hyperpolarisation (-58.7 ± 0.97 vs -64.1 ± 1.15 mV, respectively; Table 3). However, in the presence of acidic buffer ouabain (-59.3 ± 1.31 mV) but not Ba^{2+} (-57.5 ± 0.26 mV) alone, diminished isoprenaline induced hyperpolarisation (Table 3). Isoprenaline mediated hyperpolarization was partially inhibited by exposure of Ba^{2+} and TEA combined (-60.5 ± 0.85 vs -65.5 ± 1.70 mV, respectively), or Ba^{2+} and ouabain combined (-58.7 ± 0.97 vs -64.1 ± 1.15 mV, respectively) (Table 3). Isoprenaline-induced hyperpolarisation was similarly affected by Ba^{2+}, ouabain and TEA together in regular (-55.9 ± 0.86 vs -57.7 ± 0.81 mV, respectively) or acidic buffer (-56.4 ± 0.54 vs -58.3 ± 0.62 mV, respectively; Table 3).

Table 3. Membrane potential (mV) in vascular smooth muscle cells of rat pulmonary ring preparation with (intact) and without (denuded) endothelium in absence or presence of isoprenaline (1.0 μM) after various treatments.

	Regular diet (+E)			Regular diet (-E)		
	Control	*Treatment*	*Isoprenaline*	*Control*	*Treatment*	*Isoprenaline*
No treatment	-62.2 ± 1.27 (8)(34)		-70.6 ± 0.72[a] (8)(41)	-68.2 ± 0.27[b] (6)(25)		-69.6± 1.18[b] (6)(35)
Acidic buffer	-61.6 ± 1.04 (5)(24)	-61.3 ± 1.24 (5)(24)	-64.2 ± 1.29 (5)(21)	-67.1 ± 0.52[b] (9) (44)	-66.2 ± 1.41 (7) (31)	-63.9 ± 1.84 (4) (23)
TEA	-61.8 ± 0.72 (4)(17)	-60.5 ± 1.32 (4)(24)	-64.2 ± 1.37[c] (4)(26)	-68.5 ± 1.25[b] (8)(30)	-64.9 ± 0.95 (7)(34)	-66.5 ± 1.19 (4)(22)
$BaCl_2$	-60.7 ± 1.21 (4)(23)	-57.7 ± 0.38[a] (4)(18)	-64.1 ± 1.56[a,c] (4)(25)	-68.1 ± 0.45[b] (8)(36)	-66.4 ± 1.01 (6)(31)	-64.2 ± 1.72 (4) (22)
Ouabain	-60.4 ± 0.56 (4)(24)	-58.7 ± 0.53 (4)(25)	-64.9 ± 0.51[c] (4)(23)	-69.1 ± 1.43[b] (5) (12)	-61.7 ± 1.14[a] (4) (22)	-59.2 ± 1.79[a] (8) (22)
Acidic buffer + TEA	-60.1 ± 0.99 (4)(15)	-58.3 ± 1.12 (4)(19)	-59.5 ± 1.38 (4)(19)	-66.8 ± 0.67[b] (5)(35)	-61.9 ± 1.78[a] (4)(23)	-62.4 ± 1.92[a] (4)(22)

Acidic buffer + $BaCl_2$	-61.5 ± 0.78 (4)(23)	-57.5 ± 0.26[a] (4)(23)	-64.8 ± 0.69[a,c] (4)(18)	67.2 ± 0.72[b] (7)(35)	-63.9 ± 0.32[a] (5)(26)	-62.6 ± 1.27[a] (4) (19)
Acidic buffer + Ouabain	-60.8 ± 0.49 (4)(24)	-59.3 ± 1.31 (4)(24)	-63.5 ± 1.64 (4)(22)	-68.5 ± 1.85[b] (4)(19)	-61.7 ± 1.76[a] (4)(22)	-59.3 ± 1.88[a] (4)(19)
$BaCl_2$ + TEA	-60.6 ± 0.28 (4)(17)	-60.5 ± 0.85 (4)(17)	-65.5 ± 1.70[a,c] (4)(24)	-67.4 ± 1.69[b] (4)(11)	-63.1 ± 1.48[a] (4)(19)	-61.7 ± 1.82[a] (4)(19)
$BaCl_2$ + Ouabain	-61.2 ± 0.48 (4)(22)	-58.7 ± 0.97[a] (4)(22)	-64.1 ± 1.15[a,c] (4)(20)	-70.3 ± 1.32[b] (4)(11)	-58.3 ± 1.20[a,e] (4)(23)	-60.7 ± 1.19[a] (4)(22)
Acidic buffer + $BaCl_2$ + TEA	-61.8 ± 0.49 (4)(10)	-57.6 ± 0.94[a,d] (4)(19)	-60.1 ± 1.82 (4)(14)	-68.5 ± 1.89[b] (3)(10)	-63.0 ± 1.39[a] (4)(19)	-61.3 ± 1.41[a] (4)(19)
Acidic buffer + $BaCl_2$ + Ouabain	-62.2 ± 0.21 (4)(22)	-58.2 ± 0.35[a,d] (4)(21)	-61.3 ± 1.55 (4)(22)	-68.7 ± 2.19[b] (4)(9)	-58.5 ± 0.73[a,e] (4)(23)	-61.2 ± 0.87[a] (4)(23)
$BaCl_2$ + Ouabain + TEA	-60.8 ± 1.37 (4)(16)	-55.9 ± 0.86[a,d] (4)(26)	-57.7 ± 0.81 (4)(24)	-66.5 ± 0.50[b] (4)(9)	-56.8 ± 1.67[a,e] (4)(27)	-61.3 ± 1.38[a] (4)(23)
Acidic buffer + $BaCl_2$ + Ouabain + TEA	-61.3 ± 1.01 (4)(15)	-56.4 ± 0.54[a,d] (4)(21)	-58.3 ± 0.62[a] (4)(24)	-67.3 ± 1.01[b] (4)(7)	-58.6 ± 1.59[a,e] (4)(21)	-61.7 ± 1.35[a] (4)(15)

Acidic Buffer (pH 6.4 ± 0.2); Tetraethylammonium (TEA; 3.0 mM); $BaCl_2$ (100 μM); Ouabain (100 μM)

Each value is the mean ± s.e.m. with the number of cells and number of rats indicated in parentheses

[a]Significantly different from respective control; $p<0.05$

[b]Significantly different from control (intact); $p<0.05$

[c]Significantly different from respective treatment; $p<0.05$

[d]Significantly different from acidic buffer (intact); $p<0.05$

[e]Significantly different from acidic buffer (denuded); $p<0.05$

3.1.2.2. Denuded tissue

The resting E_m of smooth muscle cells of denuded blood vessels (-E) was significantly different from that of intact blood vessels (+E) (-62.2 ± 1.27 vs -68.2 ± 0.27 mV, respectively; Table 3). The cells were hyperpolarized by approximately 6-8 mV in denuded compared to intact blood vessels. Neither acidic buffer (-67.1 ± 0.52 vs -66.2 ± 1.41 mV, respectively) nor Ba^{2+} alone (-68.1 ± 0.45 vs -66.4 ± 1.01 mV, respectively) influenced the resting E_m. In contrast, ouabain (-69.1 ± 1.43 vs -61.7 ± 1.14 mV, respectively) but not TEA alone (-68.5 ± 1.25 vs-64.9 ± 0.95 mV, respectively) caused significant depolarization of the vascular smooth muscle cells (Table 3). However, the inclusion of Ba^{2+} (-67.2 ± 0.72 vs -63.9 ± 0.32 mV, respectively) TEA (-66.8 ± 0.67 vs -61.9 ± 1.78 mV, respectively) or ouabain alone (-68.5 ± 1.85 vs -61.7 ± 1.76 mV, respectively) in acidic buffer also caused significant depolarization of the E_m. As well, the combination of Ba^{2+} and TEA, Ba^{2+} and ouabain, and Ba^{2+}, TEA and ouabain in regular or acidic buffer also depolarized the smooth muscle cells (Table 3). Isoprenaline-induced hyperpolarization was notably absent in these tissues with or without any intervention (Table 3).

Table 4. Systolic and diastolic blood pressure (BP; mmHg); heart rate (HR; beats/min); body weight (BW; g) and ratio of right ventricle dry (RV_d) to wet (RV_w) weight; ratio of left ventricle + septum, dry ($LV + S_d$) to wet ($LV + S_w$) weight of rats fed a regular and 4% salt (NaCl) diets for 18-23 days.

	BP	**HR**	**BW**	**RV_d/RV_w**	**$LV+S_d/LV+S_w$**
Regular diet	94/70 ± 0.61/0.78 (77)	368 ± 4.20 (77)	295 ± 3.23 (77)	0.21 ± 0.01 (41)	0.29 ± 0.01 (41)
4% Salt diet	106/72 ± 0.94[a]/0.68[a] (83)	378 ± 3.77 (83)	282 ± 3.74[a] (83)	0.21 ± 0.01 (41)	0.28 ± 0.01 (41)

[a]Significantly different from respective value in regular diet group; $p < 0.05$.

Each value represents a mean ± s.e.m.; number of rats indicated in parenthesis.

3.2. Study II. Influence of high salt diet on β-adrenoceptor signalling

Blood pressure (systolic and diastolic) of rats on high salt diet was modestly but significantly higher than rats on regular diet while heart rates among the two groups were not significantly different (Table 4). A significantly lower body weight was recorded for rats on high salt diet compared to those on a regular diet (Table 4). The ratio of wet to dry weight of left and right ventricles was found not to be different between the two groups of rats (Table 4) i.e. no left or right ventricular hypertrophy was apparent. The values of serum electrolytes were also not different between the two groups of rats (Table 5).

3.2.1. Mechanical Function

Relaxations produced by isoprenaline in pulmonary arteries from rats on regular and high salt diets were of similar magnitude in terms of E_{max} and pEC_{50} (84.16 ± 1.08% and 7.24± 0.03 vs. 83.34 ± 1.09% and 7.23 ± 0.03; n = 65), respectively. Addition of twice distilled water did not affect the cumulative concentration-response curves to isoprenaline in tissues from rats fed a regular compared to the salt diet (Figure 9A, 10A, 11A). Acidic buffer (pH 6.4) attenuated isoprenaline-induced responses, significantly reducing E_{max} and pEC_{50} (Figure 9B, 10B, 11B). The latter intervention produced a significantly greater inhibition of the relaxant response to isoprenaline in blood vessels obtained from rats on high salt compared to regular diet (Figure 11B, Table6). The effects of the cAMP antagonist, Rp-cAMP, on relaxant responses to isoprenaline were similar as in the previous findings

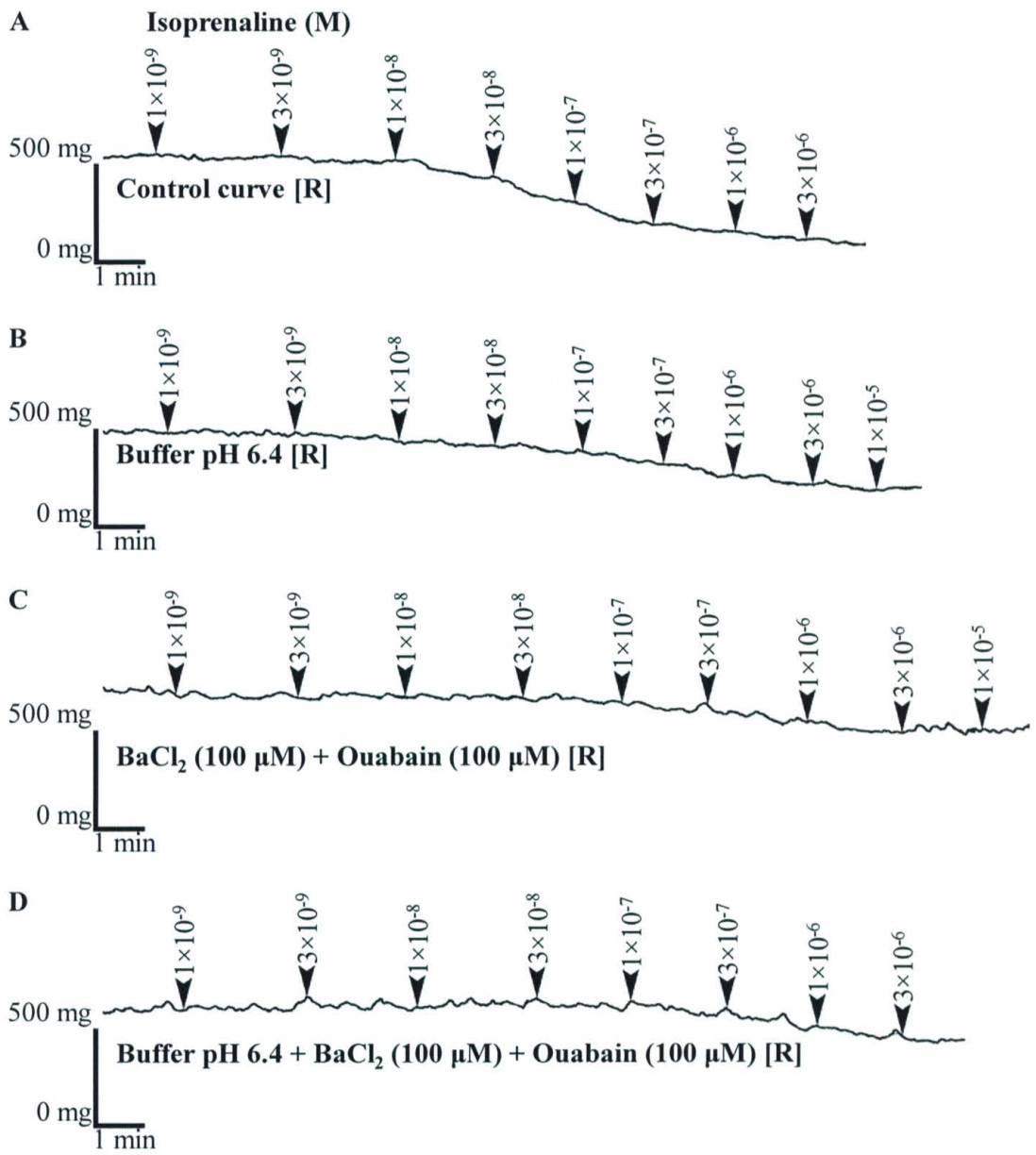

Figure 9: Concentration-response curve for isoprenaline in main pulmonary artery ring preparation from rats fed regular diet [R] of **A)** in regular buffer (control), **B)** in buffer pH 6.4, **C)** in the presence barium chloride (100 µM) plus ouabain (100 µM) and, **D)** buffer pH 6.4 plus barium chloride (100 µM) plus ouabain (100 µM).

Table 5. Serum electrolytes (mmol/l) of the rats fed regular diet and 4% salt diet for 18-23 days.

	Regular diet	**4% Salt diet**
Na^+	137.69 ± 0.39	136.81 ± 0.38
K^+	4.48 ± 0.08	4.67 ± 0.10
Cl^-	98.44 ± 0.47	98.19 ± 0.33
Ca^{2+}	2.49 ± 0.02	2.53 ± 0.02

Each value represents a mean ± s.e.m., n=16

from our laboratory (Bieger *et al*, 2006), and of similar magnitude in tissues from rats on a high salt or a regular diet.

L-NAME (10 μM) significantly reduced E_{max} and pEC_{50} of isoprenaline concentration-response curves in tissues from rats on regular diet. In contrast, L-NAME only caused a modest but significant reduction in E_{max} without affecting the pEC_{50} in tissues from rats on high salt diet (Table 6; Figure 12A). The inclusion of L-NAME in acidic buffer resulted in a significant reduction in E_{max} and pEC_{50} for isoprenaline. The inhibitory effect of L-NAME in acidic buffer was modestly but significantly greater in tissues from rats on high salt compared to the regular diet (Table 6; Figure 12B). The presence of Ba^{2+} (100 μM) significantly attenuated isoprenaline-induced relaxations and this effect was of similar magnitude in blood vessels from rats fed either regular or a high salt diet (Table 6; Figure 13A). The inclusion of Ba^{2+} in the acidic buffer caused a significantly greater inhibition of the isoprenaline relaxations in pulmonary arteries from rats on high salt compared to those on a regular diet (Table 6; Figure 13B).

The presence of Ba^{2+} and ouabain together produced additional inhibition of isoprenaline-induced relaxations when compared to Ba^{2+} alone in the tissues from rats on a high salt diet (Table 6; Figure 9C, 10C, 13A and 14A). It was apparent that Ba^{2+} and ouabain together produced a greater inhibitory effect in tissues from rats fed a high salt in comparison to the regular diet (Figure 14A). However, the inclusion of Ba^{2+} and ouabain together in acidic buffer resulted in similar inhibition of isoprenaline responses in tissues from both rats on a high salt diet and those on a regular diet (Table 6; Figure 9D, 10D,

14B). Together, TEA (3.0 mM), Ba^{2+} and ouabain produced significantly greater inhibition of isoprenaline

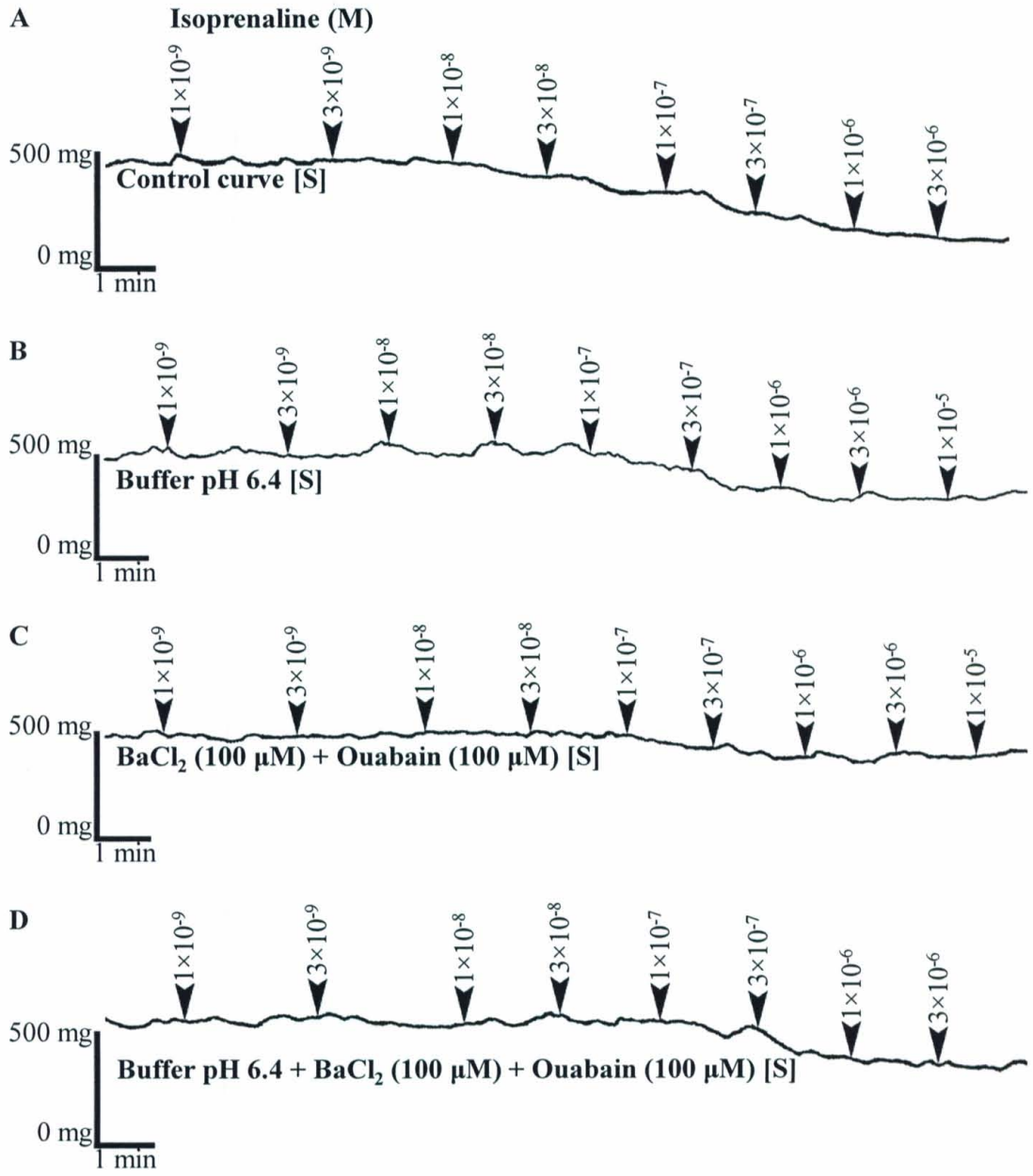

Figure 10: Concentration-response curve for isoprenaline in main pulmonary artery ring preparation from rats fed 4% salt diet [S] **A)** in regular buffer (control), **B)** in buffer pH 6.4, **C)** in the presence of barium chloride (100 µM) plus ouabain (100 µM) and, **D)** buffer pH 6.4 plus barium chloride (100 µM) plus ouabain (100 µM).

evoked relaxation in tissues from rats on high salt compared to regular diet, while in the acidic buffer, these pharmacological agents (i.e. TEA, Ba^{2+} & ouabain, together) produced similar inhibition of isoprenaline-induced relaxations in blood vessels of rats on either diet (Figure 15A and B).

Table 6. Percent maximal response (E_{max}) and pEC_{50} determined from individual concentration-response curves for isoprenaline in main pulmonary artery ring preparations from rats fed regular diet and 4% salt diets for 18-23 days.

	Regular diet		4% Salt diet	
	pEC_{50}	E_{max}	pEC_{50}	E_{max}
Control	7.21 ± 0.06	85.00 ± 3.02	7.11 ± 0.15	88.14 ± 3.25
200 µL dd H_2O	7.16 ± 0.07	79.29 ± 2.01[c,e]	7.42 ± 0.09[f]	86.86 ± 5.35[d,f]
Control	7.24 ± 0.11	77.00 ± 3.81	7.29 ± 0.02	79.17 ± 3.48
Acidic buffer	6.91 ± 0.08 [a]	54.33 ± 4.42[a]	6.70 ± 0.19[a]	43.00 ± 3.53[a,b]
Control	7.36 ± 0.15	87.86 ± 4.06	7.50 ± 0.05	88.43 ± 2.92
L-NAME	6.88 ± 0.11[a]	66.71 ± 4.40[a,c,e]	7.18 ± 0.09	73.71 ± 3.19[a,d,f]
Control	7.25 ± 0.06	87.14 ± 2.32	7.25 ± 0.05	79.83 ± 5.89
Acidic buffer + L-NAME	6.74 ± 0.081[a]	53.00 ± 5.26[a]	6.52 ± 0.23[a]	39.33 ± 3.64[a,b]
Control	7.26 ± 0.05	80.33 ± 5.76	7.15 ± 0.07	78.17 ± 2.26
$BaCl_2$	6.87 ± 0.09[a]	42.00 ± 4.14[a]	6.98 ± 0.05	48.50 ± 3.03[a]
Control	7.15 ± 0.14	85.50 ± 1.91	7.18 ± 0.11	85.67 ± 1.14
Acidic buffer + $BaCl_2$	6.69 ± 0.12[a]	58.00 ± 4.12[a,e]	6.52 ± 0.06[a,f]	47.33 ± 2.73[a,b]

Control	7.19 ± 0.07	85.38 ± 3.66	7.30 ± 0.05	87.88 ± 2.05
$BaCl_2$ + Ouabain	6.77 ± 0.11[a]	44.00 ± 5.01[a]	6.83 ± 0.14[a]	27.88 ± 3.62[a,b,d,f]
Control	7.19 ± 0.08	80.50 ± 2.95	7.25 ± 0.07	81.67 ± 3.58
Acidic buffer + $BaCl_2$ + Ouabain	6.62 ± 0.08[a]	33.00 ± 5.39[a,c]	6.46 ± 0.13[a,d,f]	37.83 ± 2.83[a,d,f]
Control	7.35 ± 0.06	87.33 ± 2.94	7.26 ± 0.09	81.71 ± 3.05
$BaCl_2$ + Ouabain + TEA	6.72 ± 0.17[a]	43.83 ± 8.87[a]	6.63 ± 0.07[a]	23.86 ± 5.63[a,b,d,f]
Control	7.29 ± 0.06	84.17 ± 3.89	7.14 ± 0.04	79.71 ± 4.17
Acidic buffer + $BaCl_2$ + Ouabain + TEA	6.32 ± 0.17[a,c]	26.00 ± 5.03[a,c,e]	6.32 ± 0.19[a,d,f]	21.86 ± 2.93[a,d,f]

Acidic buffer (pH 6.4 ± 0.2); N^{ω}-nitro-L-arginine-methyl ester (L-NAME; 10 μM); $BaCl_2$ (100 μM); Ouabain (100 μM); Tetraethylammonium (TEA, 3.0 mM).

Each value is the mean ± s.e.m., n= 6-8

[a]Significantly different from respective control; $p<0.05$

[b]Significantly different from treatment (regular diet); $p<0.05$

[c]Signifcantly different from acidic buffer (regular diet); $p<0.05$

[d]Significantly different from acidic buffer (4%Salt diet); $p<0.05$

[e]Significantly different from $BaCl_2$ (regular diet); $p<0.05$

[f]Significantly different from $BaCl_2$ (4%Salt diet); $p<0.05$

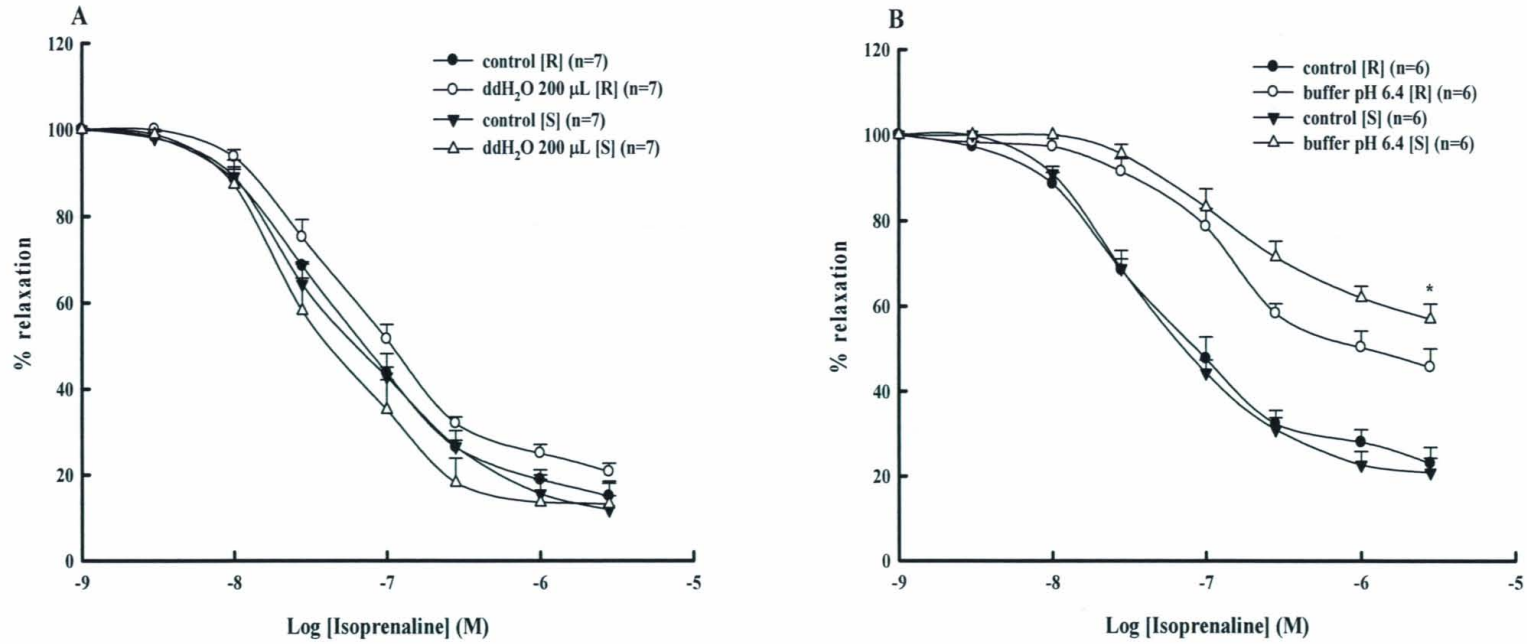

Figure 11. Concentration-response curve for isoprenaline (% relaxation remaining from phenylephrine induced sub-maximal contractions) in main pulmonary artery ring preparations from rats fed a regular [R] or 4% salt [S] diets for 18-23 days in **A)** regular buffer (control) and twice-distilled water and; **B)** regular buffer (control) and acidic buffer. Each point represents a mean ± s.e.m. of six to seven experiments. *Significantly different from respective value of regular diet; $p<0.05$.

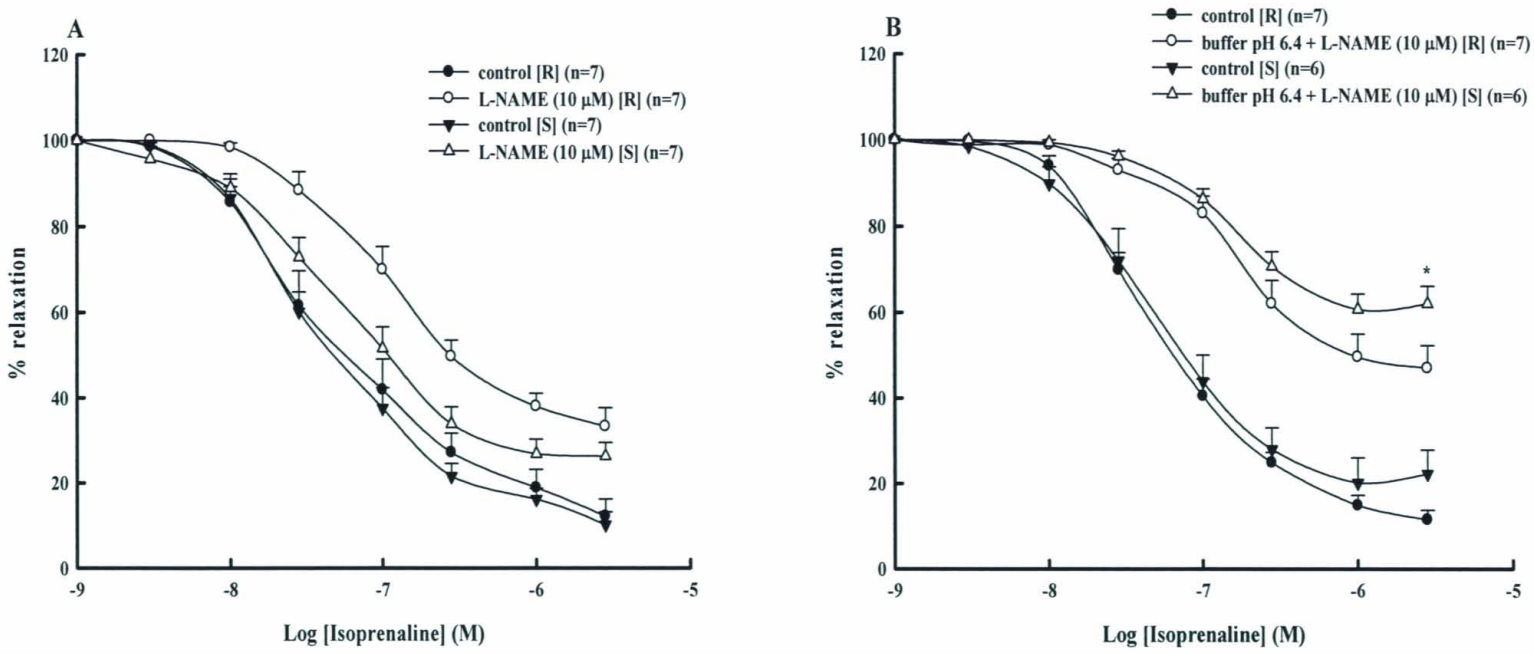

Figure 12. Concentration-response curve for isoprenaline (% relaxation remaining from phenylephrine induced sub-maximal contractions) in main pulmonary artery ring preparations from rats fed a regular [R] or 4% salt [S] diets for 18-23 days in **A)** regular buffer absence (control) or presence of L-NAME and; **B)** regular buffer (control) or acidic buffer plus L-NAME. Each point represents a mean ± s.e.m. of six to seven experiments. *Significantly different from respective value of regular diet; $p<0.05$.

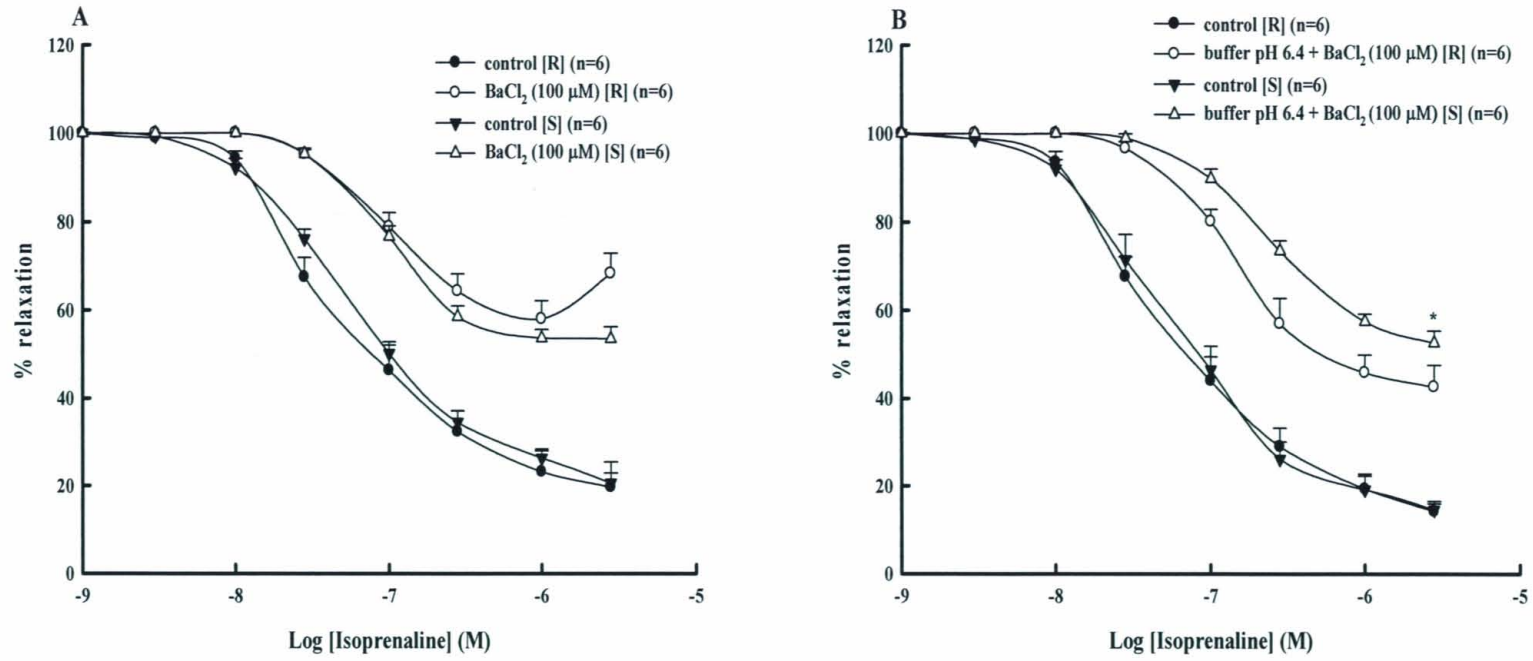

Figure 13. Concentration-response curve for isoprenaline (% relaxation remaining from phenylephrine induced sub-maximal contractions) in main pulmonary artery ring preparations from rats fed a regular [R] or 4% salt [S] diets for 18-23 days in **A)** regular buffer absence (control) or presence of $BaCl_2$ and; **B)** regular buffer (control) or acidic buffer plus $BaCl_2$. Each point represents a mean ± s.e.m. of six experiments. *Significantly different from respective value of regular diet; $p<0.05$.

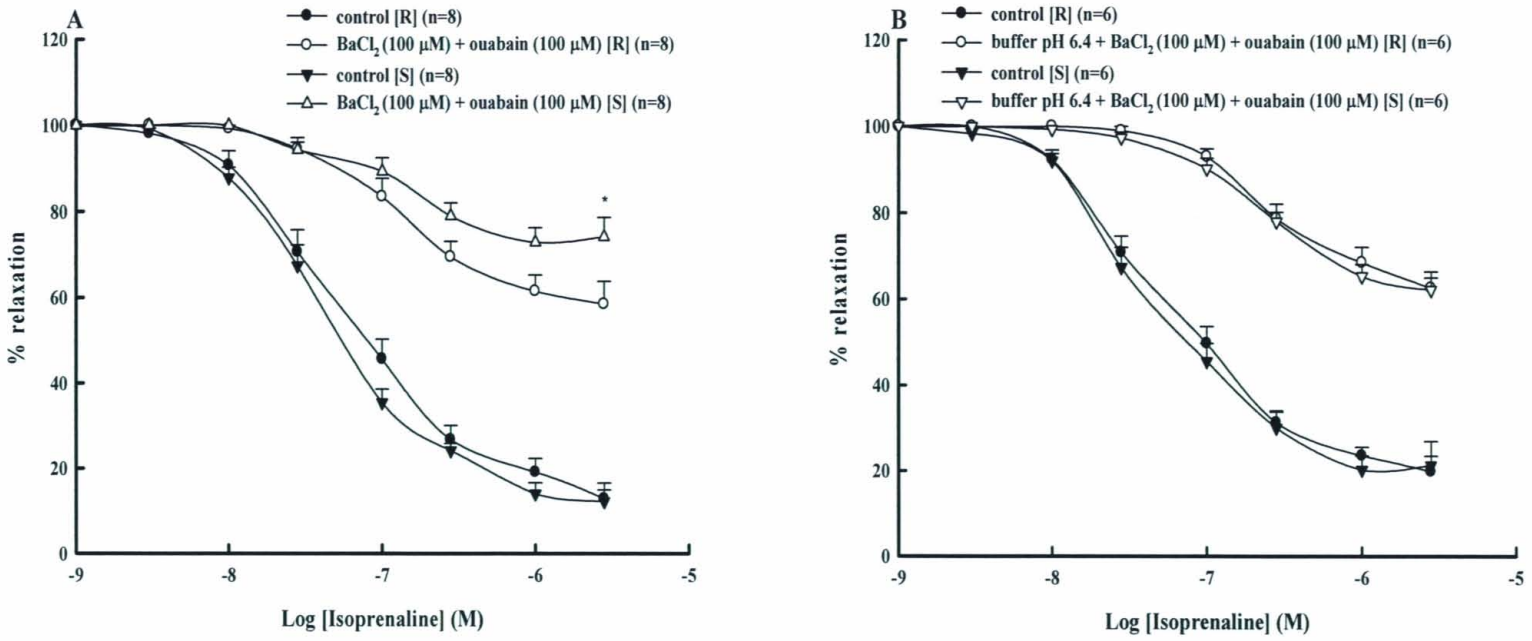

Figure 14. Concentration-response curve for isoprenaline (% relaxation remaining from phenylephrine induced sub-maximal contractions) in main pulmonary artery ring preparations from rats fed a regular [R] or 4% salt [S] diets for 18-23 days in **A)** regular buffer absence (control) or presence of $BaCl_2$ and ouabain together and; **B)** regular buffer (control) or acidic buffer plus $BaCl_2$ and ouabain together. Each point represents a mean ± s.e.m. of six to eight experiments. *Significantly different from respective value of regular diet; $p<0.05$.

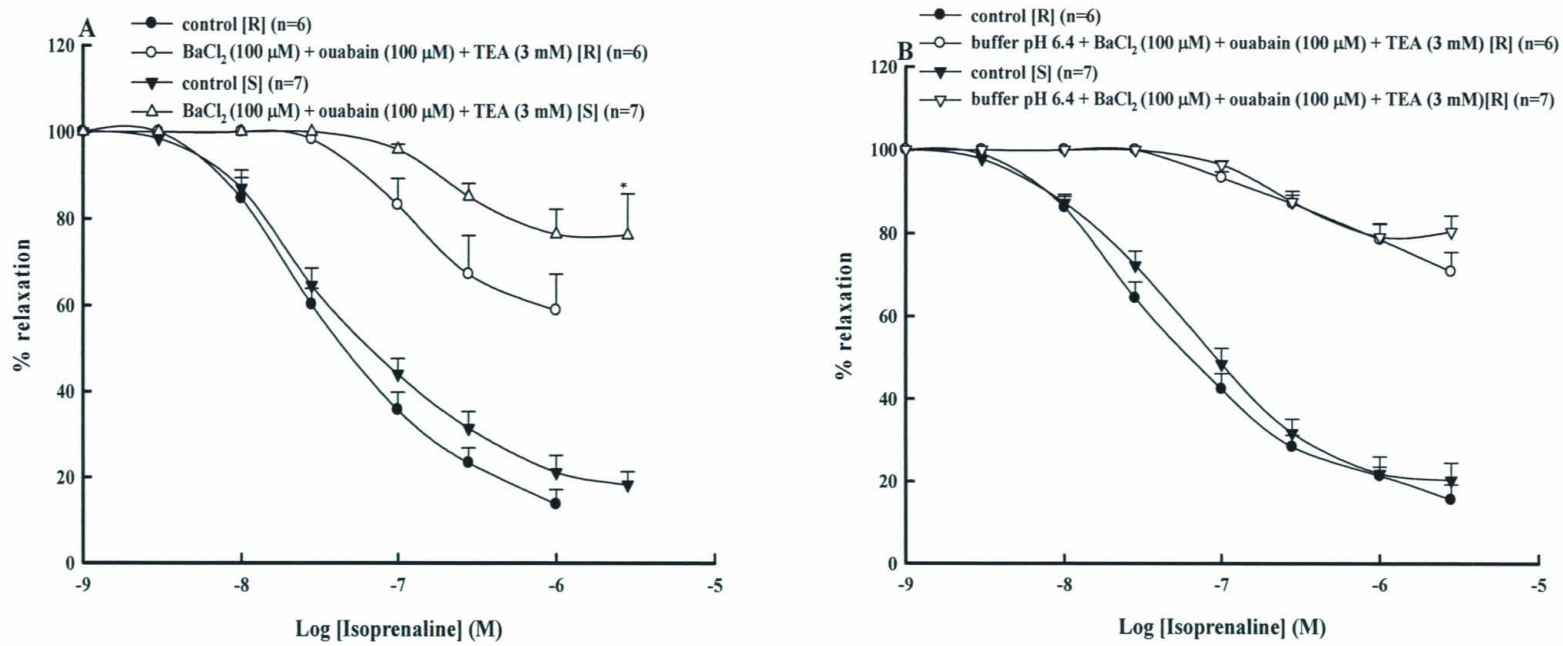

Figure 15. Concentration-response curve for isoprenaline (% relaxation remaining from phenylephrine induced sub-maximal contractions) in main pulmonary artery ring preparations from rats fed a regular [R] or 4% salt [S] diets for 18-23 days in A) regular buffer, absence (control) or presence of $BaCl_2$, ouabain and TEA together; B) regular buffer (control) or acidic buffer plus $BaCl_2$ ouabain and TEA together. Each point represents a mean ± s.e.m. of six to seven experiments. *Significantly different from respective value of regular diet; $p<0.05$.

3.2.2. Membrane potential

The resting E_m of smooth muscle cells of the pulmonary arteries of rats on a high salt diet was significantly more negative compared to rats on a regular diet (-62.2 ± 1.27 vs -66.9 ± 0.96 mV, respectively; Table 7, Figure 16 AC). Addition of isoprenaline (1.0 μM) induced significant hyperpolarization of smooth muscle cells of pulmonary arteries of rats on the regular diet but not of those on the high salt diet (-62.2 ± 1.27 vs -70.6 ± 0.72 mV, respectively; Table 7; Figure 16 BD, 17B). Exposure to acidic buffer (pH 6.4) resulted in a significant depolarization of smooth muscle cells of pulmonary arteries of rats on high salt diet (-68.2 ± 1.01 vs -63.4 ± 1.11 mV, respectively) but not of those on a regular diet (-61.6 ± 1.04 vs -61.3 ± 1.24 mV, respectively; Figure 17A). The presence of acidic buffer significantly impaired hyperpolarization caused by isoprenaline (Table 7; Figure 17B).

L-NAME alone had no influence on the resting E_m of smooth muscle cells in blood vessels from rats fed either regular (-61.3 ± 0.49 vs -60.4 ± 1.27 mV, respectively) or high salt diet (-67.9 ± 1.41 vs -67.2 ± 1.36 mV, respectively). In the presence of acidic buffer, did not produce any additional effects on the resting E_m of smooth muscle cells of pulmonary arteries from rat fed either diet (Table 7; Figure 17B). Addition of isoprenaline to acidic buffer containing L-NAME produced similar effects to those of acidic buffer alone (Table 7). While Ba^{2+} alone did not have an effect, combined with ouabain it produced a significantly greater degree of depolarization in smooth muscle cells of blood vessels from rats on salt compared to those on a regular diet (Figure 17A). In the presence of Ba^{2+} alone, isoprenaline produced a similar degree of hyperpolarization

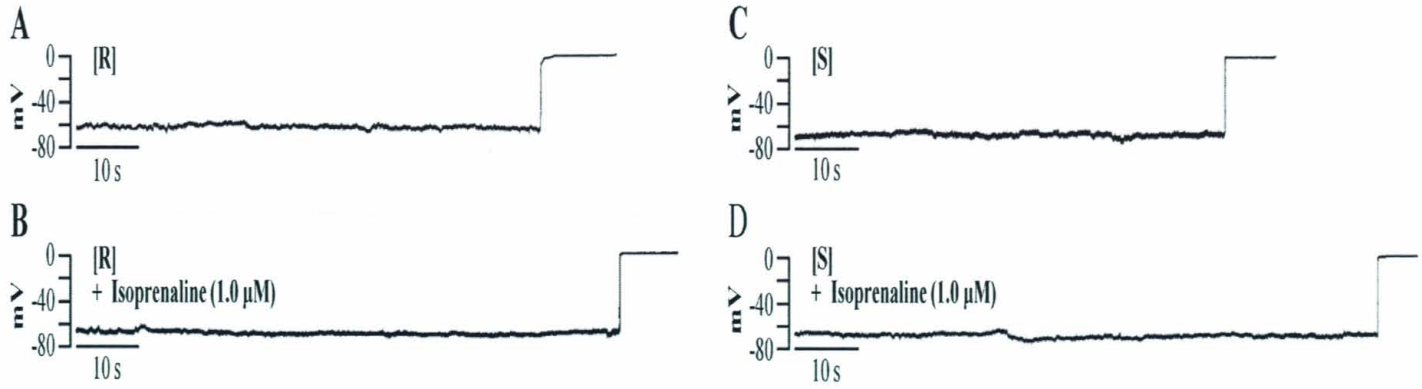

Figure 16. Membrane potential recordings of pulmonary arteries from rat fed regular [R] or 4% salt diet [S] in the presence of **A)** regular buffer (control), **B)** isoprenaline (1.0 µM), **C)** regular buffer (control) and, **D)** isoprenaline (1.0 µM).

of smooth muscle cells of pulmonary arteries from rats on either high salt (-61.5 ± 1.84 vs -65.9 ± 0.45 mV, respectively) or regular diet (-57.7 ± 0.38 vs -64.1 ± 1.56 mV, respectively; Table 7). In contrast, hyperpolarization evoked by isoprenaline was affected more in the presence of Ba^{2+} and ouabain in the smooth muscle cells from rats on a high salt diet (-60.8 ± 0.46 vs -62.8 ± 0.19 mV, respectively) compared to those on a regular diet (-58.7 ± 0.97 vs -64.1 ± 1.15 mV, respectively; Table 7; Figure 17B).

The extent of the depolarization by inclusion of Ba^{2+} and ouabain in acidic buffer, but not by Ba^{2+} alone in acidic buffer, was significantly different in smooth muscle cells of blood vessels from rats on a high salt diet compared to a regular diet (Table 7; Figure 17A). Isoprenaline was able to produce a significant hyperpolarization of the smooth muscle cells in acidic buffer containing Ba^{2+} alone in blood vessels from rats on a regular diet (-57.5 ± 0.26 vs -64.8 ± 0.69 mV, respectively) but not those on a high salt diet (-60.6 ± 1.21 vs -62.8 ± 0.22 mV, respectively; Table 7; Figure 17B). However, in acidic buffer, the combined presence of Ba^{2+} and ouabain inhibited isoprenaline-induced hyperpolarization in blood vessels from rats on either regular (-58.2 ± 0.35 vs -61.3 ± 1.55 mV, respectively) or high salt diet (-60.2 ± 1.02 vs -60.9 ± 1.79 mV, respectively; Table 7; Figure 17B).

The presence of TEA with Ba^{2+} and ouabain in regular buffer in comparison to acidic buffer produced a similar degree of depolarization in smooth muscle cells of pulmonary arteries from rats on either diet (Table 7; Figure 17A). Isoprenaline-induced hyperpolarization was between 1 to 3 mV in tissues with the latter treatments (Figure 17B).

Table 7. Membrane potential (mV) of smooth muscle cells in main pulmonary artery ring preparations from rats fed regular or 4% salt diets for 18-23 days.

	Regular diet			4% Salt diet		
	Control	*Treatment*	*Isoprenaline*	*Control*	*Treatment*	*Isoprenaline*
No treatment	-62.2 ± 1.27 (8)(34)		-70.6 ± 0.72[a] (8)(41)	-66.9 ± 0.96[b] (11)(53)		-68.3 ± 0.73[b] (11)(56)
Acidic buffer	-61.6 ± 1.04 (5)(24)	-61.3 ± 1.24 (5)(24)	-64.2 ± 1.29 (5)(21)	-68.2 ± 1.01[b] (5)(22)	-63.4 ± 1.11[a] (5)(22)	-65.8 ± 0.97 (5)(21)
L-NAME	-61.3 ± 0.49 (7)(25)	-60.4 ± 1.27 (7)(32)	-66.7 ± 0.68[a,c] (7)(37)	-67.9 ± 1.41[b] (6)(24)	-67.2 ± 1.36 (6)(27)	-68.9 ± 0.82 (6)(29)
Acidic buffer + L-NAME	-61.9 ± 1.19 (7)(21)	-61.1 ± 1.27 (7)(32)	-64.2 ± 1.05 (7)(34)	-67.8 ± 1.91[b] (6)(19)	-62.9 ± 0.80[a] (6)(21)	-65.4 ± 1.17 (6)(20)
$BaCl_2$	-60.7 ± 1.21 (4)(23)	-57.7 ± 0.38[a] (4)(18)	-64.1 ± 1.56[a,c] (4)(25)	-66.8 ± 0.66[b] (4)(24)	-61.5 ± 1.84[a] (4)(24)	-65.9 ± 0.45[c] (4)(24)
$BaCl_2$ + Ouabain	-61.2 ± 0.48 (4)(22)	-58.7 ± 0.97[a] (4)(22)	-64.1 ± 1.15[a,c] (4)(20)	-67.6 ± 0.50[b] (4)(24)	-60.8 ± 0.46[a] (4)(24)	-62.8 ± 0.19[a] (4)(15)
Acidic buffer + $BaCl_2$	-61.5 ± 0.78	-57.5 ± 0.26[a]	-64.8 ± 0.69[a,c]	-67.2 ± 0.70[b]	-60.6 ± 1.21[a]	-62.8 ± 0.22[a]

	(4)(23)	(4)(23)	(4)(18)	(4)(24)	(4)(24)	(4)(21)
Acidic buffer + $BaCl_2$ + Ouabain	-62.2 ± 0.21 (4)(22)	-58.2 ± 0.35[a] (4)(21)	-61.3 ± 1.55 (4)(22)	-66.9 ± 1.05[b] (4)(23)	-60.2 ± 1.02[a] (4)(23)	-60.9 ± 1.79[a] (4)(17)
$BaCl_2$ + Ouabain + TEA	-60.8 ± 1.37 (4)(16)	-55.9 ± 0.86[a,d] (4) (26)	-57.7 ± 0.81 (4)(24)	-66.6 ± 0.06[b] (4)(16)	-57.9 ± 0.50[a,e] (4)(23)	-58.9 ± 1.79[a] (4)(13)
Acidic buffer + $BaCl_2$ + Ouabain + TEA	-61.3 ± 1.01 (4)(15)	-56.4 ± 0.54[a,d] (4)(21)	-58.3 ± 0.62[a] (4)(24)	-68.6 ± 0.83[b] (4)(15)	-57.2 ± 1.20[a,e] (4)(23)	-58.6 ± 0.70[a] (4)(20)

Acidic buffer (pH 6.4 ± 0.2); N^{ω} nitro-L-arginine-methyl ester (L-NAME; 10 μM); $BaCl_2$ (100 μM); Ouabain (100 μM); Tetraethylammonium (TEA; 3.0 mM)

Each value is the mean ± s.e.m., with the number of rats and of cells indicated in parentheses.

[a]Significantly different from respective control; $p<0.05$

[b]Significantly different from respective control (regular diet); $p<0.05$

[c]Significantly different from respective treatment; $p<0.05$

[d]Significantly different from acidic buffer (regular diet); $p<0.05$

[e]Significantly different from acidic buffer (salt diet); $p<0.05$

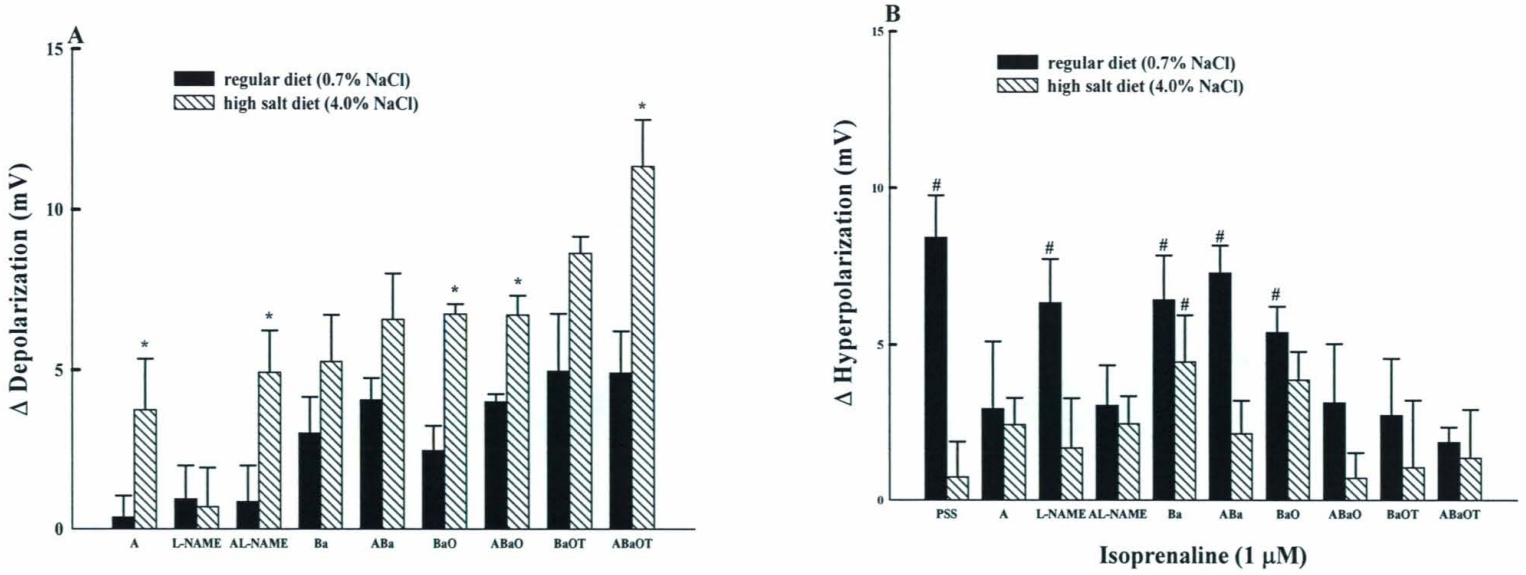

Figure 17. Effect of regular buffer (RB) and acidic buffer (A; pH 6.4) in absence and presence of various pharmacological agents on **A)** membrane potential (E_m) and **B)** isoprenaline elicited hyperpolarization in vascular muscle of main pulmonary arteries from rats on a regular or a high salt diet. N^{ω} nitro-L-arginine methyl ester; (L-NAME; 10 μM), Ba^{2+}; (Ba; 100 μM), Ouabain;(O; 100 μM), TEA (T; 3 mM). Resting E_m: regular diet: 61.5 ± 0.32 mV (mean ± s.e.m., n=51 rats & 221 cells); high salt diet: 67.4 ± 0.36 mV (mean ± s.e.m., n=52 rats & 244 cells). *Significantly different from respective value of regular diet; $p<0.05$. #Significantly different from respective treatment; $p<0.05$.

3.3. Study III. Effect of denudation of endothelium on vascular β-adrenoceptor signalling in rats fed a high salt diet

3.3.1. Mechanical Function

3.3.1.1. Intact tissues

Isoprenaline-induced responses for intact tissues with E_{max} and pEC_{50} in absence or presence of various treatments were described in section 3.2.1. For purposes of comparison, the data for vehicle (ddH_2O 200 µL), acidic buffer (pH 6.4), Ba^{2+}, ouabain or the combination of these interventions is mentioned again in Table 8.

3.3.1.2. Denuded tissues

Relaxant responses to isoprenaline were significantly attenuated in denuded tissues compared to intact tissues as evidenced by their respective E_{max} and pEC_{50} values (66.25 ± 1.59% and 7.07 ± 0.03; n = 40 vs 83.79 ± 1.23% and 7.27 ± 0.03; n = 39). The removal of endothelium significantly reduced isoprenaline mediated maximal responses (Table 8; Figure 18A). The presence of acidic buffer did not affect inhibition of isoprenaline-induced vasorelaxation (Table 7; Figure 18B). Ba^{2+} alone and in combination with ouabain in both regular buffer and acidic buffer significantly reduced isoprenaline-elicited relaxations (Table 8; Figure 19A and 19 B, 20A and B). However, inhibition of isoprenaline-induced relaxations were noticeably less with Ba^{2+} alone and in combination with ouabain in acidic buffer compared to in regular buffer.

Table 8. Percent maximal response (E_{max}) and pEC_{50} determined from individual concentration–response curves for isoprenaline in main pulmonary artery ring preparations with (+E) and without (-E) endothelium from rats fed a 4% salt diet for 18-23 days.

	4% Salt diet (+E)		4% Salt diet (-E)	
	pEC_{50}	E_{max}	pEC_{50}	E_{max}
Control	7.29 ± 0.02	79.17± 3.48	6.99 ± 0.08	65.86 ± 3.43
Acidic buffer	6.70 ± 0.19[a]	43.00 ± 3.53[a]	6.78 ± 0.09[a]	66.29 ± 4.73[c,e]
Control	7.15± 0.07	78.17± 2.26	7.06 ± 0.04	63.14 ± 6.05
$BaCl_2$	6.98± 0.05	48.50 ± 3.03[a]	6.68 ± 0.09[a]	28.29 ± 3.42[a,c]
Control	7.18 ± 0.11	85.67 ± 1.14	7.02 ± 0.07	65.83 ± 5.33
Acidic buffer + $BaCl_2$	6.52 ± 0.06[a]	47.33 ± 2.73[a]	6.64 ± 0.16	39.17 ± 5.53[a,c]
Control	7.30 ± 0.05	87.88 ± 2.05	7.05 ± 0.05	64.71 ± 1.66
$BaCl_2$ + ouabain	6.83 ± 0.14[a]	27.88 ± 3.62[a,b,d]	6.81 ± 0.07[a]	17.57 ± 2.54[a,c]
Control	7.25 ± 0.07	81.67 ± 3.58	7.09 ± 0.04	69.17 ± 2.04
Acidic buffer + $BaCl_2$ + ouabain	6.46 ± 0.13[a]	37.83 ± 2.83[a,b,d]	6.47 ± 0.09[a]	33.00 ± 6.32[a,c]

Acidic Buffer (pH 6.4) $BaCl_2$ (100 μM); ouabain (100 μM)

Each value is the mean ± s.e.m., n= 6-8

[a]Significantly different from respective control; $p<0.05$

[b]Significantly different from acidic buffer (+E); $p<0.05$

[c]Significantly different from acidic buffer (-E); $p<0.05$

[d]Significantly different from $BaCl_2$ (+E); $p<0.05$

[e]Significantly different from $BaCl_2$ (-E); $p<0.05$

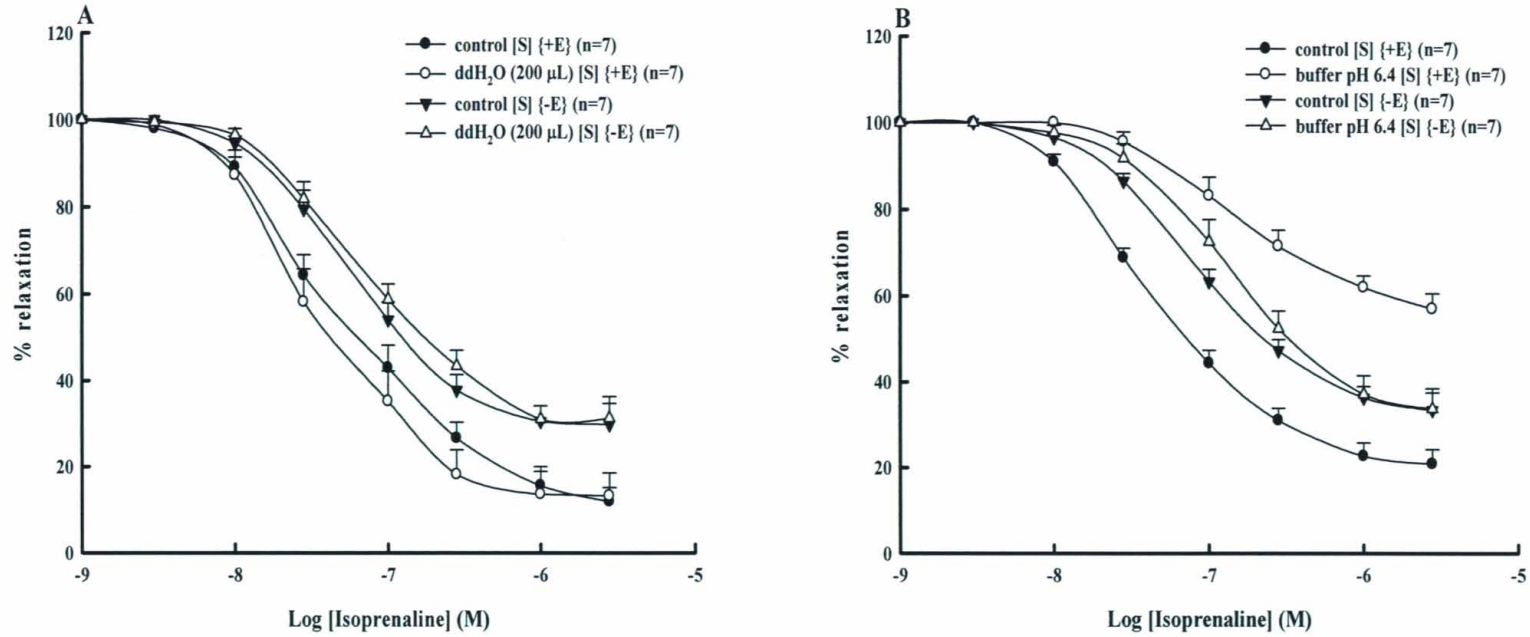

Figure 18. Concentration-response curve for isoprenaline (% relaxation remaining from phenylephrine induced sub-maximal contractions) in intact {+E} and denuded {-E} main pulmonary artery ring preparations from rats fed a high salt diet [S] in **A)** regular buffer (control) and twice-distilled water and; **B)** regular buffer (control) and acidic buffer. Each point represents a mean ± s.e.m. of seven experiments.

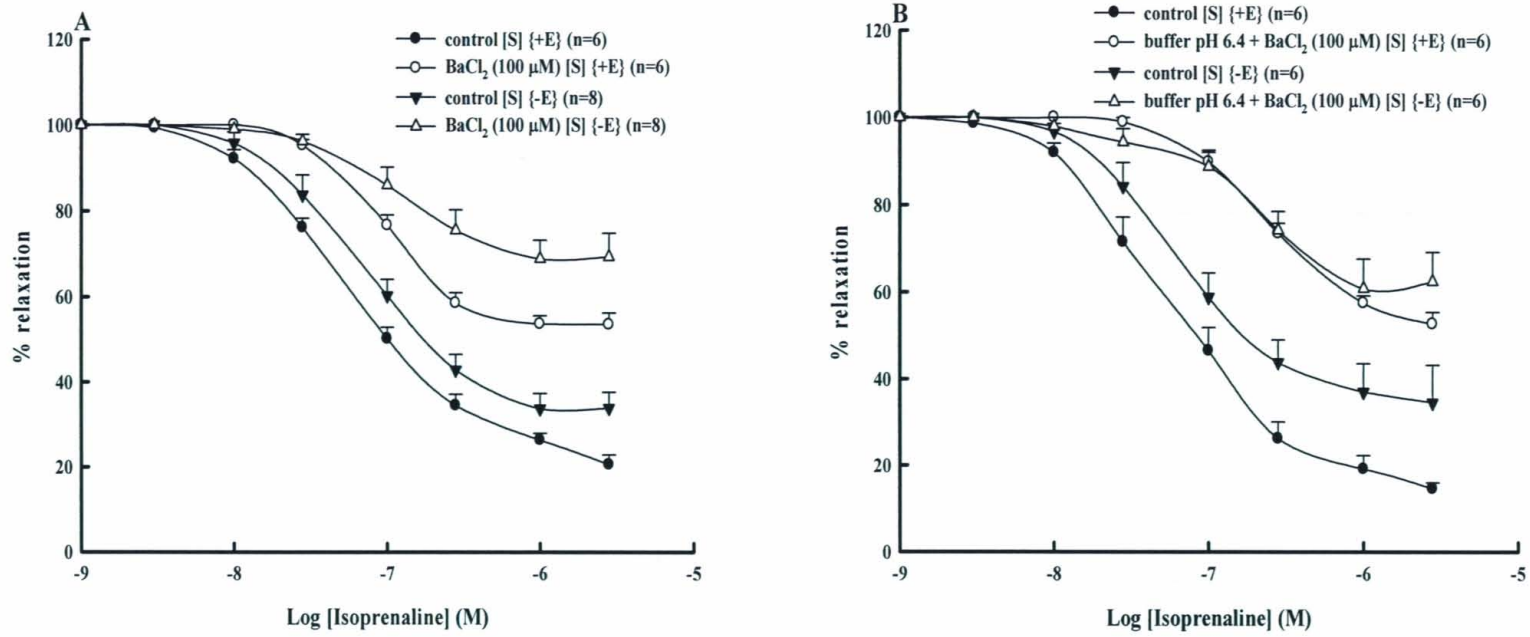

Figure 19. Concentration-response curve for isoprenaline (% relaxation remaining from phenylephrine induced sub-maximal contractions) in intact {+E} and denuded {-E} main pulmonary artery ring preparations from rats fed a high salt diet [S] in **A)** regular buffer absence (control) or presence of $BaCl_2$ and; **B)** regular buffer (control) or acidic buffer plus $BaCl_2$. Each point represents a mean ± s.e.m. of six to eight experiments.

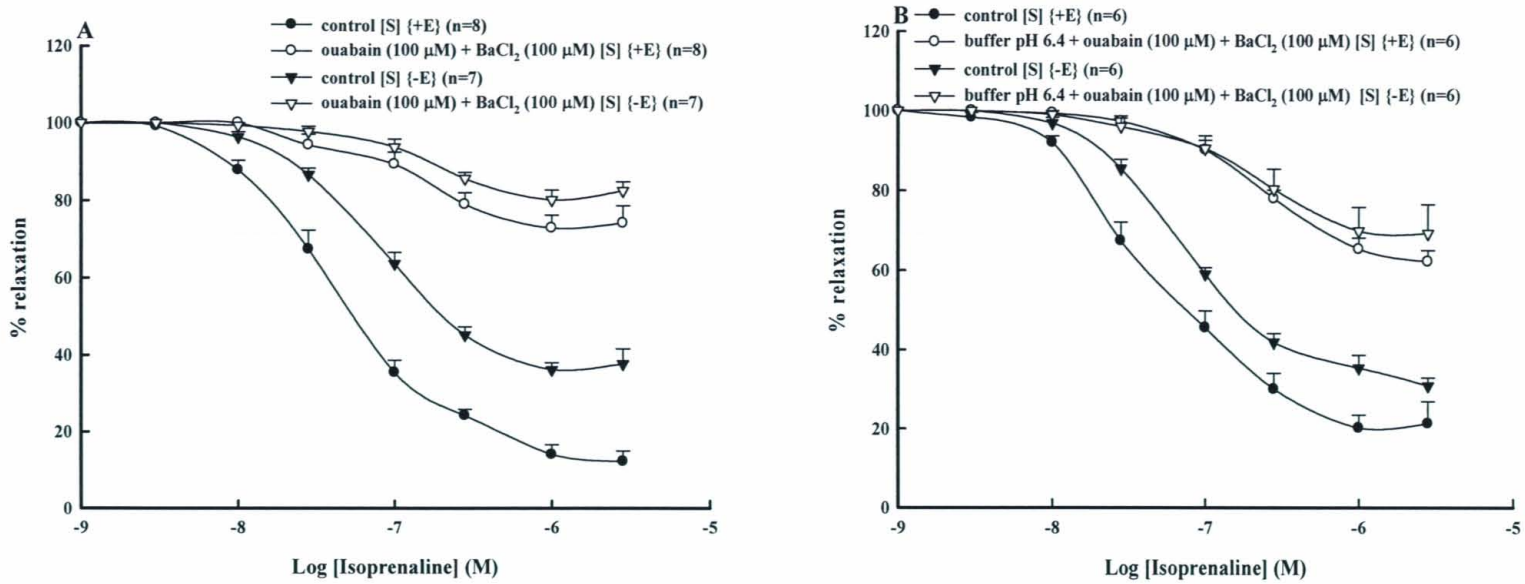

Figure 20. Concentration-response curve for isoprenaline (% relaxation remaining from phenylephrine induced sub-maximal contractions) in intact {+E} and denuded {-E} main pulmonary artery ring preparations from rats fed a high salt diet [S] in **A)** regular buffer absence (control) or presence of $BaCl_2$ and ouabain together and; **B)** regular buffer (control) or acidic buffer plus $BaCl_2$ and ouabain together. Each point represents a mean ± s.e.m. of six to eight experiments.

Table 9. Membrane potential (mV) of smooth muscle cells in main pulmonary artery ring preparations with (+E) and without (-E) endothelium from rats fed a 4% salt diet for 18-23 days.

	4% Salt diet (+E)			4% Salt diet (-E)		
	Control	*Treatment*	*Isoprenaline*	*Control*	*Treatment*	*Isoprenaline*
No treatment	-66.9 ± 0.96 (11)(53)		-68.3 ± 0.73 (11)(56)	-69.6± 0.98 (5)(16)		-69.7± 2.17 (5)(11)
Acidic buffer	-68.2 ± 1.01 (5)(22)	-63.4 ± 1.11[a] (5)(22)	-65.8 ± 0.97 (5)(21)	-69.2 ± 0.65 (5)(20)	-66.3 ± 2.88 (5)(17)	-66.7± 2.88 (5)(12)
$BaCl_2$	-66.8 ± 0.66 (4)(24)	-61.5 ± 1.84[a] (4)(24)	-65.9 ± 0.45[b] (4)(24)	-69.5 ± 1.28 (4)(15)	-59.9 ± 1.41[a,d] (4)(19)	-61.1 ± 1.23[a] (4)(12)
Acidic buffer + $BaCl_2$	-67.2 ± 0.70 (4)(24)	-60.6 ± 1.21[a] (4)(24)	-62.8 ± 0.22[a] (4)(21)	-68.2 ± 1.07 (4)(15)	-57.9 ± 1.46[a,d] (4)(18)	-60.5 ± 1.29[a] (4)(15)
$BaCl_2$ + ouabain	-67.6 ± 0.50 (4)(24)	-60.8 ± 0.46[a] (4)(24)	-64.7 ± 1.12[a,b] (4)(19)	-69.4 ± 1.38 (4)(17)	-58.1 ± 1.13[a,d] (4)(22)	-60.3 ± 1.95[a] (4)(13)
Acidic buffer + $BaCl_2$ + ouabain	-66.9 ± 1.05 (4)(23)	-60.2 ± 1.02[a,c] (4)(23)	-60.1 ± 2.40[a] (4)(18)	-68.4 ± 1.15 (4)(15)	-59.9 ± 1.73[a,d] (4)(19)	-61.8 ± 1.55[a] (4)(13)

Acidic Buffer (pH 6.4 ± 0.2); $BaCl_2$ (100 μM); ouabain (100 μM)

Each value is the mean ± SE. with the number of rats (= n) and number of cells indicated in parentheses.

[a]Significantly different from respective control; $p<0.05$

[b]Significantly different from respective treatment; $p<0.05$

[c]Significantly different from acidic buffer (+E); $p<0.05$

[d]Significantly different from acidic buffer (-E); $p<0.05$

3.3.2. Membrane potential

3.3.2.1. Intact tissues

The E_m for intact tissues was stated in section 3.2.2. For evaluation purposes, the data for no treatment, acidic buffer (pH 6.4), Ba^{2+}, ouabain or combination of these interventions is mentioned again in Table 9.

3.3.2.2. Denuded tissue

Hyperpolarization recorded in smooth muscle cells was of similar magnitude in pulmonary arteries with (-66.9 ± 0.96 vs -68.3 ± 0.73 mV, respectively) or without endothelium (-69.6± 0.98 vs -69.7± 2.17 mV, respectively) from rats fed a high salt diet (Table 9). The presence of acidic buffer did not affect resting E_m of smooth muscle cells (-69.2 ± 0.65 vs -66.3 ± 2.88 mV, respectively; Table 9). Ba^{2+} (-69.5 ± 1.28 vs -59.9 ± 1.41 mV, respectively) alone and together with ouabain in absence (-69.4 ± 1.38 vs -58.1 ± 1.13 mV, respectively) and presence of acidic buffer(-68.4 ± 1.15 vs -59.9 ± 1.73 mV, respectively) significantly depolarized vascular smooth muscle cells compared to acidic buffer alone (Table 9). The extent of isoprenaline-induced hyperpolarization was noticeably reduced in the absence or presence of various treatments such as acidic buffer (pH 6.4), Ba^{2+}, ouabain or combination of these interventions.

4.0. DISCUSSION

Removal endothelium produced hyperpolarization of vascular smooth muscle cells in blood vessels obtained from rats fed regular diet. The hyperpolarisation due to denudation was the result of the activation of K^+ channels and Na^+/K^+ATPase. β-adrenoceptor-evoked hyperpolarization appeared to be mutually dependent on the presence of endothelial cells. Isoprenaline-induced hyperpolarization was absent in denuded tissues. β-adrenoceptor-mediated relaxation was found to be partly dependent on the presence of endothelium. Isoprenaline produced similar relaxations in intact pulmonary arteries obtained from animals fed a high salt compared to normal diet. However, the E_m was found to be hyperpolarized in intact pulmonary arteries of animals fed a high salt diet compared to those fed a normal diet. The isoprenaline evoked hyperpolarization was notably absent in the pulmonary arteries. In addition, in intact versus denuded blood vessels obtained from rats on high salt diet E_m was not significantly different. Collectively, the studies concluded that the presence of the endothelium is essential for the propagation of β-adrenoceptor-mediated relaxation. Moreover, the evidence seem to also imply that consumption of high salt leads to the activation of K^+ channels and Na^+/K^+ATPase channels preserving β-adrenoceptor-mediated relaxations in pulmonary arteries.

A substantial amount of evidence suggests that a link between excessive consumption of Na^+ in the form of dietary salt and development of hypertension (Intersalt, 1988). This observation stands true for both human and animal populations (Tobian, 1991; Meneton *et al.*, 2005; He and MacGregor, 2010). In animals, this is particularly marked in the

genetically selected Dahl salt sensitive rats (Dahl *et al.*, 1962), but it also occurs to a smaller extent in non-selected animals including Sprague-Dawley rats (Sofola *et al.*, 2002), Wistar rats (Huang & Johns, 2000), and chimpanzees (Denton *et al.*, 1995). In the latter mentioned animal population, high salt consumption has been linked with causing adverse cardiovascular effects (i.e. ventricular or vascular hypertrophy) without altering the blood pressure (Tobian, 1991; Menton *et al.*, 2005; He and MacGregor, 2010). The mechanisms linking consumption of high salt diet and hypertension/ adverse cardiovascular effects appears to be complex and to involve alteration in the cardiovascular system i.e. heart, kidney and vascular system (Tobian, 1991; Menton *et al.*, 2005; He and MacGregor, 2010).

The alteration of adrenergic responsiveness due to the consumption of high salt diet could be an important factor in the pathogenesis and maintenance of the hypertension and thus cardiovascular disease (Feldman, 1990 a; Michel *et al.*, 1990; Brodde and Michel, 1992). One of the possible mechanisms that is a contributor to the increased in peripheral vascular resistance is an increase in α-adrenoceptor mediated vasoconstriction (Michel *et al.*, 1990; Brodde and Michel, 1992). However, an alteration in other vascular adrenoceptors i.e. β-adrenoceptor responsiveness could also be a contributing factor to the overall increase in peripheral resistance in the hypertension. Blunted β-adrenoceptor responses have been reported in hypertensive animals and humans (Field and Soltis, 1985; Naslund *et al.*, 1988; Feldman, 1990b; Borkowski *et al.*, 1992). Further, high salt diet has inconsistent effects on β-adrenoceptor-mediated vasorelaxation. In humans, a reduction in salt intake has been associated with improved β-adrenoceptor-mediated

vasodilation (Naslund *et al.*, 1988; Feldman, 1990b) and improved vascular distensibility in hypertensive and elderly patients (Avolio *et al.*, 1986; Gates *et al.*, 2004). In Dahl-salt sensitive rats fed a high salt diet, β-adrenoceptor-mediated responses to isoprenaline were attenuated in aortic ring (Soltis and Katovich, 1991) but were unchanged in pulmonary arteries (Ford *et al.*, 2011). This discrepancy could be associated with variability in the vascular beds (i.e. systemic *vs* pulmonary) and different intracellular signalling pathway activated as a consequence of β-adrenoceptor stimulation i.e. cAMP, PKA, cGMP and potassium channels (Scheid *et al.*, 1979; Rubanyi and Vanhoutte, 1985; Nakashima and Vanhoutte, 1995; Bieger *et al.*, 2006). Against this background, the current thesis research was undertaken to assess the electrical and mechanical differences in the low pressure segment of the circulatory system (pulmonary artery) from Sprague-Dawley rats on regular and high salt diet.

4.1. Role of endothelium in β-adrenoceptor signalling

β-adrenoceptor-mediated responses in the rat pulmonary artery have both endothelial dependent and independent components. In an intact tissue, β-adrenoceptor stimulation produces hyperpolarization of smooth muscle cells via the activation of an acid-sensitive channel (i.e. TASK). This latter response was absent in the endothelial denuded tissues. Bieger *et al*, (2006) demonstrated that isoprenaline-evoked hyperpolarization in the isolated rat main pulmonary artery was not only sensitive to inhibitory actions of acidic buffer but also to the anandamide (TASK channel antagonist). Anandamide also produced concentration dependent inhibition of the relaxation and inhibition of

hyperpolarization elicited by isoprenaline (Bieger *et al.*, 2006). TASK channels are present in rat pulmonary artery (Gardener *et al.*, 2004) and were found to be sensitive to inhibition by anandamide (Maingret *et al.*, 2001; Gurney *et al.*, 2003). In the present study, hyperpolarization induced by stimulation of β-adrenoceptors was significantly inhibited by the presence of acidic buffer but not Ba^{2+}, ouabain or TEA alone. The latter suggests that TASK plays an integral role in β-adrenoceptor-mediated hyperpolarization. In the pulmonary artery, Ba^{2+} and ouabain together inhibited β-adrenoceptor-mediated mechanical response in the absence and presence of endothelium. Ba^{2+} and ouabain together modestly inhibited isoprenaline-evoked hyperpolarization in intact arteries compared to denuded arteries. Moreover, acidic buffer combined with Ba^{2+} and ouabain, did not cause further inhibition of the relaxation when compared to Ba^{2+} and ouabain combined. In accordance to the mentioned observation, it is plausible to suggest that the stimulation of β-adrenoceptor caused the activation of TASK channels, likely located on the smooth muscle cells, leading to modest efflux of K^+ which then perpetuated the opening $K_{ir2.1}$ channels and/or the over-activation of Na^+/K^+ATPase leading to a well defined sustained hyperpolarization of approximately 8.0 mV in an intact blood vessel. Alternatively, it is also be possible that TASK, $K_{ir2.1}$, Na^+/K^+ATPase were activated in parallel and worked in concert to produce the isoprenaline mediated hyperpolarization. Evidently, isoprenaline mediated vasorelaxant responses were similar in the presence of acidic buffer and Ba^{2+} suggesting that the latter might be acting as an inhibitor of TASK channels in addition to its effect on $K_{ir2.1}$. Nonetheless, Ba^{2+} is believed to be a relatively selective inhibitor of $K_{ir2.1}$ channels (Sabirov *et al.*, 1997; Owen *et al.*, 1999).

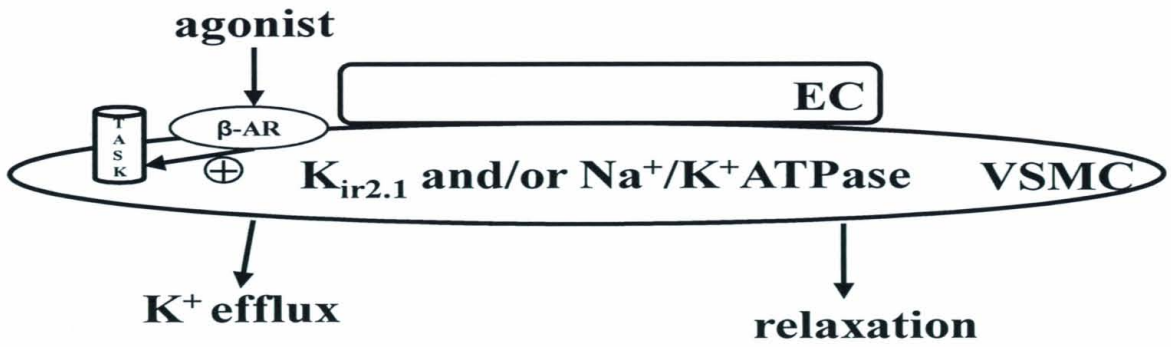

Figure 21. A schematic demonstrating β-adrenoceptor (β-AR) signalling in intact rat main pulmonary artery.

Inward rectifier potassium channels, ($K_{ir2.1}$); electrogenic pump, (Na^+/K^+ATPase); endothelial cell, (EC); vascular smooth muscle cell, (VSMC); two-pore domain acid sensitive K^+ channels, (TASK).

Yet, another hypothesis could be that stimulation of β-adrenoceptors resulted in endothelium dependent hyperpolarization. This event would have had to predominantly occur via the activation of acid-sensitive channels, and a portion of the electrical response may have been brought on as a result of the so called "potassium clouds" i.e. activation of Na^+/K^+ATPase causing the $K_{ir2.1}$ channels to open (Edwards and Weston, 2004). Hyperpolarization of vascular smooth muscle cells is believed to occur under certain conditions due to K^+ efflux via $K_{ir2.1}$ and/or Na^+/K^+ATPase as a consequence of the activation of the intermediate Ca^{2+}-activated K^+ channels located on the endothelial cells (Félétou and Vanhoutte, 2009). However, this does not explain the finding that the acidification of the buffer and anandamide were both capable of inhibiting hyperpolarization and relaxation induced by isoprenaline in such blood vessels (Bieger *et al.*, 2006). As well, in the present investigation, a combination of Ba^{2+} and ouabain inhibited isoprenaline-induced relaxation in both intact and denuded tissue to a similar degree.

It was also observed that TEA partially attenuated hyperpolarization induced by isoprenaline in intact pulmonary arteries. TEA caused a modest but not significant inhibition of the relaxation in the intact tissues. The lack of complete inhibition of hyperpolarization by TEA in the presence of isoprenaline may support the view that the latter intervention unlike acidic buffer is not able to substantially inhibit the K^+ channels that are activated by the stimulation of β-adrenoceptors in isolated rat pulmonary arteries. In the rat abdominal aorta, β-adrenoceptor-mediated relaxation has been reported to be, in part, dependent on the activation of large-conductance, Ca^{2+}-sensitive K^+ channels (BK)

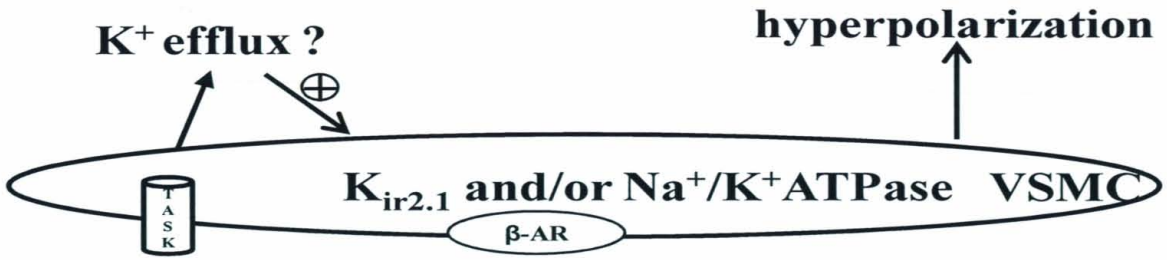

Figure 22. A schematic demonstrating denudation-induced hyperpolarization in rat main pulmonary artery.

Inward rectifier potassium channels, ($K_{ir2.1}$); electrogenic pump, ($Na^+/K^+ATPase$); vascular smooth muscle cell, (VSMC); two-pore domain acid sensitive K^+ channels, (TASK)

(Matsushita *et al.*, 2006) while in the isolated rat aorta, relaxant responses to isoprenaline were partly inhibited by TEA (Kang *et al.*, 2007). It had been previously reported that charybdotoxin but not apamin also partially attenuated relaxant responses to isoprenaline in intact rat main pulmonary artery (Bieger *et al.*, 2006). Furthermore, relaxant responses to isoprenaline in rat pulmonary artery could be completely abolished by elevation in the extracellular concentration of K^+ (30 mM) (Bieger *et al.*, 2006). Collectively, the evidence seems to suggest that the relaxant responses due to the stimulation of β-adrenoceptors in vasculature are partly dependent upon the activation of K^+ channels.

It is evident that denudation can abolish hyperpolarization caused by isoprenaline in rat main pulmonary artery. We did observe that the removal of the endothelial cells resulted in the E_m shifting to a more negative setting of approximately -67 mV. One simple explanation could be that the substantial hyperpolarization following denudation rendered the TASK channels inefficient to cause further hyperpolarization upon stimulation of β-adrenoceptor. The E_K in single vascular muscle cell has been reported to be about -75 mV (Russell *et al.*, 1992). It is possible, that since denudation of blood vessels caused an increase in E_m (i.e. hyperpolarization) of the smooth muscle cells, this subdued the participation of the acid-sensitive channels following the application of isoprenaline as E_m was brought close to E_K (Figure 22).

Altogether, the study suggests that the denudation of blood vessels causes hyperpolarization of smooth muscle cells as a result of the activation of $K_{ir2.1}$ and Na^+/K^+ATPase. Moreover, inclusion of Ba^{2+} and ouabain in acidic buffer does not produce any additive depolarization of denuded pulmonary arteries. It is also apparent

that this hyperpolarization is brought on in part via the activation of K^+ channels, since TEA was able to produce modest depolarization in denuded tissues without having any impact in the intact blood vessels. Therefore, it is reasonable to assume that the participation of Na^+/K^+ATPase and K^+ channels including the $K_{ir2.1}$ in increasing the E_m meant that the electrical responses caused by the stimulation of β-adrenoceptors seem no longer a viable trigger in generating hyperpolarization in a denuded blood vessel.

4.2. Influence of high salt diet on β-adrenoceptor signalling.

In the current investigation, hyperpolarization observed in vascular muscle of blood vessels from animals on a high salt diet is in accord with previous work from this laboratory (Bieger *et al.*, 2004; Parai and Tabrizchi, 2005). An approximately 6.0 mV of hyperpolarization was recorded in the resting E_m of smooth muscle cells of the main pulmonary arteries of rats that consumed a high salt diet in comparison to a regular diet. This hyperpolarization can be attributed to the opening of K^+ channels and the activation of Na^+/K^+-ATPase. It is also apparent that isoprenaline-induced relaxations were not perturbed in pulmonary arteries from rats fed a high salt diet compared to those on a regular diet. The latter observation could be taken to indicate that hyperpolarization of smooth muscle cells of the pulmonary arterial vessels played a pivotal role in preserving isoprenaline-mediated relaxations in tissues obtained from animals on a high salt diet. Support for this view stems from a recently published report by Garland and colleagues (2011). These investigators have purposed the view that hyperpolarization of smooth

muscle cells evoked by the stimulation of β-adrenoceptors is instrumental in spreading of vasodilation in blood vessels (Garland *et al.*, 2011). Therefore, hyperpolarization may be a compensatory mechanism to restore the normal relaxant responses to β-adrenoceptor stimulation in tissues from animals that consumed a high salt diet.

A modest but significant increase in blood pressure was recorded in normotensive rats after consumption of high salt diet in our present study. Similarly, Sofola *et al.* (2002) also reported a significant increase in blood pressure of Sprague-Dawley rats fed a high salt diet compared to those on a regular diet. In systemic arteries, an increase in blood pressure has been reported to attenuate β-adrenoceptor-mediated functional and membrane potentials (Asano *et al.*, 1982; Borkowski *et al.*, 1992; Goto *et al.*, 2001). Previously evidence from this laboratory indicated that salt-induced systemic hypertension in Dahl rats did not lead to elevation in pulmonary arterial pressure (Bieger *et al.*, 2004). However, it was noted that alteration in receptor-mediated signal transduction evolved in pulmonary arterial vascular bed of Dahl hypertensive rats fed a high salt diet independent of elevation in systemic arterial pressure (Bieger *et al.*, 2004).

It was observed that the electrolyte levels were similar in plasma collected from the animals fed either regular or high salt diets. Even though we did not detect any significant changes in plasma levels of Na^+ in animals fed a high salt diet, it is plausible that alteration in β-adrenoceptor-mediated signal transduction noted in our present investigation may have occurred as a consequence of small daily fluctuations in plasma levels of Na^+ due to the consumption of high salt diet. Data in the literature supports the view that a rise in Na^+ concentration greater than 2 mmol/L induces changes in vascular

response independent of the increase in blood pressure (de Wardener *et al.*, 2004). This may have been the case in the current investigation.

Dietary salt intake has also been shown to have a variable influence on β-adrenoceptor-mediated vascular responses. An intake of high salt in the diet has been implicated as a cause for an impaired β-adrenoceptor-mediated response (Feldman, 1990 a). For instance, in borderline hypertensive patients or elderly normotensive individuals, a reduction in salt intake results in normalized β-adrenoceptor-mediated venodilation (Feldman, 1990 b, 1992). However, Naslund and colleagues (1990), in forearm brachial artery, have found that the potency of isoprenaline-induced vasodilation increased in salt insensitive individuals after six days on high salt diet (250 mmol Na^+ per day) compared to individuals on a low salt diet (10 mmol Na per day). Experimental evidence for the influence of high salt diet on vascular β-adrenoceptor-mediated responses varies in rat. For example, Soltis and Katovich (1991) demonstrated that isoprenaline evoked relaxant responses were attenuated in aortic rings from of Dahl-salt sensitive rats following three weeks on high salt diet. In contrast, isoprenaline mediated relaxant responses were not different in pulmonary artery from Dahl-salt sensitive rat fed a high salt diet (Ford *et al.*, 2011). This noted variance between the former and latter findings as to the effect of high salt diet on β-adrenoceptor-mediated relaxations could be due to differences in the nature of the vessels i.e. systemic arteries that cater high blood pressure as compare to marginally lower pressure by pulmonary arteries.

Here, it is also evident that isoprenaline-induced hyperpolarization in tissues from rats on the high salt diet was absent. Based on the electrophysiological data in our current study,

the hyperpolarization recorded in vascular smooth muscle cells of pulmonary arterial vessels from rats fed a high salt diet compared to regular diet is mainly due to the activation of the leak K^+ current, opening of the $K_{ir2.1}$ channels, the activation of Na^+/K^+ATPase and to a modest degree the opening of the TEA-sensitive voltage-gated K^+ channels. Thus, it is plausible that since consumption of the high salt diet had already caused the opening of K^+ channels and activation of Na^+/K^+ATPase, leading to the hyperpolarization of vascular muscle cells, isoprenaline was no longer capable of producing a further decrease in the E_m. Alternatively, it is possible that E_m of smooth muscle cells of the pulmonary arterial vessels of rats fed a high salt diet was close to the K^+ reversal potential and further hyperpolarization by isoprenaline did not ensue. As mentioned earlier, E_k of single smooth muscle cell has been reported to be approximately -75 mV (Russell *et al.*, 1992)

Stimulation of β-adrenoceptors in vascular muscle leads to the activations of the adenylyl cyclase with a subsequent increase in intracellular cAMP concentration and vasorelaxation (Scheid *et al.*, 1979). In rat pulmonary artery, TASK channels have been reported to be present in vascular muscle (Gardener *et al.*, 2004). In addition, it seems that the activation of these acid-sensitive leak channels can occur independently of adenylyl cyclase/cAMP/PKA pathway (Bieger *et al.*, 2006). This study seem to also reveal that cAMP antagonist, Rp-cAMP, did not display any differential inhibitory response on isoprenaline-mediated relaxations in tissues from animals fed a high salt diet versus those on a regular diet. This would argue against a modified role for cAMP in tissues from animals on high salt compared to a regular diet.

The β-adrenoceptor-mediated relaxation in part is mediated via eNOS/NO/cGMP pathway (Priest *et al.*, 1999; Bieger *et al.*, 2006). In the current investigation, the latter pathway does not seem to play any role in β-adrenoceptor-mediated hyperpolarization. However, previous evidence from our laboratory demonstrated that consumption of high salt resulted in hyperpolarization of mesenteric artery of Dahl salt-resistant rats. This hyperpolarization was reversed by L-NAME (Parai and Tabrizchi, 2005). In contrast, isoprenaline-induced relaxation of the pulmonary artery of Dahl salt-sensitive rats (Ford *et al.*, 2011) was found to be insensitive to inhibition by L-NAME. In our present study, hyperpolarization of vascular smooth cell brought on by consumption of the high salt diet was found to be insensitive to the action of L-NAME. Taken together, our current observations suggest that an altered role for eNOS/NO/cGMP pathway in relation to isoprenaline-induced hyperpolarization and relaxation in pulmonary arterial vessels from rats fed a high salt seems tenuous.

Interestingly, the opening of $K_{ir2.1}$ (Ba^{2+}-sensitive) and the activation of Na^+/K^+ATPase (ouabain-sensitive) appear to be an integral part of the resting E_m in the pulmonary arterial vessels from animals fed a high salt diet. The association between the opening of $K_{ir2.1}$ and activation of Na^+/K^+ATPase (via potassium cloud) and the ensuing hyperpolarization of vascular smooth muscle in an intact blood vessel is well documented in the literature (Edwards *et* al., 1998; Weston *et* al., 2002). It is also clear that Ba^{2+} together with ouabain but not alone produced a much greater inhibitory effect of isoprenaline evoked relaxations in tissues from rats fed a high salt diet compared to those on a regular diet. Moreover, inhibition of TASK channels by acidic buffer also produced

a greater inhibition of relaxation caused by isoprenaline in pulmonary arterial vessels from rat on a high salt diet compared to a regular diet. Taken together, such evidence could imply that TASK and the combined effects of $K_{ir2.1}$ and Na^+/K^+ ATPase act in parallel in manifestation of the hyperpolarization due to the activation of β-adrenoceptors by isoprenaline. Furthermore, it is also possible that opening of TASK might act as trigger for the activation of potassium cloud to preserve β-adrenoceptor mediated relaxations in blood vessels from animals on the high salt diet. However, based on our current electrophysiological and mechanical data, it is also clear that a limited, if any, modification of the role for TEA-sensitive channels has occurred in association with β-adrenoceptor-mediated response in tissues from rats fed a high salt diet. This is in contrast to the changes observed for the role of TASK and $K_{ir2.1}$ channels and Na^+/K^+ATPase on both mechanical and electrical functions due to the stimulation of β-adrenoceptors in blood vessels from rats on a high salt in comparison to regular diet.

In conclusion, vascular β-adrenoceptor signalling has clearly multiple electrical components in rat main pulmonary arterial vessels. The sustained hyperpolarization observed with chronic consumption of high salt diet involves the opening of leak channels (TASK) and $K_{ir2.1}$ channels and the activation of Na^+/K^+ATPase. This hyperpolarization preserves isoprenaline evoked relaxant responses in the main pulmonary artery. The vasodilatory responses to isoprenaline were not significantly changed in rats fed a high salt diet. However, they appeared to be mediated mainly by K^+ channels and Na^+/K^+ATPase rather than by NO as in the regular diet animals.

4.3. Effect of denudation of endothelium on vascular β-adrenoceptor signalling in rats fed a high salt diet.

The removal of endothelium resulted in hyperpolarization of similar magnitude in smooth muscle cells of pulmonary artery from rats fed a high salt diet compared to endothelium intact tissues from rats fed the same diet. Ba^{2+} but not acidic buffer depolarized the smooth muscle cells from denuded pulmonary artery from rats fed a high salt diet. The E_m of smooth muscle cells, following denudation of pulmonary artery of rats on either regular or high salt diet was also comparable (Table 3 and 9). In agreement with previous findings, isoprenaline did not further hyperpolarize the smooth muscle cells with E_m ranging from -67 mV to -70 mV (Table 3, 7 and 9). Isoprenaline-induced maximal response was attenuated in denuded tissues compared to intact tissues in rats fed a high salt diet. The residual isoprenaline-mediated relaxation was not mediated by TASK channels but by Ba^{2+} sensitive $K_{ir2.1}$ channels.

Replotting figures from previously mentioned data suggests that the extent of inhibition of maximal response was similar in denuded tissues from rats fed either a regular or high salt diet (Figure 23A). Despite producing significantly greater inhibition in intact pulmonary arteries from rats on a high salt diet, the inhibitory effects of acidic buffer were diminished in denuded pulmonary arteries from rats fed a regular or a high salt diet (Figure 23B). We found that, L-NAME produced similar inhibition of isoprenaline-mediated relaxant responses in tissues from rats fed a regular compared to high salt diet. The presence of L-NAME in acidic buffer produced additive inhibition that was greater in pulmonary arteries from rats on a high salt diet compared to a regular diet. This additive

effect observed in tissues from rats fed a high salt diet could be attributed to inhibition of TASK channels. Hyperpolarization recorded in the endothelium intact pulmonary artery of rats fed a high salt diet was sensitive to acidic buffer. Interestingly, in the absence of endothelium, smooth muscle cells were hyperpolarized and this event was sensitive to Ba^{2+} but not to acidic buffer in rats fed a high salt diet. Considering this evidence, it would be fair to argue that the presence of endothelium is necessary for the TASK-mediated mechanical and electrical responses in pulmonary artery of rats on either regular or high salt diet.

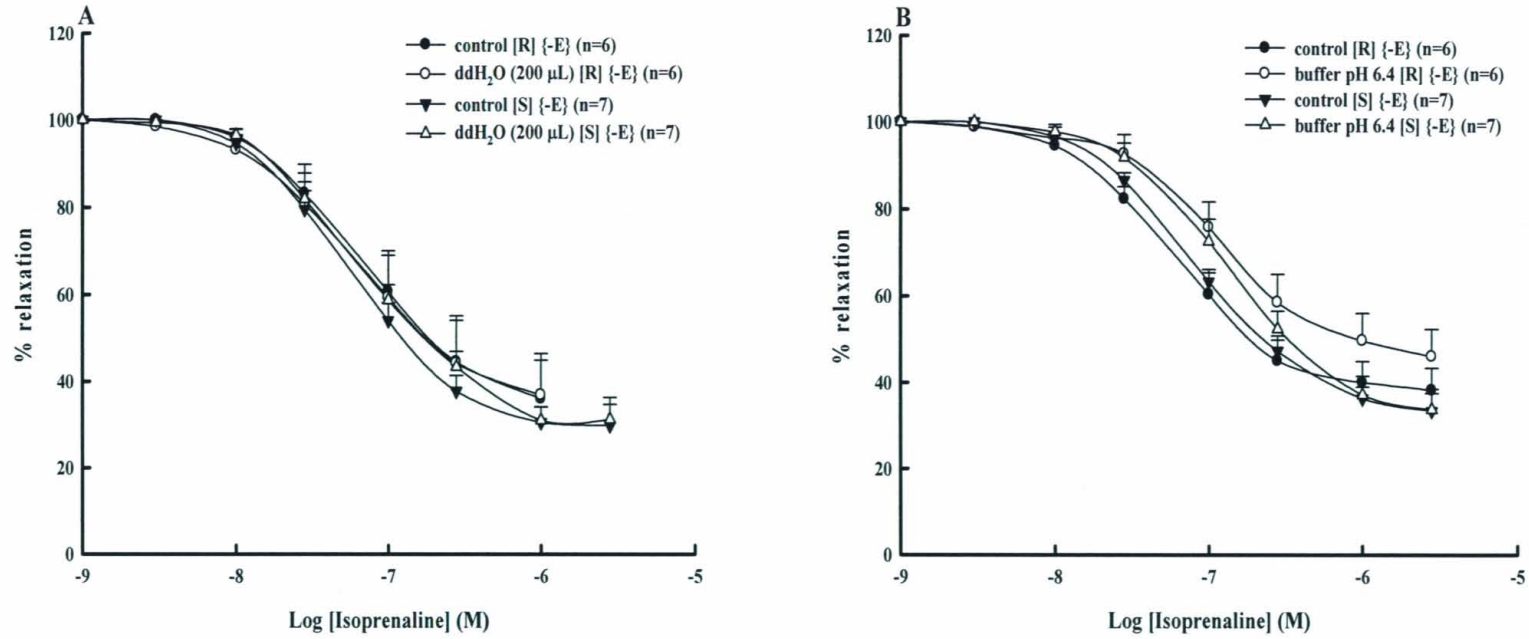

Figure 23. For discussion purposes the following figures are replotted from previously mentioned data. Concentration-response curves for isoprenaline (% relaxation remaining from phenylephrine induced sub-maximal contractions) in intact {+E} and denuded {-E} main pulmonary artery ring preparations from rats fed a regular [R] and high salt diet [S] in **A)** regular buffer (control) and twice-distilled water and; **B)** regular buffer (control) and acidic buffer. Each point represents a mean ± s.e.m. of six to seven experiments.

A major component of β-adrenoceptor signalling is Ba^{2+} sensitive alone and mutually with ouabain. It was observed that the high salt diet played a role in the over-stimulation of Ba^{2+} sensitive component of isoprenaline signalling. In study I, removal of the endothelium attenuated the Ba^{2+}-mediated inhibition of isoprenaline relaxant responses. As previously mentioned, it is possible that Ba^{2+} might also inhibit TASK channels involved in β-adrenoceptor signalling. Inhibition produced by acidic buffer and Ba^{2+} was comparable in intact and denuded pulmonary artery from rats fed a regular diet. In study II, reduction of maximal responses to isoprenaline by Ba^{2+} was of similar magnitude in intact blood vessels from rats fed a regular or high salt diet. It is also evident that the degree of inhibition of isoprenaline-induced relaxant responses with Ba^{2+} was greater accentuated in denuded pulmonary arteries from rats fed a high salt diet compared to tissue from rats fed the regular diet (Figure 24A). The presence of Ba^{2+} in acidic buffer also resulted in an additive effect in intact blood vessels from rats a fed high salt diet, and in endothelium denuded tissues from rats on either, high salt diet or regular diet (Figure 13B and 24B). It is evident that high salt diet has increased the participation of Ba^{2+} sensitive $K_{ir2.1}$ channels in β-adrenoceptor-mediated relaxation in the pulmonary artery (Figure 13AB. and 24 AB). These finding also seem to support the view that TASK channels are insensitive to the inhibitory action of Ba^{2+} (Lesage and Lazdunski, 2000).

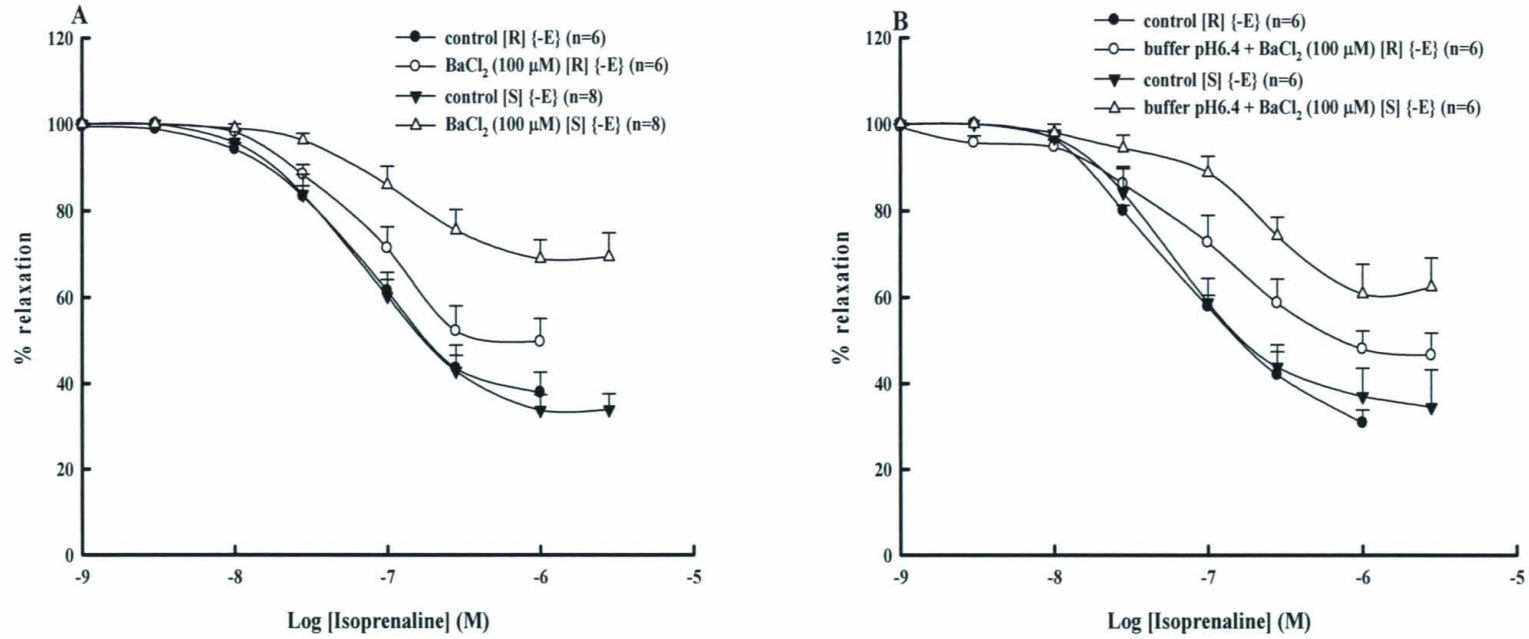

Figure 24. For discussion purposes the following figures are replotted from previously mentioned data. Concentration-response curves for isoprenaline (% relaxation remaining from phenylephrine induced sub-maximal contractions) in intact {+E} and denuded {-E} main pulmonary artery ring preparations from rats fed a regular [R] and high salt diet [S] in **A)** regular buffer absence (control) or presence of $BaCl_2$ and; **B)** regular buffer (control) or acidic buffer plus $BaCl_2$. Each point represents a mean ± s.e.m. of six to eight experiments.

It is consistent with study I and II that the "potassium clouds" theory could play an important role in vascular smooth muscle relaxation and hyperpolarization (Edward *et al.*, 1998; Weston *et al.*, 2002). The latter process may play a modest role in β-adrenoceptor mediated responses in endothelium intact pulmonary arteries of Sprague Dawley rats fed a regular diet. However, it is evident that the contribution of the $K_{ir2.1}$ and Na^+/K^+ATPase significantly increased in the pulmonary arteries from rats fed a high salt diet compared to rats fed a regular diet. Furthermore, no difference was observed in the degree of attenuation of isoprenaline mediated responses in pulmonary artery with or without endothelium from rats fed either a regular or high salt diet (Figure 25A). It is also evident that addition of ouabain and Ba^{2+} combined in acidic buffer resulted in inhibition of a similar magnitude in tissues with or without endothelium from rats fed a high salt diet (Figure 24B). Accordingly, this could suggests that removal of endothelium leads to over-activation of $K_{ir2.1}$ further causing hyperpolarization of smooth muscle cells from pulmonary arteries of rats fed a high salt diet.

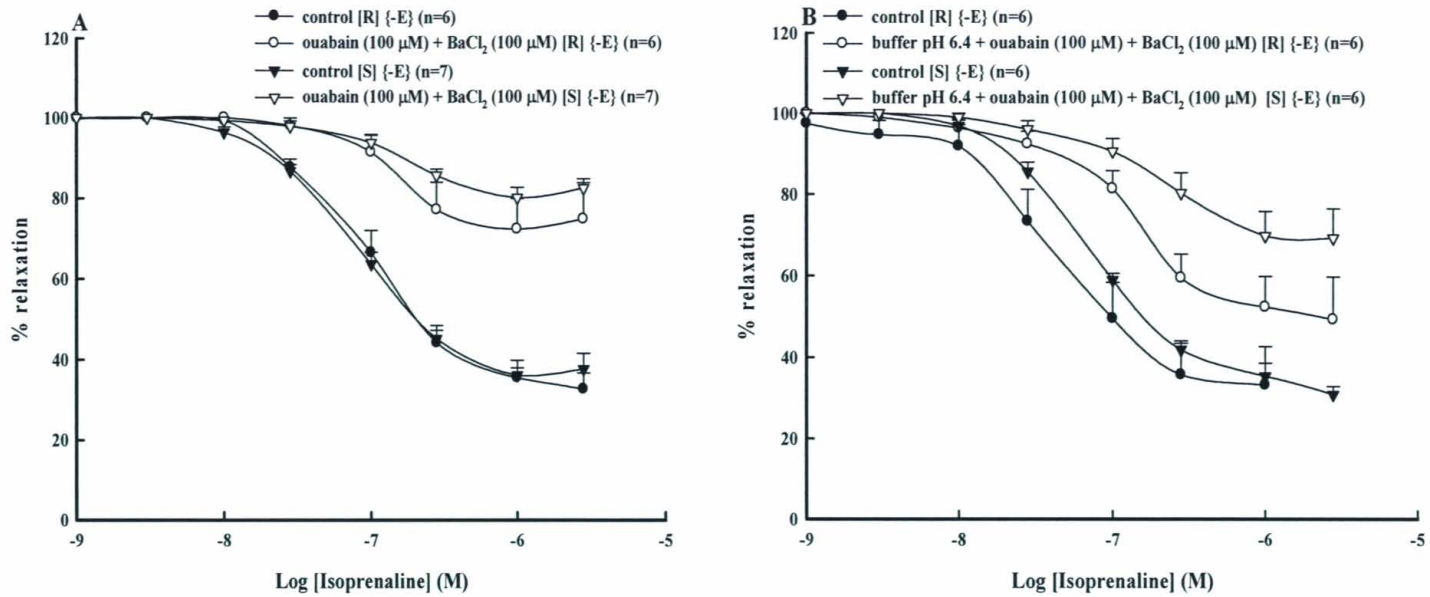

Figure 25. For discussion purposes the following figures are replotted from previously mentioned data. Concentration-response curves for isoprenaline (% relaxation remaining from phenylephrine induced sub-maximal contractions) in intact {+E} and denuded {-E} main pulmonary artery ring preparations from rats fed a regular diet [R] in **A)** regular buffer absence (control) or presence of $BaCl_2$ and ouabain together and; **B)** regular buffer (control) or acidic buffer plus $BaCl_2$ and ouabain together. Each point represents a mean ± s.e.m. of six to eight experiments.

4.4. Conclusion

The stimulation of β-adrenoceptors causes hyperpolarization of vascular smooth muscle cells. Isoprenaline-induced hyperpolarization in part contributes to the β-adrenoceptor mediated relaxation of the rat main pulmonary artery. The vascular β-adrenoceptor mediated signal transduction consists of multiple components with a sequence of events that include the activation of K^+ channels (TASK and $K_{ir2.1}$) and Na^+/K^+ATPase. The evidence implies that in the presence of endothelium, the hyperpolarization of vascular smooth muscle cells via the activation of $K_{ir2.1}$ and/or Na^+/K^+ ATPase can occur with the source of the "potassium clouds" being the smooth muscle cells, i.e. as a consequence of the activation of K^+ channels that propagate to derive the subsequent events.

Either of the treatment i.e. removal of endothelium or consumption of high salt diet shifts the E_m toward hyperpolarized state. The change of E_m to more negative potential substantially diminishes the hyperpolarization induced by isoprenaline. This suggests that there is a ceiling for isoprenaline-induced hyperpolarization i.e. if the tissue is in the range of E_K; further hyperpolarization with a β-adrenoceptor agonist may not occur.

Consumption of high salt diet has a direct impact on β-adrenoceptor signalling in rat main pulmonary arterial vessels. This change is not due to pressure nor is it seems to be dependent on electrolytes but could be due to changes in neurohumoral factors. The sustained hyperpolarization observed with chronic consumption of a high salt diet involves the opening of leak channels (TASK), $K_{ir2.1}$ channels and the activation of Na^+/K^+ATPase. This hyperpolarization preserves isoprenaline-evoked relaxant responses in the main pulmonary artery.

Stimulation of β-adrenoceptors initiates the formation of "potassium cloud" that spreads along the artery with the aid of endothelium. Endothelium plays a role of conduit for electrical coupling. The smooth muscle cell layer has been implicated as an alternative conduction pathway in the event of damage to the endothelium (Budel *et al.*, 2003). Thus it is possible that in the absence of endothelium attenuates the extent of β-adrenoceptor-mediated relaxant responses. TASK channels play an important role in β-adrenoceptors signalling in rat pulmonary artery. These leak conductances have been shown to be an important component in regulating pulmonary vascular tone (Olschewski *et al.*, 2010). This project showed that the presence of endothelium is necessary for participation of TASK in β-adrenoceptor-mediated responses. Identification of these mechanisms increases our understanding of the process involved in the blood flow to the organs and could explain how these processes can be compromised in diseases in which endothelial function is reduced.

5.0. LIMITATIONS

As with all experimental designs, a number of limitations can be identified with the present work. For example, it would have been ideal, if membrane potential was recorded simultaneously with mechanical function for complete concentration-response curve to isoprenaline. This would allow for greater understanding of the relationship between vascular hyperpolarization and relaxation. Evaluation of TASK expression on endothelial and vascular smooth muscle cells from rats fed either diet (regular or a high salt diet) could have provided additional evidence for the increased participation of the channel following high salt consumption. It would be meaningful to measure the quantity of salt consumed by rats.As well, it would be interesting to study and compare the β-adrenoceptor signalling in smaller vessels such as mesentery artery which contributes in the total peripheral vascular resistance in the systemic circulatory bed. To this end, future experiments could be aimed at investigating the changes in β-adrenoceptor-mediated electrical and mechanical responses in systemic blood vessels.

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