THE FUNCTIONAL STUDY OF Na⁺/Ca²⁺ EXCHANGER IN VASCULAR SMOOTH MUSCLE CELLS

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by

Jun Zhao

Bachelor Degree of Medicine,
Master Degree of Physiology

School of Medical Sciences
Royal Melbourne Institute of Technology
Bundoora, Victoria,
Australia

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Summary

Na⁺/Ca²⁺ exchanger (NCX) is a membrane protein which can mediate either Ca²⁺ entry (reverse mode) or exit (forward mode) in cells depending on the Na⁺ electrochemical gradient and membrane potential. The coupling ratio for NCX1 in vascular smooth muscle cell is: 3 Na⁺:1 Ca²⁺. The NCX molecule comprises nine transmembrane segments and a large intracellular hydrophilic loop. NCX can be activated by cytoplasmic Ca²⁺, and inhibited by cytoplasmic Na⁺; it can also be modulated by protein kinases and ATP on the intracellular hydrophilic loop. In vascular smooth muscle cells, the final trigger for contraction is an increase in intracellular calcium, and thus the regulation of cytosolic calcium is crucial for the cell activity. As one of the major Ca²⁺ transport systems, NCX is postulated to play a critical role in the vascular smooth muscle cell. The aims of the present study are to firstly demonstrate the functional existence of NCX in vascular smooth muscle (including aorta and arteriole); to clarify the modulation of NCX; to explore the selectivity of NCX inhibitor KB-R7943; and lastly to investigate the role of NCX in the myogenic response.

Exploration of the physiological significance of NCX requires tools to inhibit the exchanger selectively. KB-R7943 has been widely used as a NCX inhibitor. Given its mixed and contradictory reports of selectivity for NCX, the study in chapter 2 was to assess its pharmacological actions in rat aorta on a variety of Ca²⁺ dependent systems. Rat aortic rings were used. In endothelium-denuded and intact aorta with normal physiological salt solution containing Ca²⁺, KB-R7943 (10 µM) inhibited phenylephrine-
induced constriction. The phenylephrine-induced constriction in the absence of Ca\(^{2+}\) was not significantly affected by KB-R7943 (10 µM) in both endothelium-denuded and intact vessels. In endothelium denuded rat aortic rings, KB-R7943 (10 µM) significantly attenuated the constriction induced by Bay K8644, high K\(^{+}\), cyclopiazonic acid, ouabain and low-Na\(^{+}\) respectively. However, KB-R7943 at lower concentration (1 µM) exhibited negligible effects in all cases except the low Na\(^{+}\) constriction. The constriction to low Na\(^{+}\) is a functional response mediated by NCX operating in reverse mode to increase Ca\(^{2+}\) entry. The data demonstrate that 10 µM KB-R7943 affected L-type Ca\(^{2+}\) channel, the capacitative Ca\(^{2+}\) entry and α adrenergic receptor pathway. Nevertheless, KB-R7943 can be used as a selective inhibitor of NCX at the concentration of 1 µM in rat aortic rings.

Given the important role of the vascular endothelium in modulating vascular smooth muscle contractility, the aim of the study in chapter 3 was to investigate whether the operation of NCX could be affected by the endothelium in rat aortic rings using the organ bath technique. Lowering extracellular [Na\(^{+}\)] to 1.18 mM induced immediate constriction and vessel motion in endothelium denuded rat aortic rings, but only a small constriction in endothelium intact rat aortic rings. In endothelium intact rat aortic rings, the guanylate cyclase inhibitor ODQ (1 µM) and nitric oxide synthase inhibitor L-NAME (50 µM) greatly amplified the vasoconstriction to lowering extracellular Na\(^{+}\), but had no effect when the endothelium was removed. The adenylate cyclase blocker: SQ 22536 (100 µM) and the COX inhibitor indomethacin (10 µM), showed no significant effect on low-Na\(^{+}\) induced vasoconstriction in either endothelium denuded or intact aortic rings. The results suggest that endothelium modulated the NCX operation via the guanylate cyclase, not the
adenylate cyclase system; the release of nitric oxide, not prostacyclin was involved. Thus NCX may be another mechanism by which the endothelium-derived nitric oxide modulates contractility in vascular smooth muscle. The interaction between nitric oxide and NCX was further explored using the nitric oxide donor sodium nitroprusside. Endothelium denuded rat aortic rings were preconstricted to the same extent with either low Na\(^+\) (1.18 mM), or the thromboxane A\(_2\) agonist U46619 (0.1 \(\mu\)M) or high K\(^+\) (80 mM). The vasorelaxation of SNP (30 nM) in low Na\(^+\) constriction was significantly larger compared to other agents. This indicates that NO has a special antagonism of low Na\(^+\) constriction and a hypothesis is proposed involving Na\(^+\)/K\(^+\) ATPase.

The chapters above demonstrated the pharmacological functionality of NCX in arterial smooth muscle. However, the investigation of NCX is mainly conducted in large vessels; much less evidence is available for small resistance vessels. Small vessels such as arterioles exhibit a myogenic constriction in response to a pressure rise. The aim of study in chapter 4 was to explore the role of NCX on myogenic response. First order cremaster muscle arterioles were isolated, cannulated and pressurized to 70 mmHg. Reducing extracellular Na\(^+\) from 137 to 75 and then 25 mM resulted in graded vasoconstriction which was inhibited by NCX inhibitor SEA0400 (1 \(\mu\)M). Myogenic responses were induced by a transmural pressure increase from 70 to 120 mmHg, the diameter was significantly decreased and this effect was prevented by NCX inhibitor: 1\(\mu\)M SEA0400. In fura 2-AM loaded arterioles the increase of transmural pressure from 70 to 120 mmHg was accompanied by a rise in intracellular [Ca\(^{2+}\)] and this was attenuated by SEA0400 (1 \(\mu\)M). In conclusion, the myogenic response in cremaster arteriolar includes multiple
mechanisms. The underlying pathways in the literature suggest that these may involve stretch-activated channel, L-type calcium channel, store-operated Ca$^{2+}$ channel; the results of the present study suggest that the contribution from NCX through the reverse mode needs to be especially considered in cremaster arteriole.
Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; and, any editorial work, paid or unpaid carried out by a third party is acknowledged.

Jun Zhao
School of Medical Sciences
Royal Melbourne Institute of Technology
Bundoora, Victoria,
Australia
13 August, 2007
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<td>Na^{+}/Ca^{2+} exchanger</td>
</tr>
<tr>
<td>NHE</td>
<td>Na^{+}/H^{+} exchanger</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>nNOS</td>
<td>neuronal NOS</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>NSAID_{s}</td>
<td>nonsteroidal antiinflammatory drugs</td>
</tr>
<tr>
<td>NSCC</td>
<td>nonselective cation channel</td>
</tr>
<tr>
<td>ODQ</td>
<td>1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one</td>
</tr>
<tr>
<td>OO^{-} or O_{2}^{-}</td>
<td>superoxide anion</td>
</tr>
<tr>
<td>PDE</td>
<td>phosphodiesterase</td>
</tr>
</tbody>
</table>
PE = phenylephrine
PGI₂ = prostacyclin
PIP₂ = phosphatidylinositol-4,5 bisphosphate
PKA = protein kinase A
PKC = protein kinase C
PKG = protein kinase G
PLA₂ = phospholipase A₂
PLC = phospholipase C
PM = plasma membrane
PMCA pump = plasma membrane Ca²⁺ pump
PSS = physiological salt solution
ROC = receptor-operated channel
RyR = ryanodine receptor
SAC₃ = stretch-activated channels
SEA0400 = 2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline
SERCA pump = sarcoplasmic reticulum Ca²⁺ pump
sGC = soluble guanylyl cyclase
SMC = smooth muscle cell
SNP = sodium nitroprusside
SOC = store-operated Ca²⁺ channel
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>SQ 22536</td>
<td>9-(2-tetrahydrofuryl) adenine</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>TXA₂</td>
<td>thromboxane A₂</td>
</tr>
<tr>
<td>U46619</td>
<td>11α-methanoepoxy prostaglandin F₂α</td>
</tr>
<tr>
<td>VGCC</td>
<td>voltage-gated calcium channel</td>
</tr>
<tr>
<td>VSMC</td>
<td>vascular smooth muscle cell</td>
</tr>
<tr>
<td>XIP</td>
<td>exchanger inhibitory peptide</td>
</tr>
</tbody>
</table>
CHAPTER 1 GENERAL INTRODUCTION

1.1 CYTOSOLIC CALCIUM HOMEOSTASIS

1.1.1 Summary

In vascular smooth muscle cells (VSMC), vasoreactive substances exert their effects through affecting intracellular [Ca\textsuperscript{2+}] level as well as the Ca\textsuperscript{2+} sensitivity, and thus the regulation of cytosolic Ca\textsuperscript{2+} and Ca\textsuperscript{2+} sensitivity is crucial for the cell activity (Blaustein, 1989; Karaki et al., 1997; Marin et al., 1999).

Under resting conditions, the primary messenger cytosolic Ca\textsuperscript{2+} concentration is probably not higher than 100 nM, which is 10,000 times lower than that in extracellular space (Poburko et al., 2004). Upon stimulation intracellular [Ca\textsuperscript{2+}] rises up to 1-10 μM. This is partly as a result of an influx of extracellular Ca\textsuperscript{2+} through ion channels, and partly due to the release of Ca\textsuperscript{2+} from internal stores. Ca\textsuperscript{2+} activates a variety of cellular processes to induce contraction. Principal among these is the binding of Ca\textsuperscript{2+} to calmodulin (CaM) and the activation of myosin light-chain kinase (MLCK), see 1.2.2. The modulation of Ca\textsuperscript{2+} sensitivity has been shown to play a critical role in the contraction/relaxation of smooth muscle cell (refer to 1.1.4).

To induce relaxation, removal of Ca\textsuperscript{2+} from the contractile machinery is accomplished by Ca\textsuperscript{2+} resequestration into the SR, and by extrusion of Ca\textsuperscript{2+} across the plasma membrane. There are two main mechanisms to remove Ca\textsuperscript{2+} from cytoplasm. The first relies on the direct utilization of energy from ATP (the plasma membrane Ca\textsuperscript{2+} pump, and the
sarcoplasmic reticulum Ca$^{2+}$ pump), and the second uses the Na$^+$ electrochemical gradient to drive the extrusion of Ca$^{2+}$ in exchange for Na$^+$ entry (NCX) in the plasma membrane. The term superficial buffer barrier is suggested to describe the function of sarcoplasmic reticulum which is located between the plasma membrane and contractile systems and it serves as a barrier to the Ca$^{2+}$ influx as it accumulates large amount of Ca$^{2+}$ which enters the cell (Marin et al., 1999). The Ca$^{2+}$ buffering function of the cytosolic high-affinity calcium-binding proteins (e.g. calmodulin, troponin C, calpain, the annexin family and parvalbumin) is thought to play a minor role in cytosolic calcium homeostasis, and therefore will not be covered here (Carafoli, 1987).

The Ca$^{2+}$ transport systems which have been demonstrated in the plasma membrane (Carafoli, 1987; Marin et al., 1999) are the voltage-gated calcium channel, the Na$^+$/Ca$^{2+}$ exchanger (NCX), the calcium release-activated calcium channel (store-operated Ca$^{2+}$ entry), the plasma membrane Ca$^{2+}$ pumps, the nonselective cation channel and calcium-activated potassium channel (BK$_{Ca}$); and in the intracellular organelles which involve mainly the endoplasmic (sarcoplasmic) reticulum Ca$^{2+}$ pump and also Ca$^{2+}$ release from internal store via IP$_3$-receptors and ryanodine-receptors (See reviews Carafoli, 1987; Marin et al., 1999). The major systems are discussed below.

1.1.2 Ca$^{2+}$ transport systems on the plasma membrane

The principle pathways involved in the movement of Ca$^{2+}$ into and out of the cell membrane are shown in Figure 1.1.
Figure 1.1 The calcium movement across the plasma membrane in a smooth muscle cell

The major calcium transport mechanisms across the cell membrane in a smooth muscle cell are shown. $A$ indicates the Na$^+/\text{Ca}^{2+}$ exchanger (forward mode) which is discussed in 1.4; $B$ indicates the calmodulin-regulated ATP-driven plasma membrane Ca$^{2+}$ pump which is discussed in 1.1.2.4; $C$ indicates the voltage-gated calcium channel which is discussed in 1.1.2.1, see reviews (Carafoli, 1987; Marin $et$ $al.$, 1999).
1.1.2.1 Voltage-gated calcium channel (VGCC)

1.1.2.1.1 General characteristics

As implied by its name, the opening of any type of voltage-gated calcium channel (VGCC) is controlled by membrane potential. Membrane depolarization causes a structural change in the channel pore allowing $\text{Ca}^{2+}$ to flow down its concentration gradient into excitable cells. (See reviews Carafoli, 1987; Meininger et al., 1992; Wang et al., 2004). Repolarization of the membrane closes the channel. Once open, VGCC mediates the calcium influx which initiates the intracellular response such as contraction, secretion, neurotransmission and gene expression (Eisenberg et al., 2004; Godfraind, 1988; Moreno Davila, 1999). In smooth muscle cells, VGCC is well known as a critical determinant of vascular tone (Beech, 1997; Carafoli, 1987; Carbone et al., 2001; Knot et al., 1998; Meininger et al., 1992).

The VGCC family shares a general structure of a heteromultimer that is formed of four or five distinct subunits ($\alpha_1$, $\beta$, $\alpha_2$$\delta$, and $\gamma$) (Bielefeldt, 1999; Keef et al., 2001; Wang et al., 2004), and which are encoded by multiple genes (see Table 1.1). The $\alpha_1$ subunit, the largest subunit (about 190-240 kDa), controls the properties of conduction pore, voltage sensor, and drug/toxin binding site (Birnbaumer, 1998; Halling et al., 2005; Striessnig, 1999; Wang et al., 2004). The accessory (auxiliary) subunits include cytoplasmic globular $\beta$ subunit and
extracellular $\alpha_2,\delta$ subunit (Keef et al., 2001). In skeletal muscle cells, a distinctive $\gamma$ subunit is exclusively expressed. (See reviews Black, 2003; Striessnig, 2004). All the subunits work in concert as a whole functional channel complex.

Calcium currents recorded in different cell types have diverse physiological and pharmacological properties, and an alphabetical nomenclature has evolved for the distinct classes: L-type, T-type, N-type, P/Q-type, and R-type (Carbone et al., 2001; Striessnig, 1999). Each class has distinct $\alpha$ subunits (Keef et al., 2001). The properties of different calcium channels are summarized in Table 1.2.

1.1.2.1.2 Subtypes of VGCC

The predominant type in smooth muscle cell is the long lasting L-type calcium channel (LTCC); the isoform for smooth muscle cell is Ca$_{\alpha,1.2}$ (Table 1.1). LTCC requires a strong depolarization for activation; for example, the activation voltage is -4 mV (in 15 mM Ba$^{2+}$, HEK cells) for Ca$_{\alpha,1.2}$ (see review Catterall et al., 2005). LTCC differs from other types of VGCC (non-LTCC) in the unique feature that the organic L-type calcium channel antagonists, including dihydropyridine (DHP), phenylalkylamine, and benzothiazepine can block it (Striessnig, 2004). There are four isoforms (Ca$_{\alpha,1.1}$-Ca$_{\alpha,1.4}$), their electrophysiological properties and tissue expressions are summarized in Table 1.1. N-type, P/Q-type, and R-type calcium currents do not exist in smooth muscle cells, they are expressed primarily in neurons (Beech, 1997). They also require strong depolarization for activation, for example, the activation voltage is +7.8 mV for N-type in HEK 293 cells; -5
mV for native P-type; -11 mV for native Q-type; and +3.5 mV for R-type in HEK 293 cells (see review Catterall et al., 2005). They are blocked by specific polypeptide toxins from snail and spider venoms, such as ω-conotoxin GVIA, ω-agatoxin IVA & IVB, ω-conotoxin MVIIC, Ni^{2+}, SNA-482 etc.

The transient T-type calcium current is also expressed in smooth muscle cells (Ca_{3.1} and Ca_{3.2} only) (see reviews Bielefeldt, 1999; Catterall et al., 2005), as well as many other types of tissues (see Table 1.2). It is activated by weak depolarization, for example, the activation voltage is -44 ~ -46 mV for three isoforms of T-type VGCC: Ca_{3.1} Ca_{3.2} and Ca_{3.3}. In vascular smooth muscle cell, T-type calcium channels do not contribute considerably to vasoconstriction (Cribbs, 2006).

1.1.2.1.3 Regulation of VGCC

VGCC is critical in the activity of cells and as such are tightly modulated by a variety of intracellular signaling processes which is briefly introduced below. Because the predominant type in smooth muscle cells is the L-type calcium channel, we focus our attention on this type of VGCC.

Ca^{2+} has dual effects on VGCC, Ca^{2+}-dependent facilitation (CDF) is a process by which increased basal Ca^{2+} or repeated transient depolarization induce increased channel opening (Anderson, 2001; Halling et al., 2005). The repolarization closes the channel which can takes place in a voltage-dependent or Ca^{2+}-dependent manner (Halling et al., 2005; Sanchez et al., 2001). Ca^{2+}-dependent inactivation (CDI) is the mechanism that closes the channel during
Table 1.1 The features of distinctive L-type calcium channel

<table>
<thead>
<tr>
<th>Channel name</th>
<th>α1 subunit isoform</th>
<th>Tissue expression</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca_{1.1}</td>
<td>α1S</td>
<td>Skeletal muscle</td>
<td>Excitation-contraction (EC) coupling and Ca(^{2+}) homeostasis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>transverse tubule</td>
<td></td>
</tr>
<tr>
<td>Ca_{1.2}</td>
<td>α1C</td>
<td>Cardiomyocyte, SMC, neuron, sensory and endocrine cell</td>
<td>EC coupling, synaptic plasticity, hormone secretion, action potential propagation</td>
</tr>
<tr>
<td>Ca_{1.3}</td>
<td>α1D</td>
<td>Sensory and endocrine cells, low density in heart and VSMC, neuron</td>
<td>Neurotransmitter release, synaptic plasticity, hormone secretion, control cardiac rhythm and conductance at rest</td>
</tr>
<tr>
<td>Ca_{1.4}</td>
<td>α1F</td>
<td>Retinal photoreceptor and ganglion cell</td>
<td>Neurotransmitter release in retinal cell</td>
</tr>
</tbody>
</table>

For the references in the Table 1.1 above, please refer to the review papers (Halling et al., 2005; Striessnig, 1999) and the tenth edition of 1999 Receptor & Ion Channel Nomenclature Supplement (compiled by S.P.H. Alexander & J. A. Peters).
<table>
<thead>
<tr>
<th>Type of VGCC</th>
<th>T(Ca,3.1-Ca,3.3)</th>
<th>L(Ca,1.1-Ca,1.4)</th>
<th>N(Ca,2.2)</th>
<th>P/Q(Ca,2.1)</th>
<th>R(Ca,2.3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current</td>
<td>Ir</td>
<td>I_L</td>
<td>I_N</td>
<td>I_P/Q</td>
<td>I_R</td>
</tr>
<tr>
<td>Conductance (pS)</td>
<td>5-9 pS</td>
<td>25 pS</td>
<td>20 pS</td>
<td>9, 14, 19</td>
<td>-</td>
</tr>
<tr>
<td>Agonists</td>
<td>-</td>
<td>DHP, phenylalkylamine, benzothiazepine</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Blockers</td>
<td>Mibefradil, Ni^{2+}, kurtoxin</td>
<td>Nifedipine, diltiazem, verapamil, calciseptine</td>
<td>toxins from snail and spider venoms</td>
<td>toxins from snail and spider venoms</td>
<td>toxins from snail and spider venoms</td>
</tr>
<tr>
<td>Activation Voltage</td>
<td>Low</td>
<td>High</td>
<td>High</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Location</td>
<td>Various types of tissues</td>
<td>Mainly muscle and endocrine cells</td>
<td>Mainly neurons</td>
<td>Mainly neurons</td>
<td>Mainly neurons</td>
</tr>
</tbody>
</table>

For the content in the Table 1.2 above, please refer to the review papers (Grassi et al., 2004; Halling et al., 2005; Striessnig, 1999) and the tenth edition of 1999 Receptor & Ion Channel Nomenclature Supplement (compiled by S.P.H. Alexander & J. A. Peters).
sustained depolarization (Halling et al., 2005; Keef et al., 2001; Pott et al., 2006). CaM is involved in both Ca\(^{2+}\)-dependent inactivation and Ca\(^{2+}\)-dependent facilitation processes (Anderson, 2001; Halling et al., 2005).

In cardiac myocytes, L-type calcium channel opening can be dramatically potentiated by sympathetic nerve or sympathomimetic drugs stimulation via \(\beta\)-adrenergic receptors and related cAMP-dependent phosphorylation by protein kinase A (cAMP/PKA) (Carbone et al., 2001; Godfraind, 1988; Schroder et al., 2003; van der Heyden et al., 2005). However, the action of \(\beta\)-adrenergic receptors on L-type calcium channel in smooth muscle cell remains a matter of debate (Beech, 1997; Keef et al., 2001), and mainly inhibitory, as recently reported in guinea-pig detrusor smooth muscle cells (Kobayashi et al., 2003).

Activation of cGMP-dependent protein kinase (PKG) in smooth muscle cells was reported to produce inhibitory effects on L-type calcium channel in rat pulmonary artery, the A7\(\gamma\)5 SMC line, and rabbit portal vein. (See reviews Beech, 1997; Keef et al., 2001). Since NO stimulates cGMP production, this process is a potential mechanism whereby NO induces vasorelaxation. (See reviews Fischmeister et al., 2005; Grassi et al., 2004; Mironov et al., 2006; Schroder et al., 2003).

Protein kinase C (PKC) was reported to mostly stimulate VGCC, and it was suggested to be the underlying mechanism of a variety of hormones (including endothelin, norepinephrine, serotonin, arginine-vasopressin and angiotensin II), as well as intracellular ATP to cause
vasoconstriction. (See reviews Beech, 1997; Keef et al., 2001; Striessnig, 1999; van der Heyden et al., 2005). For example, PKC was reported to be activated by the Ca$^{2+}$ entry through VGCC, and in turn stimulates the activity of VGCC in porcine coronary artery (Korzick et al., 2004). However, the inhibitory effects of PKC on VGCC were also demonstrated, such as a recent study in rat pheochromocytoma cell line (PC12 cells) (Fontainhas et al., 2005) (see also reviews Beech, 1997; Striessnig, 1999).

The conflicting effect of each modulator might be due to the intracellular calcium concentration (Anderson, 2001; Beech, 1997; Halling et al., 2005; Sanchez et al., 2001), the species, and the experimental condition (Beech, 1997).

1.1.2.2 Na$^{+}$/Ca$^{2+}$ exchanger (NCX)

The plasma membrane Na$^{+}$/Ca$^{2+}$ exchanger is discussed at length in section 1.4.8. It can transport Ca$^{2+}$ in one direction coupled with the transport of Na$^{+}$ in the opposite direction. It operates in parallel with several Ca$^{2+}$ transport systems in the plasma membrane (e.g., the plasma membrane Ca$^{2+}$ pump and voltage-gated Ca$^{2+}$ channels), and in series with other Ca$^{2+}$ transport systems that are located in organellar membranes (e.g., SR/ER Ca$^{2+}$ pumps, mitochondrial Ca$^{2+}$ transport) in the regulation of calcium level (see reviews Annunziato et al., 2004; Blaustein, 1989).
1.1.2.3 Store-operated Ca\(^{2+}\) entry

Store-operated Ca\(^{2+}\) entry involves the regulation of plasma membrane Ca\(^{2+}\) channels by the filling state of intracellular Ca\(^{2+}\) stores in the sarcoplasmic reticulum (SR) or endoplasmic reticulum (ER). In other words, the depletion of intracellular stores due to the action of IP\(_3\) or other Ca\(^{2+}\)-releasing signals activates a signaling pathway leading to the opening of some plasma membrane Ca\(^{2+}\) channels. Please refer to 1.3 for details and references.

1.1.2.4 Plasma membrane Ca\(^{2+}\) pump (PMCA pump)

The plasma membrane Ca\(^{2+}\) pump, also called plasma membrane Ca\(^{2+}\)-ATP\(_\text{ase}\) has the function to pump Ca\(^{2+}\) out of the cell and is driven by ATP (Furukawa et al., 1988). It is a multigene family consisting of at least four gene products: PMCA1, PMCA\(^2\), PMCA3, PMCA4. The plasma membrane Ca\(^{2+}\)-ATP\(_\text{ase}\) has the role of counteracting the Ca\(^{2+}\) influx due to electrochemical gradient in order to maintain a steady state with regard to the total intracellular Ca\(^{2+}\) content. The electroneutral Ca\(^{2+}\) efflux of this pump is accomplished via the coupling influx of two H\(^+\) (see review Carafoli, 1987).

1.1.2.5 Other Ca\(^{2+}\) transport channels on the plasma membrane

Ca\(^{2+}\)-activated potassium channels are believed to play an important role in the modulation of vascular smooth muscle cell (VSMC) relaxation (see reviews Ghatta et al., 2005; Ledoux et al., 2006). Ca\(^{2+}\)-activated potassium channel is classified into three categories according to
its conductance: large-conductance (100-300 pS, BKCa), intermediate-conductance (25-100 pS, IKCa) and small-conductance (2-25 pS, SKCa) (see also review Ghatta et al., 2005; Sollini et al., 2002). BKCa exists in vascular smooth muscle, and is activated by increased intracellular Ca2+ or membrane depolarization (see reviews Cox, 2002; Quast et al., 1994). In VSMC, Ca2+ entry via the opening of VGCC has the potential to activate BKCa which induces membrane hyperpolarization and the inhibition of VGCC as a negative feedback system (see reviews Ghatta et al., 2005; Ledoux et al., 2006)(Nishimura, 2006).

Calcium permable channels include not only voltage-dependent Ca2+ channels, but voltage-independent Ca2+ channels as well, such as the nonselective cation channel (NSCC) which is poorly selective for Ca2+, Na+ and other cations (Albert et al., 2002b; Matsuoka et al., 1997b; Trepakova et al., 2001). The store-operated channel (SOC), stretch-activated channels (SAC) and receptor-operated channel (ROC) are members of the superfamily of voltage-independent Ca2+ channels which are believed to be encoded by transient receptor potential (TRP) genes (See reviews Belkacemi et al., 2005; Dietrich et al., 2006; Inoue et al., 2002; Okuhara et al., 2007; Remillard et al., 2006)(Tiruppathi et al., 2006; Tiruppathi et al., 2002). The voltage-independent Ca2+ channels also contribute to the control of vascular smooth muscle contraction, cell proliferation and migration (Okuhara et al., 2007; Remillard et al., 2006; Yao, 2007).
1.1.3 Intracellular Ca\textsuperscript{2+} transport systems

1.1.3.1 Calcium release from the sarcoplasmic reticulum (SR)

The sarcoplasmic reticulum in excitable cells or the endoplasmic reticulum (ER) in nonexcitable cells are the main intracellular Ca\textsuperscript{2+} storage organelles from which Ca\textsuperscript{2+} can be released to increase the [Ca\textsuperscript{2+}]\textsubscript{i} (Marin et al., 1999). In the SR, there are two types of Ca\textsuperscript{2+} channels: one is sensitive to IP\textsubscript{3}, and the other sensitive to ryanodine and caffeine.

(1) Inositol 1,4,5-trisphosphate receptor (IP\textsubscript{3}R)

The inositol 1,4,5-trisphosphate (IP\textsubscript{3}) is produced through the classical inositol lipid pathway (PLC-IP\textsubscript{3} pathway). Plasma membrane phospholipase C (PLC) hydrolyzes the phosphatidylinositol-4,5 bisphosphate (PIP\textsubscript{2}) that presents in the inner cell membrane of lipid bilayer and forms two important secondary messengers: the diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP\textsubscript{3}) (Rosendorff, 1997). The hydrophilic IP\textsubscript{3} diffuses into the cytoplasm and interact with the IP\textsubscript{3}-sensitive Ca\textsuperscript{2+} channel in the SR membrane causing the calcium release from the SR or ER (Marin et al., 1999). The hydrophobic DAG remains in the plasma membrane, and together with Ca\textsuperscript{2+}, activates protein kinase C. The major steps of PLC-IP\textsubscript{3} pathway is depicted in Figure 1.2.

The activation of cell-surface receptors in the plasma membrane by agonists, such as noradrenaline, acetylcholine, or serotonin evokes interdependent and closely coupled
biphasic increase in cytosolic free $\text{Ca}^{2+}$. The rapid phase reflects $\text{Ca}^{2+}$ release from internal stores upon stimulation of IP$_3$R, whereas the sustained phase is due to calcium influx into the cell (Elliott, 2001; Laher et al., 1989). The quantal $\text{Ca}^{2+}$ release from ER or SR store through IP$_3$R causes depletion of the store and induces $\text{Ca}^{2+}$ entry from the extracellular space, is referred to store-operated $\text{Ca}^{2+}$ influx (formerly capacitative $\text{Ca}^{2+}$ entry, CCE), please refer to 1.3 for details and references.

(2) Ryanodine receptor (RyR)

Ryanodine is a neutral plant alkaloid and a naturally occurring insecticide. RyR forms a family of intracellular $\text{Ca}^{2+}$ release channels on the ER/SR that can be activated and opened by ryanodine, caffeine and micromolar order of $\text{Ca}^{2+}$. Once open, it induces calcium release from internal store, and it is responsible for $\text{Ca}^{2+}$--induced $\text{Ca}^{2+}$ release in some tissues (Bolton, 2006; Dabertrand et al., 2006; Karaki et al., 1997; Wang et al., 2004; Zhang et al., 2006). Its function can be inhibited by ruthenium red and millimolar $\text{Ca}^{2+}$ (Marin et al., 1999).
Figure 1.2  The modulation of contractile system by the classical inositol lipid pathway (PLC-IP₃ pathway)

This above graph depicts the PLC-IP₃ pathway which is discussed in section 1.1.3.1(1). PLC breaks down PIP₂ into IP₃ and DAG. IP₃ acts on the IP₃ receptor on the SR and induces the Ca²⁺ release from SR. DAG activates PKC in the presence of Ca²⁺, and then regulates the activity of contractile system.
1.1.3.2 The sarcoplasmic reticulum Ca\(^{2+}\) pump (SERCA pump)

The SERCA pump is a protein which exists in the SR membrane, has a high affinity for Ca\(^{2+}\) and can transport significant amounts of intracellular Ca\(^{2+}\) into the lumen of the SR in an ATP-dependent manner. This is the principal way in which Ca\(^{2+}\) is sequestered into internal stores. For each ATP molecule hydrolyzed, two Ca\(^{2+}\) ions are sequestered to the SR. The activity of the SERCA pump is tightly controlled by phosphorylation of phospholamban (Marin et al., 1999). Phospholamban is believed to attach to the pump with the stoichiometry of 1:1, and to inhibit it. Upon phosphorylation of phospholamban by cAMP-dependent and/or Ca\(^{2+}\)-calmodulin dependent protein kinases, phospholamban detaches from the pump and activates it. (See review Carafoli, 1987).

The inhibitors of the SERCA pump, such as thapsigargin, cyclopiazonic acid (CPA), and caffeine can block the pump, deplete the internal Ca\(^{2+}\) store, and therefore increase the cytosolic Ca\(^{2+}\) concentration (Holzapfel, 1968; Luo et al., 2000; Mitamura et al., 2002; Seidler et al., 1989).

1.1.3.3 Mitochondrion

The role of mitochondria in the regulation of calcium homeostasis is less important and not well elucidated. However, there are reported activities for mitochondria in the calcium
transport. There is uptake of Ca$^{2+}$ into the matrix of mitochondria through a Ca$^{2+}$ uniporter (Crompton, 1985; Gunter et al., 1990). There are also suggested mechanisms by which calcium efflux from the mitochondrial matrix may occur: Na$^+$-Ca$^{2+}$ carrier, Na$^+$-independent efflux mechanism, and a Ca$^{2+}$-induced increase of membrane permeability (Crompton, 1985; Gunter et al., 1990). It is generally believed that mitochondria play no central role in the regulation of cytosolic Ca$^{2+}$ homeostasis (Marin et al., 1999).

1.1.4 Ca$^{2+}$ sensitivity of the contractile apparatus

The contraction induced by some agonists (such as phenylephrine and U46619) can occur without a change in [Ca$^{2+}$], indicating that their action involves an increased sensitivity of the contractile process for Ca$^{2+}$ (See reviews Himpens, 1992; Salamanca et al., 2005). The ratio of myosin light chain kinase to myosin light chain phosphatase activity affects the sensitivity to Ca$^{2+}$. The higher the ratio the more sensitive the contractile apparatus to Ca$^{2+}$. It is suggested that the underlying mechanism for increasing Ca$^{2+}$ sensitivity might include Rho-kinase and PKC (See reviews Dallas et al., 2003; Himpens, 1992; Salamanca et al., 2005) (Rikitake et al., 2005).

The protein kinase C (PKC) family includes three major groups. The conventional PKCs (cPKC): $\alpha$, $\beta$I, $\beta$II, and $\gamma$, are Ca$^{2+}$-dependent; the novel PKCs (nPKC): $\delta$, $\epsilon$, $\eta$(L) and $\theta$, are Ca$^{2+}$-independent and the atypical PKCs (aPKC): $\zeta$ and $\iota$, are also Ca$^{2+}$-independent (Horowitz et al., 1996; Salamanca et al., 2005). A variety of excitatory agonists bind to G-protein coupled receptors and induces biphasic tonic constriction in smooth muscle. The initial rapid phasic component is believed to be induced by Ca$^{2+}$ released from SR by inositol 1,4,5-trisphosphate (IP$_3$) after cleavage of PIP$_2$ by phospholipase C. The sustained phasic
contraction is maintained by the increased Ca^{2+} sensitivity via the inhibition of myosin light chain kinase phosphatase (MLCP) by PKC and Rho kinase (See reviews Dimopoulos et al., 2007; Hirano et al., 2004; Lincoln, 2007; Salamanca et al., 2005).

MLCP is comprised of a catalytic subunit, classified as a PP1c δ isoform; a myosin binding regulatory subunit known as MYPT1 and a third subunit whose function remains unclear (See reviews Dimopoulos et al., 2007; Lincoln, 2007). There are two pathways that inhibit MLCP, one is the phosphorylation of MYPT1 (Thr696 and Thr853 sites), and the other is the phosphorylation of MLCP inhibitor protein CPI-17 (See review Hirano et al., 2004). Several kinases were proposed to mediate the above pathways, the most investigated are protein kinase C (See reviews Dallas et al., 2003; Dimopoulos et al., 2007; Himpens, 1992; Lincoln, 2007; Salamanca et al., 2005; Walsh et al., 1996) and the small molecular weight G-protein, RhoA which activates Rho-activated kinase (ROK) (See reviews Dimopoulos et al., 2007; Lincoln, 2007; Murthy, 2006)(Hu et al., 2005).

On the other hand, the process of desensitization is believed to be mediated by cGMP which activates PKG leading to the dephosphorylation of CPI-17 and the phosphorylation of MYPT1 (S695 site) (See reviews Dimopoulos et al., 2007; Lincoln, 2007).
1.2 MECHANISM OF CONTRACTION AND RELAXATION IN VASCULAR SMOOTH MUSCLE CELLS

1.2.1 Structure of smooth muscle cells (SMC)

Smooth muscle locates chiefly in the walls of hollow organs, and is controlled involuntarily. Smooth muscle fibers are small, spindle shaped uninucleate cells and display no striation. They contain thick filament (myosin), thin filament (actin) and non-contractile intermediate filament (Ham, 1969; Shepro et al., 1974; Tortora et al., 1981).

1.2.2 Mechanism of contraction in the smooth muscle cell

The mechanism of contraction in the smooth muscle cell parallels that of skeletal muscle. The common features are: (1) The contraction is triggered by the rise of intracellular Ca\(^{2+}\); (2) actin and myosin interact via the sliding mechanism; (3) The sliding process is fueled by ATP (Allen et al., 1994; Murphy et al., 2002a; Zou et al., 1995; Zou et al., 2000).

However, the smooth muscle cell is distinctive in many ways. In SMC, Ca\(^{2+}\) triggers muscle contraction by calmodulin-dependent myosin light chain kinase (MLCK) and subsequent phosphorylation of the 20-kDa regulatory myosin light chains (Allen et al., 1994; Murphy et al., 2002a; Zou et al., 1995).

The major steps of contraction in SMC are as follows (Lincoln et al., 2001): (1) Ca\(^{2+}\) binds to calmodulin and activates it; (2) activated calmodulin activates the kinase enzyme in the
myosin filament: MLCK; (3) phosphorylation of the 20-kDa regulatory myosin light chains; (4) cross-bridge cycling and the force generation. The sequence of events during contraction is shown in Figure 1.3.

When \([\text{Ca}^{2+}]\) drops, the myosin binding sites on actin are physically blocked by tropomyosin molecules, and the resulting myosin dephosphorylation by MLC phosphatase causes relaxation (Allen et al., 1994).
Figure 1.3 The sliding filament model of muscle contraction

A. ATTACHED-myosin head (cross bridge) attaches to an actin filament.

B. FORCE GENERATING-myosin head pivots and causes the movement of the actin filament, two types of filaments slide against each other. Muscle contraction takes place.

C. RELEASED-a new ATP molecule binds to myosin head, cross bridge detaches.

D. COCKED-ATP hydrolyzes to ADP and Pi, myosin head cocks.
1.3 STORE-OPERATED Ca\(^{2+}\) CHANNELS (SOC)

It is firmly established that depletion of intracellular calcium stores in the SR/ER in a wide range of cells could activate Ca\(^{2+}\) influx into the cell through store-operated Ca\(^{2+}\) channels (SOC), which is termed capacitative Ca\(^{2+}\) influx (See reviews Arnon et al., 2000; Bolotina, 2004; Parekh et al., 1997a; Venkatachalam et al., 2002). These plasma membrane Ca\(^{2+}\) channels are regulated by the filling state of intracellular Ca\(^{2+}\) stores in the sarcoplasmic reticulum (SR) or endoplasmic reticulum (ER). This Ca\(^{2+}\) current was termed Ca\(^{2+}\)-release-activated Ca\(^{2+}\) current (I\(_{\text{CRAC}}\)) (See reviews Hoth et al., 1992; Parekh et al., 1997a).

The SOC is believed to be encoded by transient receptor potential (TRP) genes (Trepakova et al., 2001), (See also reviews Dietrich et al., 2006; Inoue et al., 2002; Okuhara et al., 2007; Remillard et al., 2006) (Tiruppathi et al., 2006; Tiruppathi et al., 2002). In comparison to the clear identity of SOC, the mechanism linking depletion of intracellular Ca\(^{2+}\) stores to the activation of plasma membrane Ca\(^{2+}\) channels remains a mystery. There is accumulating evidence for a conformational coupling theory. It suggests that inositol 1,4,5-trisphosphate (IP\(_3\)) receptor (IP\(_3\)R) is coupled to the plasma membrane Ca\(^{2+}\) entry channel. Depletion of intracellular Ca\(^{2+}\) stores induces a conformational change in IP\(_3\)R, which interacts with and activates a plasma membrane Ca\(^{2+}\) channel (See reviews Bolotina, 2004; Parekh et al., 1997b; Putney et al., 2001). This pathway is IP\(_3\)-dependent, no store depletion is needed. However, there is also a convincing body of evidence indicating that the aforesaid mechanism can not account for the activation of store-operated channels in all circumstances. Thus, the pathway by a diffusible Ca\(^{2+}\)-influx factor, Ca\(^{2+}\)-independent phospholipase A\(_2\) (iPLA\(_2\)) and its lysophospholipid products was suggested (See reviews
Putney et al., 2001; Smani et al., 2003). Apparently, IP$_3$ is not needed for this mechanism. This can explain why SOC operates normally in the triple IP$_3$R knockout DT40 cells that lack IP$_3$R1, IP$_3$R2, and IP$_3$R3 (Bakowski et al., 2001; Ma et al., 2001; Prakriya et al., 2001). Taken together, one tentative conclusion is that there is a co-existence of two functionally distinct mechanisms with regard to the activation of SOC in excitable and nonexcitable cells.

Thapsigargin, cyclopiazonic acid (CPA), and 2,5-di-(tert-butyl)-1,4-benzohydroquinone (tBHQ) are inhibitors of ATP-dependent Ca$^{2+}$ pumps of the sarcoplasmic reticulum or endoplasmic reticulum, and can thereby be employed to activate SOC by depleting the Ca$^{2+}$ stores (Gomez-Viquez et al., 2005; Liu et al., 2006; Lopes et al., 2006; Mitamura et al., 2002; Sohoel et al.).

1.3.1 Store-operated Ca$^{2+}$ channels in nonexcitable cells

In most non-excitable cells like epithelial cells, endothelium cells, lymphocytes, and hepatocytes, the activation of cell-surface receptor stimulates inositol 1,4,5-trisphosphate (IP$_3$) production and induce interdependent and closely coupled biphasic cytosolic calcium signal (Elliott, 2001). The rapid phase reflects Ca$^{2+}$ release from internal stores, whereas the sustained phase is due to Ca$^{2+}$ influx from the extracellular space. The release from ER stores is believed to be mediated by IP$_3$ receptors or other Ca$^{2+}$-releasing signals leading to the opening of plasma membrane store-operated Ca$^{2+}$ channels (See reviews Elliott, 2001; Parekh et al., 1997b; Putney et al., 2001). Therefore, the SOC is the dominant calcium entry pathway in nonexcitable cells. Growing evidence describes a highly calcium-selective current in nonexcitable cells, which is called Ca$^{2+}$-SOC (See reviews Bolotina, 2004; Hoth et
Ca\textsuperscript{2+}-SOC might be the mechanism by which nonexcitable cell maintains raised intracellular calcium level and refills the empty calcium store.

As the main calcium entry pathway in non-excitable cells such as mast cells, rat basophilic leukemia cells and Jurkat T cells, the SOC modulates diverse processes, such as, exocytosis, enzyme control, gene regulation, cell proliferation and apoptosis (See reviews Hoth et al., 1992; Parekh et al., 1997a; Parekh et al., 1997b).

1.3.2 Store-operated Ca\textsuperscript{2+} channels in excitable cells

There is very limited information with respect to the SOC in excitable cells due to the difficulty of electrophysiological recording of SOC (McFadzean et al., 2002).

In excitable cells like neurons, muscle, and endocrine cells, it is widely agreed that the dominant calcium entry pathway is the well-characterized voltage-operated calcium channel (See reviews Carafoli, 1987; Godfraind, 1988; IUPHAR, 2005; Poburko et al., 2004).

Characteristics of SOC in excitable cell: SOC in nonexcitable cell is highly selective for Ca\textsuperscript{2+}. However, the SOC in excitable cells is poorly selective to cation channel (Cat-SOC), and permeable to Ca\textsuperscript{2+} as well as Na\textsuperscript{+} (See reviews Albert et al., 2003; Bolotina, 2004). Na\textsuperscript{+} entry through Cat-SOC might act as a trigger to evoke secondary calcium influx through NCX by elevating intracellular Na\textsuperscript{+} (See reviews Arnon et al., 2000; Bolotina, 2004). In addition, Cat-SOC was shown to be activated via G-protein-coupled receptors, instead of IP\textsubscript{3} receptor on SR. In this case, Cat-SOC is therefore a receptor-operated channel (ROC)
The direct function of SOC in excitable cells is replenishing the depleted calcium store stimulated by the G-protein-coupled receptors after agonist application. Indirectly, it is involved in the regulation of contraction and cell proliferation (Albert et al., 2003; Arnon et al., 2000).
1.4 \textbf{Na}^+/{\textit{Ca}}^{2+} \textbf{EXCHANGER}

1.4.1 General introduction of Na$^+$/Ca$^{2+}$ exchanger

The Na$^+$/Ca$^{2+}$ exchanger (NCX) is an integral membrane protein responsible for the countertransport of Na$^+$ and Ca$^{2+}$ across the plasma membrane of virtually all animal cells. It is one of several mechanisms that mediate Ca$^{2+}$ flow across the plasma membrane. It acts in series with the plasma membrane ATP-driven Ca$^{2+}$ pump to extrude Ca$^{2+}$. As a reversible transporter, it can, under some circumstances, mediate Ca$^{2+}$ entry in parallel with various ion channels. The energy and direction for net Ca$^{2+}$ movement across the NCX depends on the electrochemical gradient of Na$^+$, Ca$^{2+}$, and K$^+$, the membrane potential, and the transport stoichiometry. (For review, see Philipson, 2002).

1.4.2 The structure of NCX

In 1988, the first cardiac NCX (NCX1) was isolated (Philipson \textit{et al.}, 1988). In 1990, the first NCX1 was cloned and sequenced from cardiac sarcolemma (Nicoll \textit{et al.}, 1990). Each functional NCX1 isoform comprises nine transmembrane segments and a large intracellular hydrophilic loop between the fifth and sixth transmembrane segments (Figure 1.4) (Philipson \textit{et al.}, 2000; Shigekawa \textit{et al.}, 2001). This replaces the initial suggestion of twelve transmembrane segments which include the signal peptide (Philipson \textit{et al.}, 2000). The glycosylated N-terminal includes five transmembrane segments, and the C-terminal the other four transmembrane segments. The protein contains two internal repeat sequences (α-1 and
α-2) that are highly conserved in all members of NCX family and critically involved in ion transportation (Nicoll et al., 1996a; Shigekawa et al., 2001). The mature NCX1 protein is composed of 938 amino acids after the cleaving of the first 32 amino acid-long signal peptide. The cytoplasmic loop (about 550 amino acids long) is a Ca\(^{2+}\) binding site (also called Ca\(^{2+}\) regulatory site) and does not participate in ion transportation, but is involved in the regulation of NCX by intracellular Na\(^{+}\), Ca\(^{2+}\) and PIP\(_2\) (Maack et al., 2005).

1.4.3 Isoforms of NCX

Nine genes that code for the NCX have been identified in mammals: three in the Na\(^{+}/Ca^{2+}\) exchanger family: NCX1 (Nicoll et al., 1990), NCX2 (Li et al., 1994), and NCX3 (Nicoll et al., 1996b) and six in the Na\(^{+}/Ca^{2+}\) plus K\(^{+}\) family (NCKX1 - NCKX6) (see Dong et al., 2006; Reilander et al., 1992).

NCX1 is widely expressed in diverse tissues including heart, brain, skeletal muscle, smooth muscle, kidney, spleen, and lung. The cloned cardiac NCX1 has been elucidated in heart (Nicoll et al., 1990), aorta (Nakasaki et al., 1993), and many other tissues (Iwamoto et al., 1995). Six exons (A, B, C, D, E, and F) code for the alternatively spliced NCX1 isoforms (Lee et al., 1994)(Kofuji et al., 1994; Nakasaki et al., 1993; Nicoll et al., 1990). Exon A-containing transcripts are predominantly expressed in excitable cells (e.g. NCX\(_{1,1}\) containing exons A,C,D,E and F in cardiomyocyte: NCX\(_{1,4}\) containing exons A and D (Ander et al., 2007; Dunn et al., 2002; Hurtado et al., 2006; Schulze et al., 2002) and NCX\(_{1,5}\) containing exons A, D and F in neurons of the brain) (Dunn et al., 2002; Hryshko, 2002; Sergeeva et al., 2003); Exon B-containing transcripts are mainly expressed in nonexcitable cells (e.g. NCX\(_{1,3}\)
containing exons B and D is abundant in kidney as well as vascular smooth muscle cells) (Iwamoto et al., 2004b; Karashima et al., 2007; Ruknudin et al., 2000; Szewczyk et al., 2007; Zheng et al., 2007). Comparatively, NCX2 and NCX3 are exclusively distributed in brain and skeletal muscle respectively (Quednau et al., 1997). The present study focuses on NCX1 in rat aorta. There is a high homology of the NCX1 clones in various tissues. For example, the NCX1 in rat aorta shares the same structure with canine cardiac NCX except for the central loop domain amino acid 570-631 and N-terminal portion (Nakasaki et al., 1993). There are no distinctive functional differences among tissue-specific NCX isoforms (Linck et al., 1998), (see review Blaustein et al., 1999).

1.4.4 Coupling ratios of NCX

In most cells, three Na\(^+\) ions are moved in exchange for one oppositely directed Ca\(^{2+}\) ion. Due to the difference between charges of imported and exported ions (3 Na\(^+\) : 1 Ca\(^{2+}\)), NCX is electrogenic and can be measured as an ionic current: Na\(^+\)/Ca\(^{2+}\) exchange current (I\(_{\text{NCX}}\)) (Chin et al., 1993; Kimura et al., 1986; Philipson, 2002). Both outward (Ca\(^{2+}\) entry mode) and inward current (Ca\(^{2+}\) exit mode) are detectable using the method of giant patch voltage clamp combined with internal perfusion (Hilgemann, 1989; Kimura et al., 1987; Kimura et al., 1986; Matsuoka et al., 1995). However, in vertebrate photoreceptors, some neurons, and certain other cells, K\(^+\) is transported in the same direction as Ca\(^{2+}\), with a coupling ratio of four Na\(^+\) to one Ca\(^{2+}\) plus one K\(^+\) (NCKX) (Dong et al., 2006). The exchanger kinetics are affected by nontransported Ca\(^{2+}\), Na\(^+\), protons, ATP, and other modulators. (For review, see Blaustein, 1989). Some conspicuous features of NCX are summarized in Table 1.3.
Figure 1.4  Model of Na\(^+\)/Ca\(^{2+}\) exchanger protein

The nine transmembrane segments are indicated as cylinders, numbered from 1 to 9. The α-repeat regions in transmembrane segments 2, 3, and 7 are shaded. N terminus of the protein is located in the external side of the plasma membrane; C terminus and hydrophilic loop are in the internal side. On the loop, self-inhibitory XIP region, the binding site for regulatory Ca\(^{2+}\), and alternatively spliced region are identified. GIG represents a conserved GIG motif.
Table 1.3 The properties of different types of NCX.

<table>
<thead>
<tr>
<th>TYPE OF NCX</th>
<th>COUPLING RATIO</th>
<th>ISOFORMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>K⁺-independent (Cardiac/neuronal/renal type)</td>
<td>3Na⁺ : 1Ca²⁺</td>
<td>NCX1-NCX3</td>
</tr>
<tr>
<td>K⁺-dependent (Vertebrate photoreceptor type)</td>
<td>4Na⁺ : (1Ca²⁺ : 1K⁺)</td>
<td>NCKX1-NCKX6</td>
</tr>
</tbody>
</table>

Please refer to 1.4.4 for details and references.
1.4.5  Modes of operation

The transport of ions mediated by NCX is bi-directional. NCX can move Ca\(^{2+}\) either outwardly or inwardly depending on the prevailing electrochemical driving forces (i.e. the Na\(^{+}\) and Ca\(^{2+}\) concentration gradients and the membrane potential). These two modes of Na\(^{+}\)/Ca\(^{2+}\) exchange (see Figure 1.5 and Figure 1.6) are generally referred to as "Ca\(^{2+}\) entry mode" (reverse mode) and "Ca\(^{2+}\) exit mode" (forward mode) respectively.

The net Ca\(^{2+}\) transport \(J_{\text{Ca(Na/Ca)}}\) is decided by the difference between the membrane potential \((V_M)\), the reversal potential of the exchanger \((E_{\text{Na/Ca}})\), as well as the complex kinetic parameter \((k)\) which is described in the following equation: \(J_{\text{Ca(Na/Ca)}} = k (V_M - E_{\text{Na/Ca}})\) (see review Blaustein, 1989).

For the K\(^{-}\)-independent exchanger, the coupling ratio is 3Na\(^{+}\) : 1Ca\(^{2+}\).

Eq. 1  \[ E_{\text{Na/Ca}} = 3E_{\text{Na}} - 2E_{\text{Ca}} \]

Eq. 2  \[ E_{\text{Na}} = \frac{RT}{F} \ln\left(\frac{[\text{Na}^{+}]_{o}}{[\text{Na}^{+}]_{i}}\right) \]

Eq. 3  \[ E_{\text{Ca}} = \frac{RT}{2F} \ln\left(\frac{[\text{Ca}^{2+}]_{o}}{[\text{Ca}^{2+}]_{i}}\right) \]

The parameter \(k\) is related to the number of carriers; the fractional saturation of the carrier binding sites by the activating ions and transported ions; \(V_M\); and ATP.

R: gas constant; T: absolute temperature; F: Faraday’s number

The thermodynamic driving force (\(\Delta V\)) for NCX is: \(V_M - E_{\text{Na/Ca}}\) (see reviews Blaustein et al., 1999; Sipido et al., 2002). This force is clearly demonstrated in the action potential of
cardiomyocyte. When $E_{Na/Ca}$ is more negative than $V_M$ during the early cardiac action potential, $\Delta V$ drives the NCX to mediate $Ca^{2+}$ entry. When $V_M$ is more negative than $E_{Na/Ca}$ during the later part of action potential plateau, $\Delta V$ drives the NCX to mediate $Ca^{2+}$ exit (see reviews Blaustein, 1989; Blaustein \textit{et al.}, 1999; Sipido \textit{et al.}, 2002).

1.4.5.1 $Ca^{2+}$ entry mode (reverse mode)

The $Ca^{2+}$ entry mode of exchange (Figure 1.5) is usually identified as an internal $Na^+$ ($Na_i$)-dependent $Ca^{2+}$ influx and an external $Ca^{2+}$ ($Ca_o$)-dependent, ouabain-insensitive $Na^+$ efflux (Furukawa \textit{et al.}, 1991; Kimura \textit{et al.}, 1987) \{See also review \textit{Blaustein, 1999 #7}\}.

1.4.5.2 $Ca^{2+}$ exit mode (forward mode)

$Ca^{2+}$ exit mode (Figure 1.6) exchange is defined operationally as an external $Na^+$ ($Na_o$)-dependent $Ca^{2+}$ efflux and an internal $Ca^{2+}$ ($Ca_i$)-dependent ouabain- and tetrodotoxin (TTX)-insensitive $Na^+$ influx (Furukawa \textit{et al.}, 1991; Kimura \textit{et al.}, 1987), (See also review Blaustein \textit{et al.}, 1999}).

There are also two other types of modes, namely: $Ca^{2+}/Ca^{2+}$ exchange and $Na^+/Na^+$ exchange mediated by the $Na^+/Ca^{2+}$ exchanger (Blaustein \textit{et al.}, 1977). It is assumed that these two modes of exchange are perhaps not functionally important in terms of net ion translocation. It is clear that NCX can only mediate coupled countertransport, does not mediate "uncoupled" transport of either $Na^+$ or $Ca^{2+}$ (see review Blaustein \textit{et al.}, 1999).
Figure 1.5  The model of Ca\(^{2+}\) entry (reverse mode)

From the equations Eq. 1, Eq. 2 and Eq. 3, increasing intracellular Na\(^+\) or decreasing extracellular Na\(^+\) from the physiologic level can, in some circumstances, cause the exchanger to transport Ca\(^{2+}\) into cells and this is referred as Ca\(^{2+}\) entry mode or reverse mode (Schweda et al., 2001).

Figure 1.6  The model of Ca\(^{2+}\) exit (forward mode)

From the equations Eq. 1, Eq. 2, and Eq. 3, the physiological level of Na\(^+\) inside and outside the cell favors Ca\(^{2+}\) extrusion from the cell through the exchanger and this is defined as Ca\(^{2+}\) exit mode or forward mode operation (Schweda et al., 2001).
1.4.6 Inhibitors of NCX

Inhibitors of NCX are dealt with in chapter 2.1.

1.4.7 The modulation of NCX

There is abundant evidence showing that NCX can be regulated on the intracellular hydrophilic loop that occurs at two major levels: (i) modulation by ions (Na\(^+\)-inactivation, [Ca\(^{2+}\)], activation, and proton inhibition); and (ii) modulation by signal transduction processes (DiPolo et al., 2002).

1.4.7.1 The ionic modulation of NCX by regulatory ionic ligands (Na\(^+\), Ca\(^{2+}\), and H\(^+\))

The activity of NCX can be activated by cytoplasmic Ca\(^{2+}\), and inhibited by cytoplasmic Na\(^+\) (for reviews see Annunziato et al., 2004; Blaustein, 1989; DiPolo et al., 2002; Philipson et al., 2000; Shigekawa et al., 2001).

Intracellular Ca\(^{2+}\) plays a key role for the function of NCX, as not only a transport substrate but an activator as well (DiPolo et al., 1987a; DiPolo et al., 1987b; DiPolo et al., 1987c). Independent of the mode (forward or reverse mode), cardiac NCX activity can only be switched on when the intracellular Ca\(^{2+}\) binds to the Ca\(^{2+}\) regulatory site on central hydrophilic loop (see Figure 1.4) (DiPolo et al., 2002). The loop is a regulatory domain, and
separate from the Ca$^{2+}$ transport parts of the molecule (Shigekawa et al., 2001). The intracellular Ca$^{2+}$-dependent activation is a critical feature of NCX (DiPolo et al., 2002; Linck et al., 1998; Matsuoka et al., 1995). The [Ca$^{2+}$], required for half-maximal activation ($K_{1/2}$) of Ca$^{2+}$ entry mode exchange in squid axons (DiPolo, 1979) and cardiac myocyte giant patches (Kimura et al., 1987) is of the order 1 µM under physiological conditions. As such, exchangers are only partially active at the normal resting [Ca$^{2+}$], (~100 nM) in most cells. There is a requirement of micromolar [Ca$^{2+}$], above resting level for the full NCX function. The exchanger is fully activated when [Ca$^{2+}$], is in the low micromolar range (i.e., at about the [Ca$^{2+}$], expected during peak activity in many types of excitable and secretory cells). The intracellular Ca$^{2+}$-dependent activation might be a protective mechanism allowing NCX to be turned on when [Ca$^{2+}$], is high and off when [Ca$^{2+}$], is low (DiPolo et al., 2002).

In sharp contrast with Ca$^{2+}$, high [Na$^+$], reduces the exchanger-mediated Ca$^{2+}$ transport. High [Na$^+$], was observed to induce an initial peak of the exchanger current and the subsequent decaying to steady state (Hilgemann et al., 1992). This process, which is referred to as Na$^+$-dependent inactivation, is believed to involve Na$^+$ ion interacting with the auto inhibitory region of endogenous exchanger inhibitory peptide (XIP), (see reviews DiPolo et al., 2002; Philipson et al., 2000).

The activity of NCX was demonstrated to be pH$_i$ dependent and inhibited by H$^+$. The intracellular alkalinization causes a dramatic increase in NCX affinity for Ca$^{2+}$ (DiPolo et al., 1987a; Hilgemann et al., 1992). Proton inhibition of NCX activity is assumed to occur via its competing with Ca$^{2+}$ at a Ca$^{2+}$ binding site on intracellular loop (Blaustein et al., 1977; DiPolo et al., 2002).
Given that intracellular Ca\(^{2+}\)-dependent activation is a prerequisite for NCX function, intracellular Na\(^+\) and H\(^+\) synergistic inhibition take place by competing with Ca\(^{2+}\) at a calcium-binding site on the central loop (DiPolo et al., 2002; DiPolo et al., 1999a).

1.4.7.2 The modulation of NCX by signal transduction processes

Multiple cellular signal pathways have been demonstrated to participate in the modulation on NCX, such as protein kinase C (PKC), protein kinase A (PKA), ATP, and nitric oxide. The interaction between nitric oxide and NCX is discussed in 3.1. The other modulators are discussed below.

(1) The modulation of NCX by protein kinases

With regard to the modulation of NCX by protein kinase C, some convincing data have been obtained in smooth muscle cells. The phosphorylation sites for PKC and PKA in the intracellular loop of NCX in rat aorta had been reported (Nakasaki et al., 1993). This may have functional significance. In rat aortic VSMC from the A7r5 cell line, phorbol ester stimulated the NCX activity (Vigne et al., 1988). Iwamoto and co-workers provided direct evidence that PKC-dependent phosphorylation activated NCX in both quiescent and growth factor-stimulated cultured aortic smooth muscle cells of rat (Iwamoto et al., 1995). Again, PKC-catalyzed phosphorylation up-regulated the activity of both cloned and native NCX in both NCX overexpressed canine cardiomyocytes and rat neonatal cardiomyocytes (Iwamoto et al., 1996a).
Yamanaka et al. showed that the cAMP/PKA system stimulated NCX activity in porcine coronary artery smooth muscle cells (Yamanaka et al., 2003). This is consistent with the up-regulatory effect of cAMP/PKA system on NCX in rat neurons (He et al., 1998). However, both PKA and PKG were shown to inhibit the activity of NCX in cultured human mesangial cells (Mene et al., 1993). In another case, the adenylate cyclase activator: forskolin inhibited the NCX mRNA and activity in cultured rat aortic myocyte (Smith et al., 1995). Clearly, the modulation of NCX by cyclic nucleotide depends on the tissue and the experimental condition. This needs to be further elucidated.

(2) The modulation of NCX by ATP

The operation of NCX is not ATP-driven, the hydrolysis of ATP is therefore not required to power net $\text{Ca}^{2+}$ movement and $\text{Na}^+/\text{Ca}^{2+}$ exchange can be demonstrated in ATP-depleted cells (Blaustein et al., 1969; Blaustein et al., 1977). Nevertheless, there is a growing body of evidence showing that ATP alters the exchanger kinetics (Blaustein et al., 1977; Caroni et al., 1983; Linck et al., 1998; Poburko et al., 2006), presumably by promoting phosphorylation of regulatory sites on the exchanger protein (DiPolo et al., 1987b; Hinde et al., 1999). In dialyzed squid axon, ATP activated the reverse $\text{Na}^+/\text{Ca}^{2+}$ exchange (DiPolo et al., 1987a). ATP-depletion inhibited NCX activity in both overexpressed canine cardiomyocytes and rat neonatal cardiomyocytes (Iwamoto et al., 1996a). In stably transfected BHK cells, ATP stimulated NCX1 activity and ATP-depletion inhibited NCX1 and NCX2 (Linck et al., 1998). Clearly, ATP up-regulates the invertebrate and mammal
NCX activities by changing the affinity for Na\(^+\) and Ca\(^{2+}\) at transporting and regulatory sites (DiPolo et al., 1987a; DiPolo et al., 1998; DiPolo et al., 1987b); (for review see DiPolo et al., 1999b).

NCX1 was shown to be up-regulated by the level of membrane phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) (Hilgemann et al., 1996). It was suggested that ATP exerts its indirect regulatory effect on NCX through the forming of PIP\(_2\) from phosphatidylinositol (PI). The further experimental data in favor of this hypothesis was shown by (Berberian et al., 1998).

(3) The metabolic modulation of NCX by other high energy compounds

Apart from ATP, another phosphoryl donor reported to be a regulator of NCX is phosphoarginine (Pa). Phosphoarginine, as well as ATP was reported to up-regulate the NCX activity through phosphorylation in dialysed squid axons (DiPolo et al., 1998).

1.4.8 Physiological function of NCX

1.4.8.1 NCX in vascular smooth muscle cell (VSMC)

Early investigation of NCX function in affecting contractility can be traced back to the late 1950s (Hagiwara et al., 1967; Luttgau et al., 1958). It is now widely accepted that NCX makes critical contributions to the maintenance of calcium homeostasis in a variety of tissues (Chapman et al., 1983). The primary function of NCX is removing the Ca\(^{2+}\) out of the cells.
(operating in forward mode), although it also mediates Ca$^{2+}$ influx (operating in reverse mode) in some circumstances (Linck et al., 1998). See also 2.4, 3.4 and 4.4.

Unlike the uniformly distributed plasma membrane Ca$^{2+}$ pump that serves a housekeeping role by maintaining a low [Ca$^{2+}$], (Furukawa et al., 1988), NCX was found to distribute focally in reticular pattern over the cell surface of the plasma membrane that is close to SR or ER (subsarcolammmal domain), e.g. surface sarcolemmal or disk region, in the T-tubules only at the level of the Z-line (Blaustein et al., 1992; Juhaszova et al., 1994; Juhaszova et al., 1996). The co-localization of NCX with SR may give clues about its function (Juhaszova et al., 1994; Juhaszova et al., 1996). Under resting conditions, NCX may have access to SR regulating the content of Ca$^{2+}$ stored in the SR and, be available for contractions (Busselen et al., 1991; Evans et al., 1997; Goldman et al., 1994; Lewartowski et al., 1990; Litwin et al., 1996), (see also reviews Blaustein et al., 1999; Sipido et al., 2002). When the cell is activated, much of the trigger Ca$^{2+}$ for contraction may enter the cells via voltage-gated Ca$^{2+}$-selective channels (Davis et al., 1999; Hagiwara et al., 1967; Jaggar, 2001; Wesselman et al., 1996), but some may also enter via the exchanger when the cells are depolarized and [Ca$^{2+}$]$_{\text{i}}$ rises (Bova et al., 1990; DiPolo et al., 1987c; Rebolledo et al., 2006; Zhu et al., 1994). NCX may help to keep Ca$^{2+}$ elevated during tonic contractions.

To facilitate the relaxation of smooth muscle cells after contraction is initiated, Ca$^{2+}$ must be extruded rapidly (Hill et al., 2003; Philipson, 2002). This Ca$^{2+}$ extrusion is against a tremendous electrochemical gradient, due to the free [Ca$^{2+}$]$_{\text{i}}$ at rest in arterial SMC is around 100-200 nM as opposed to 1.8 mM externally (Ashida et al., 1987). Whilst a large fraction of Ca$^{2+}$ is sequestered back into sarcoplasmic reticulum (SR), a significant portion of it is
pumped out of the cytoplasm via NCX (Liang et al., 2004; Wang et al., 2002b). NCX pumps out Ca\(^{2+}\) ions about tenfold more rapidly (>1000 sec\(^{-1}\)) (Cheon et al., 1988) than the ATP-driven Ca\(^{2+}\) extrusion pump (~50-150 sec\(^{-1}\)), (see review Blaustein et al., 1999), far fewer exchanger molecules would be required to lower \([Ca^{2+}]_i\) rapidly (Ashida et al., 1987). Based on the different Ca\(^{2+}\)-buffering ability of NCX and Ca\(^{2+}\) pump, it is broadly agreed that the exchanger plays an important role in the extrusion of Ca\(^{2+}\) in many types of cells, particularly following a rise in \([Ca^{2+}]_i\) when cell activity is increased (Ashida et al., 1987; Nazer et al., 1998; Smith et al., 1987). (See reviews Blaustein et al., 1999; DiPolo et al., 1987c; DiPolo et al., 1987d; Shigekawa et al., 2001).

In cardiomyocytes from NCX knockout (NCX\(^{-/-}\)) mice, decreased Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channel, increased plasma membrane Ca\(^{2+}\) pump activity, increased transient outward current through potassium channel and increased excitation-contraction coupling were able to compensate for the reduced Ca\(^{2+}\) extrusion capacity under basal conditions, and there were no change of any Ca\(^{2+}\) transport systems at the protein expression level (Pott et al., 2005; Pott et al., 2007; Sarai et al., 2006). However, when the cell activity is increased (e.g. after the addition of caffeine, isoproterenol), the NCX\(^{-/-}\) heart tube was unable to maintain Ca\(^{2+}\) homeostasis (Reuter et al., 2003). There have been no studies in vascular smooth muscle using NCX knockouts.

1.4.8.2 NCX in vascular endothelial cells (ECs)

The existence of endothelial NCX has been reported in cardiac microvascular ECs (Kaye et
al., 1999), in cultured rat aortic ECs (Dong et al., 2004; Juhaszova et al., 1994; Quednau et al., 1997), in cultured human pulmonary arterial ECs (Mizuno et al., 2002) and in cultured pig coronary arterial ECs (Szewczyk et al., 2007). NCX has been shown to modulate calcium homeostasis in vascular endothelial cells of rabbit aorta (Liang et al., 2004; Wang et al., 2002b), porcine aorta (Schneider et al., 2002a; Teubl et al., 1999), calf pulmonary artery (Sedova et al., 1999), human umbilical vein (Ogura et al., 2004; Paltauf-Doburzynska et al., 2000) and rat epicardial artery (Moccia et al., 2002). It is generally accepted that there is a tight interaction between endothelial NCX and endothelium-released nitric oxide (NO) (Dong et al., 2004; Kim et al., 2005; Mizuno et al., 2002; Ogura et al., 2004; Schneider et al., 2002a; Szewczyk et al., 2007). In EC, NCX can facilitate the Ca^{2+}-dependent activation of endothelial nitric oxide synthase (eNOS) and the subsequent production of NO (Dong et al., 2004; Mizuno et al., 2002; Schneider et al., 2002a; Teubl et al., 1999). Interestingly, an increase in [Ca^{2+}]_{i} in EC and SMC has opposite effect. Whilst increased [Ca^{2+}]_{i} in EC causes vasorelaxation via activating eNOS, increased [Ca^{2+}]_{i} in SMC causes vasoconstriction via controlling contractility (See reviews Ledoux et al., 2006)(Szewczyk et al., 2007).

In summary, NCX appears to play a central role in the regulation of the cytosolic [Ca^{2+}]_{i}, the SR Ca^{2+} store and the contractility, in both normal smooth muscle function and a variety of pathological conditions (Hill et al., 2003).
2.1 INTRODUCTION

The actions of the Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) in Ca\(^{2+}\) homeostasis has been discussed in Section 1.4. Exploration of the physiological significance of NCX proteins requires tools to either activate or inhibit the exchanger selectively. This has led to a variety of approaches to manipulate the exchanger, which is discussed below.

2.1.1 Inorganic cations

Ca\(^{2+}\) is a critical ion in the modulation of cellular activity and there are a variety of ion channels, membrane pumps, enzymes and other proteins which have Ca\(^{2+}\) binding or transport functions, including Ca\(^{2+}\) channels, ATP-dependent Ca\(^{2+}\) pumps, and the Na\(^+\)/Ca\(^{2+}\) exchanger (NCX). Ions with similar characteristics to Ca\(^{2+}\) may competitively inhibit Ca\(^{2+}\) binding to the Na\(^+\)/Ca\(^{2+}\) exchanger and thus act as inhibitors. These have been used in a variety of studies (Bondarenko, 2004; Deval et al., 2002; Hagiwara et al., 1967; Hinde et al., 1999; Kimura et al., 1987; Subramani et al., 2005; Trosper et al., 1983). The IC\(_{50}\) values to inhibit NCX1 in Chinese hamster lung fibroblasts are: La\(^{3+}\)(22 μM) < Cd\(^{2+}\)(33 μM) < Ni\(^{2+}\)(52 μM) < Co\(^{2+}\)(105 μM) < Mn\(^{2+}\)(166 μM) < Mg\(^{2+}\)(4.6 mM) (Iwamoto et al., 1998). LaCl\(_3\) (0.5 mM) inhibited the Na\(^+\)-induced Ca\(^{2+}\) uptake in microsomal fractions from the longitudinal smooth muscle of the guinea-pig ileum and the rat aorta which is NCX mediated (Morel et al., 1984). Ni\(^{2+}\) was used as NCX inhibitor in cultured rat aortic endothelial cells at
the concentration of 5 mM (Dong et al., 2004), in endothelium-denuded rat mesenteric artery at the concentration of 30 - 300 μM (Tsang et al., 2003), and in guinea-pig ventricular myocytes at the concentration of 10 mM (Hinde et al., 1999).

Selectivity is a major issue as many of these polyvalent cations inhibit a number of other Ca\textsuperscript{2+} transport and permeability mechanisms. La\textsuperscript{3+} is a good example, and La\textsuperscript{3+} (50 - 250 μM) suppressed 80 to 95% of active calcium extrusion through a Ca\textsuperscript{2+}-ATPase mechanism in intact human red blood cells (Sarkadi et al., 1977). Additionally, La\textsuperscript{3+} (0.1 mM) was used as a selective inhibitor of Ca\textsuperscript{2+} pump and blocked the Ca\textsuperscript{2+} efflux in human erythrocyte ghosts (Quist et al., 1975). Further, in cultured arterial myocytes, La\textsuperscript{3+} (0.25 mM) inhibited the calcium pump (Slodzinski et al., 1998a). It has in fact been suggested that low [La\textsuperscript{3+}]\textsubscript{o} (0.06-0.25 mM) inhibited the plasma membrane Ca\textsuperscript{2+} pump and not the NCX in arterial myocytes (Shimizu et al., 1997). A similar profile has been observed for other inorganic ions.

A further issue in trying to use heavy metal inhibitors is that these polyvalent cations could be transported into cells as surrogates for Ca\textsuperscript{2+} eliciting other effects by acting on intracellular membrane. For example, La\textsuperscript{3+} (0.25 mM) entered arterial myocytes (Shimizu et al., 1997). Further the intracellular transported La\textsuperscript{3+} was found to replace regulatory Ca\textsuperscript{2+} and potently activate the exchanger activity even at picomolar concentrations in Chinese hamster ovary cells expressing the bovine cardiac Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX1.1) (Reeves et al., 2003). Taken together, these results indicate that inorganic ions are unsuitable in NCX studies.
2.1.2 Amiloride

Amiloride, has been widely used as a blocker of Na\(^+\)/H\(^+\) exchanger (NHE) (Bondarenko et al., 2005; Kleyman et al., 1988; Murphy et al., 1991) but is also a weak inhibitor of Na\(^+\)/Ca\(^{2+}\) exchanger at the concentration of 1 mM in rat heart (Murphy et al., 1991), and at the concentration of 5 μM – 500 μM in guinea-pig aorta (Bova et al., 1988). Amiloride has poor selectivity for NCX. For example, a study on the inhibition of Na\(^+\) transport across tight epithelia through Na\(^+\) channel by amiloride was shown at the IC\(_{50}\) of 0.34 μM. In the same study, much weaker inhibition on NHE and NCX were demonstrated with an IC\(_{50}\) of 83.8 and 1100 μM respectively (Kleyman et al., 1988). Thus amiloride cannot be claimed to be selective. This may affect interpretation of results. Blockade of NHE by amiloride may affect NCX operation. For example, it is known that increased Na\(^+\) loading via NHE can induce NCX to operate in reverse mode by altering intracellular [Na\(^+\)] (Kuramochi et al., 2004; Palandoken et al., 2005; Schafer et al., 2001). This may give confusing results such as in ischemia-reperfusion injury where both NHE and NCX are involved. Therefore it is not clear if the cardiac protective effect by amiloride in this condition is through its inhibition on NCX or NHE (Bondarenko et al., 2005).

2.1.3 Amiloride analogues

Among approximately one thousand types of amiloride derivatives, benzamil and DCB (see Figure 2.1) have been demonstrated to inhibit the Na\(^+\)/Ca\(^{2+}\) exchanger more potently and have been used to block NCX activity (Hegde et al., 2004; Plasman et al., 1991; Siegl et al., 1984; Watano et al., 1996). In pancreatic islet cells, DCB (IC\(_{50}\): 18 μM) potently inhibited
the $^{45}$Ca uptake mediated by the reverse mode of NCX (Plasman et al., 1991); In single cardiac ventricular cells of guinea-pig, DCB (3-30 μM) depressed the inward Na$^+$/Ca$^{2+}$ exchange current with IC$_{50}$ of 17 μM (Watano et al., 1996); With the same IC$_{50}$ (17 μM), DCB was shown to be a potent inhibitor of Na$^+$-dependent Ca$^{2+}$ influx in both guinea pig cardiac membrane vesicles and papillary muscles (Siegl et al., 1984). Likewise, benzamil was used to selectively inhibit NCX at the concentration of 100 μM in intact endothelial cells from rat aorta (Bondarenko, 2004), at 20 μM in endothelium-denuded rat mesenteric artery (Tsang et al., 2003), at 10 μM in cultured rat aortic endothelial cells (Dong et al., 2004) and at 1 mM in both rat aortae and cultured porcine aortic endothelial cells (Schneider et al., 2002a). However, the DCB and benzamil may have limited specificity. For example, Suarez-Kurtz et al demonstrated that DCB dose-dependently (1 - 25 μM)) inhibited the T-type and slowly-inactivating L-type Ca$^{2+}$ channels in GH3 clonal pituitary cells (Suarez-Kurtz et al., 1988). DCB (30 μM) also inhibited the endothelin-induced constriction in intact rat aortic rings (Criscione et al., 1989). Furthermore, in cultured aortic myocytes, both DCB (IC$_{50}$ = 10 μM) and benzamil (IC$_{50}$ = 30 μM) inhibited the monovalent cation channel induced by palytoxin (van Renterghem et al., 1993). Thus these agents whilst inhibiting NCX potently are not selective.
Figure 2.1 The chemical structure of amiloride and its analogues

Panel A shows the structure of amiloride; panel B shows the structure of DCB (3', 4'-dichlorobenzamil); and panel C shows the structure of benzamil.
2.1.4 Exchanger inhibitory peptide (XIP)

As introduced in the previous chapter 1.3., the NCX protein comprises 9 transmembrane segments and a large hydrophilic loop (Figure 1.4). The cytoplasmic loop (about 550 amino acids long) is a calmodulin binding site and does not participate in ion transportation, but is involved in the regulation of NCX by intracellular \( \text{Na}^+ \), \( \text{Ca}^{2+} \) and \( \text{PIP}_2 \) (Maack *et al.*, 2005). The loop is a regulatory domain, and separate from the \( \text{Ca}^{2+} \) transport parts of the molecule (Shigekawa *et al.*, 2001). High \([\text{Na}^+]_i\) was observed to induce an initial peak of the exchanger current and the subsequent decaying to steady state (Hilgemann *et al.*, 1992). This process, which is referred to as \( \text{Na}^+ \)-dependent inactivation, is believed to interact with the endogenous XIP auto inhibitory region (Philipson *et al.*, 2000).

A synthetic polypeptide (XIP) that mimics a 20 amino acid sequence of the large intracellular loop of NCX molecule (Li *et al.*, 1991; Nicoll *et al.*, 1991), was reported to be a selective inhibitor of NCX (Chin *et al.*, 1993; Li *et al.*, 1991). This supports the notion that the calmodulin-binding site on intracellular loop serves as autoinhibitory structure.

One issue is that the binding site for this synthetic peptide on NCX is on the cytoplasmic surface of the membrane and XIP does not appear to permeate through the cell membranes, therefore XIP must be delivered to the inside of the cell to exert its effect. This limits its application to a limited range of studies where intracellular access is not an issue, for example, patch-clamp electrophysiology in cardiac sarcolemmal vesicles (Li *et al.*, 1991; Matsuoka *et al.*, 1997a), in guinea pig ventricular cell (Chin *et al.*, 1993) and in cardiac myocytes (Maack *et al.*, 2005). In these studies, intracellularly delivered XIP inhibited NCX.
2.1.5 Antisense oligonucleotides (AS-oligos)

With the aid of advanced molecular biological techniques, some research groups have attempted to synthesize antisense oligonucleotides directed against the NCX mRNA to reduce the expression of NCX. Some substantial progress has been made, although further studies are still needed.

For example, the extracellular Na\(^+\)-dependent Ca\(^{2+}\) efflux was significantly inhibited in cultured mesenteric artery myocytes after 9 days AS-oligo treatment (Slodzinski et al., 1998b). Antisense treatment (5 μM for 48 hours) also reduced exchanger activity of primary cultured rat cardiac myocyte approximately 30-40% (Takahashi K. et al., 1995). Most single cultured myocytes exposed to 3 μM antisense oligodeoxynucleotide for 2 days showed neither an NCX current nor an increase of [Ca\(^{2+}\)]\(_i\) upon extracellular Na\(^+\) removal (Lipp et al., 1995). In primary cultured neonatal rat cardiomyocytes, ~60% of the cells did not exhibit spontaneous Ca\(^{2+}\) transients or respond to Na\(^-\)free medium after 4 days treatment of AS-oligo (0.5 μM), and NCX protein was reduced by ~50% in cells treated with AS-oligos for 7 days (Slodzinski et al., 1998a).

Antisense approaches in functional tissues are more complicated compared to cell lines in that the tissues have to maintain functionality during a prolonged period of antisense incubation or methods of antisense delivery in vivo have to be developed. Neither of these approaches has been used for NCX to my knowledge.
2.1.6 SEA0400

The structure of 2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline (SEA0400) was shown in Figure 2.2. SEA0400 was found to inhibit NCX and unlike KB-R7943 (see 2.1.7) inhibits both forward and reverse mode with equal potency (Magee et al., 2003; Takahashi et al., 2003). On guinea-pig ventricular myocytes, the IC$_{50}$ value for the inward and outward current was 40 nM and 32 nM respectively (Tanaka et al., 2002). However, it has been reported to be a much better tool for NCX inhibition with higher potency and greater selectivity than KB-R7943 (table 2.1). At the concentration inhibiting NCX in cultured astrocytes, SEA0400 showed negligible effect on Ca$^{2+}$ channels, Na$^+$ channels, and K$^+$ channels, noradrenaline transporter, 14 receptors (adenosine A$_1$ and A$_2$; adrenergic $\alpha_1$, $\alpha_2$, $\beta_1$, and $\beta_2$; Glutamate AMPA, Kainate, and N-methyl-D-aspartate (NMDA); mACh; Bradykinin B$_1$, B$_2$, LTB$_4$, PAF), and did not have any effect on 5 enzymes (phospholipase A$_2$, phospholipase C, 5-lipoxygenase, iNOS, and cNOS), store-operated calcium entry, NHE, Na$^+$ pump, Ca$^{2+}$ pump (Matsuda et al., 2001). It has also been viewed as a promising therapeutic strategy for myocardial ischemia-reperfusion cell damage (Akabas, 2004; Hobai et al., 2004; Iwamoto et al., 2004a; Matsuda et al., 2001; Ogata et al., 2003; Yoshiyama et al., 2004).
Figure 2.2  Molecular structure of SEA0400
Table 2.1 Comparison of the inhibitory effect of KB-R7943 and SEA0400 on NCX activity (All data are presented as IC$_{50}$ values)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>SEA0400</th>
<th>KB-R7943</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultured neuron</td>
<td>33 nM</td>
<td>3.8 μM</td>
<td>(Matsuda et al., 2001)</td>
</tr>
<tr>
<td>Cultured astrocyte</td>
<td>5.0 nM</td>
<td>2.0 μM</td>
<td>(Matsuda et al., 2001)</td>
</tr>
<tr>
<td>Cultured microglia</td>
<td>8.3 nM</td>
<td>3.1 μM</td>
<td>(Matsuda et al., 2001)</td>
</tr>
<tr>
<td>Canine Cardiac sarcolemmal vesicles</td>
<td>90 nM</td>
<td>7.0 μM</td>
<td>(Takahashi et al., 2003)</td>
</tr>
<tr>
<td>Rat Cardiomyocyte</td>
<td>92 nM</td>
<td>9.5 μM</td>
<td>(Takahashi et al., 2003)</td>
</tr>
</tbody>
</table>
Figure 2.3  Molecular structure of KB-R7943 molecule
2.1.7 KB-R7943

As discussed early in chapter 1.4.8, under physiological conditions, NCX mainly operates in forward mode to pump calcium out of the cell. However, the activity of NCX in reverse mode may be important with regard to the disease states, such as the cardiac ischemia-reperfusion injury, where it mediates Ca\(^{2+}\) entry and the subsequent tissue hypercontracture (Philipson, 2002; Shigekawa et al., 2001; Sipido et al., 2002). This finding has prompted a search for the drug that selectively inhibits the NCX operating in reverse mode. A variety of benzyloxyphenyl derivatives were synthesized and tested. Among them, the isothiourea derivative (see Figure 2.3): 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl] isothiourea (KB-R7943) has been widely used and extensively investigated (Kim et al., 1999; Kim et al., 2005).

In most studies, it has been shown that KB-R7943 inhibits preferentially the reverse mode of NCX (Ladilov et al., 1999; Watano et al., 1996; Watano, 1998). For example, the IC\(_{50}\) value was 0.32 \(\mu\)M for the outward exchange current and 17 \(\mu\)M for the inward current in single cardiac ventricular cells (Watano et al., 1996); 1.2 - 2.4 \(\mu\)M for reverse mode and \(\geq\) 30 \(\mu\)M for forward mode in cardiomyocytes, smooth muscle cells, and NCX1-transfected fibroblasts (Iwamoto et al., 1996b).

Initial results suggested that KB-R7943 was relatively selective for NCX. KB-R7943 at 10 \(\mu\)M did not affect Na\(^+\)/H\(^+\) exchanger, passive Na\(^+\) uptake, sarcolemmal Ca\(^{2+}\)-ATPase, Na\(^+\)/K\(^+\)-ATPase in cardiomyocyte (Iwamoto et al., 1996b), and it has been used as a putative
selective inhibitor of NCX in some studies (Bondarenko, 2004; Chau et al., 2003; Iwamoto et al., 1996b; Ladilov et al., 1999; Watano et al., 1996).

However, the selectivity of KB-R7943 was questioned by further studies. Both SEA0400 (0.1 μM) and KB-R7943 (5 μM) were shown to depress the Ca\(^{2+}\) transient even in the absence of NCX in heart tube of knock out mouse (NCX\(^{-/-}\)) (Reuter et al., 2002). Additionally, in acutely isolated rat hippocampal neurons, KB-R7943 blocked two distinct N-methyl-D-aspartate (NMDA) channels, IC\(_{50}\) values were 0.8 μM and 11 μM respectively (Sobolevsky et al., 1999). In cultured neurons and astrocytes, it inhibited the store-operated Ca\(^{2+}\) entry at the IC\(_{50}\) value of 7 μM (Arakawa et al., 2000), and in guinea-pig ventricular myocytes, it attenuated the sodium current, L-type calcium current, delayed rectifier potassium current and inwardly rectifying potassium current by more than 50% at the concentration of 10 μM (Tanaka et al., 2002). Furthermore, in single cardiac ventricular cells, it inhibited the voltage-gated Na\(^{+}\) current, Ca\(^{2+}\) current, and the inward rectifier K\(^{+}\) current with IC\(_{50}\) value of approximately 14, 8, and 7 μM respectively (Watano et al., 1996); and it blocked native and oocyte-expressed neuronal nicotinic receptors on bovine adrenal chromaffin cells at the IC\(_{50}\) value of 6.5 and 0.4 μM respectively (Pintado et al., 2000).

Recently, a thorough investigation by Matsuda et al (Matsuda et al., 2001) has provided the full-spectrum view of the effects of KB-R7943 in cultured astrocytes. The inhibitory concentrations of KB-R7943 on the major ion channels (L-type calcium channel, N-type calcium channel, Na\(^{+}\) channel, and K\(^{+}\) channel), 4 ion transporters (NCX, NHE, NE and calcium pump), 14 receptors (adenosine A\(_1\) and A\(_2\); adrenergic α\(_1\), α\(_2\), β\(_1\), and β\(_2\); Glutamate

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AMPA, Kainate, and NMDA; mACh; Bradykinin B₁, B₂, LTB₄, PAF) and 5 enzymes (phospholipase A₂, phospholipase C, 5-lipoxygenase, iNOS, and eNOS) were about 30 μM or even higher. At the concentration of 3 μM, it affected only L-type Ca²⁺ channel; muscarinic acetylcholine, LTB₄, and platelet-activating factor receptors; and noradrenaline transporter.

The above discussion highlights some contradictory results with KB-R7943 in that in some tissues at concentrations in excess of 3 μM it appears to have some other biological actions than inhibition of NCX. In the present study, KB-R7943 was the most selective inhibitor that was routinely available as the relevant pharmaceutical company refused SEA0400 supplies. To determine whether KB-R7943 could be used as a tool in cardiovascular tissue such as aorta, it was decided to investigate the effects of both 1 and 10 μM on a variety of Ca²⁺ mediated constrictor actions in the aorta. This was considered necessary as the majority of non-selective effects were obtained in non-cardiovascular tissue and on systems that are not present in blood vessels (see Table 2.2).

As summarized in table 2.3, KB-R7943 was used as a selective inhibitor at the concentration up to 10 μM in various types of VSMC. However, the selectivity of KB-R7943 was not tested in those studies; it remains still questionable if it is selective to NCX inhibition.
Table 2.2  Nonselective effect of KB-R7943 in variety of tissues (All data are presented as IC$_{50}$ values)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>[KBR] for NCX inhibition</th>
<th>[KBR] causing nonselective inhibition</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reverse mode</td>
<td>Forward mode</td>
<td>0.8 μM (high affinity) &amp; 11 μM (low affinity)</td>
</tr>
<tr>
<td>acutely isolated rat hippocampal neurons</td>
<td>3 μM</td>
<td>7 μM</td>
<td>(Arakawa et al., 2000)</td>
</tr>
<tr>
<td>cultured neurons and astrocytes</td>
<td>457 nM</td>
<td>10 μM</td>
<td>(Tanaka et al., 2002)</td>
</tr>
<tr>
<td>guinea-pig ventricular myocytes</td>
<td>5.5 μM</td>
<td>6.5 and 0.4 μM</td>
<td>(Pintado et al., 2000)</td>
</tr>
<tr>
<td>bovine adrenal chromaffin cells</td>
<td>5 μM</td>
<td>≥3 μM</td>
<td>(Reuter et al., 2002)</td>
</tr>
<tr>
<td>heart tube of NCX$^{-/-}$ mouse</td>
<td>3.8; 2.0; 3.1 μM</td>
<td>≥7 μM</td>
<td>(Watano et al., 1996)</td>
</tr>
</tbody>
</table>

[KBR] indicates the concentration of KB-R7943. The 2nd column shows the concentration of KB-R7943 for inhibiting NCX; and the 3rd column shows the concentration of KB-R7943 for inhibiting other ion transportors other than NCX (nonselective inhibition).
Table 2.3  The inhibition of NCX (reverse mode) by KB-R7943 in a variety of smooth muscle cells

<table>
<thead>
<tr>
<th>Tissue tested</th>
<th>[KBR]</th>
<th>(μM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human umbilical artery</td>
<td>5</td>
<td></td>
<td>(Rebolledo et al., 2006)</td>
</tr>
<tr>
<td>Rat carotid artery</td>
<td>3.5 (IC₅₀)</td>
<td></td>
<td>(Takai et al., 2004)</td>
</tr>
<tr>
<td>Rat aorta &amp; cultured porcine aortic endothelial cells</td>
<td>1</td>
<td></td>
<td>(Schneider et al., 2002a)</td>
</tr>
<tr>
<td>Cultured rat mesenteric and aortic smooth muscle cells</td>
<td>1-10</td>
<td></td>
<td>(Dong et al., 2006)</td>
</tr>
<tr>
<td>Mouse aorta &amp; mouse aortic endothelial cell</td>
<td>10</td>
<td></td>
<td>(Kim et al., 2005)</td>
</tr>
<tr>
<td>Human pulmonary artery</td>
<td>10</td>
<td></td>
<td>(Zhang et al., 2005b)</td>
</tr>
</tbody>
</table>

[KBR] indicates the concentration of KB-R7943.
In conclusion, there are contradictory results regarding the selectivity of KB-R7943 in non-smooth muscle cells. Also, there are some gaps in our knowledge about the selectivity of KB-R7943 in smooth muscle cells, which awaits further elucidation.

2.1.8 Aims of the study

One of the principal aims of the project was to develop insights into the operation of NCX in vascular tissue. Whilst we did receive some initial supplies of SEA0400, it became impossible to obtain ongoing supplies and we decided to use KB-R7943. However given its mixed and contradictory reports of selectivity for NCX (see 2.1.7), this present section was to assess its pharmacological actions in rat aorta on a variety of Ca$^{2+}$ dependent systems including: voltage-gated calcium channels; store-operated channels; and $\alpha$ adrenergic receptors. The hypothesis to be investigated was that an appropriate concentration of KB-R7943 could be found which was selective for NCX and which did not affect other contractile and Ca$^{2+}$ transport mechanisms.
2.2 METHODS

2.2.1 Tissue preparation for functional studies

Male Sprague Dawley rats (5-7 weeks old, weighing 200-350 g) were anaesthetized with Pentobarbital sodium (60mg/kg, i.p. Sigma, St Louis, USA) and then killed by decapitation. The thoracic aorta was removed, placed in a cold physiological salt solution (see 2.2.2.1), cleaned of surrounding fat and connective tissue and cut into equal-size ring segments (4 mM in length) with scissors. Two thin stainless-steel hooks were inserted through the lumen of aorta ring. The lower hook was connected to a tissue holder and the upper to an isometric force Grass FT03 displacement transducer. Each ring was suspended in an organ bath containing 2ml of physiological salt solution (PSS: A-F, see 2.2.2). Tissues were washed thoroughly by replacing the PSS repeatedly and were then allowed to equilibrate for a period of 45 minutes under 2g of resting tension. Supply reservoirs and organ baths were maintained at 37°C and were bubbled continuously with a mixture of 95%O₂ and 5%CO₂ to give rise to a pH of 7.4. Drugs and reagents were added directly to the bathing fluids using a micro syringe, and mixed thoroughly by repeated syringing with the bath solution and were allowed to reach a steady concentration in the incubation fluid within the tissue chamber. Baseline tone was readjusted when necessary.

In some cases, the endothelium was removed by gentle rubbing of the luminal surface with a plastic rod.
2.2.2 Composition of physiological salt solution (PSS) (in mM):

2.2.2.1 For functional studies (PSS-A):

\[
\text{NaCl 118, KCl 4.7, KH}_2\text{PO}_4 1.03, \text{NaHCO}_3 25, \text{D-} (+)-\text{glucose 11.1, MgSO}_4 1.2, \text{CaCl}_2 2.5 \text{ (mM).}
\]

2.2.2.2 For phenylephrine studies (PSS-B):

\[
\text{NaCl 118, KCl 4.7, KH}_2\text{PO}_4 1.03, \text{NaHCO}_3 25, \text{D-} (+)-\text{glucose 11.1, MgSO}_4 1.2, \text{CaCl}_2 2.5, \text{ L-ascorbic acid 0.14, Na}_2\text{EDTA 0.067 (mM). Ascorbic acid and EDTA were added to prevent oxidation of phenylephrine.}
\]

2.2.2.3 For Ca\(^{2+}\) free studies (PSS-C):

\[
\text{NaCl 118, KCl 4.7, KH}_2\text{PO}_4 1.03, \text{NaHCO}_3 25, \text{D-} (+)-\text{glucose 11.1, MgSO}_4 0.6, \text{EGTA 10, L-ascorbic acid 0.14, Na}_2\text{EDTA 0.067 (mM). Ascorbic acid and EDTA were added to prevent oxidation of phenylephrine.}
\]

2.2.2.4 For studies using Bay K8644 (PSS-D):

\[
\text{NaCl 108, KCl 14.7, KH}_2\text{PO}_4 1.03, \text{NaHCO}_3 25, \text{D-} (+)-\text{glucose 11.1, MgSO}_4 1.2, \text{CaCl}_2 2.5
\]
(mM). The higher \([K^+]_o\) was to partially constrict the vessel in order to facilitate the Bay K8644 action.

2.2.2.5 For studies of 80 mM KCl (PSS-E):

\[\text{NaCl 43.73, KCl 78.97, KH}_2\text{PO}_4 1.03, \text{NaHCO}_3 25, \text{D-}(+)-\text{glucose 11.1, MgSO}_4 1.2, \text{CaCl}_2 2.5 \text{ (mM). A depolarizing concentration of K}^+ (80 \text{ mM}) \text{ was used to elicit vasoconstriction.}\]

2.2.2.6 For 25 mM Na\(^+\) and ouabain studies (PSS-F):

\[\text{Normal Na}^+ \text{ PSS-F: NaCl 118, KCl 4.7, KH}_2\text{PO}_4 1.03, \text{NaHCO}_3 25, \text{D-}(+)-\text{glucose 11.1, MgSO}_4 1.2, \text{CaCl}_2 2.5 \text{ (mM).}\]

\[\text{Lower Na}^+ \text{ PSS-F: Choline chloride 118, KCl 4.7, KH}_2\text{PO}_4 1.03, \text{NaHCO}_3 25, \text{D-}(+)-\text{glucose 11.1, MgSO}_4 1.2, \text{CaCl}_2 2.5 \text{ (mM). Lower Na}^+ \text{ PSS-F was obtained by replacing 118 mM NaCl with iso-osmolar amounts of Choline Chloride.}\]

2.2.3 The effect of KB-R7943 on the vascular responsiveness to phenylephrine in the presence of extracellular Ca\(^{2+}\) in rat aortic rings

See 2.2.2.2 for the composition of bathing solution for this set of experiments. Following the
45-minute equilibration period, the viability of the tissues was assessed. Tissues which failed to produce 0.5 g increase in tension to phenylephrine (1 µM) were rejected. Successful removal of the endothelial cells was verified by lack of relaxant response to acetylcholine (1 µM) in the presence of phenylephrine (1 µM) at the beginning of each experiment (<5% relaxation) (see Figure 2.4).

Tissues were washed and allowed to equilibrate for a further 45 minutes, after which the first of two cumulative concentration-response curves to phenylephrine (1 nM – 10 µM) were performed, with approximately three minutes between subsequent additions of phenylephrine or until a steady-state constriction was achieved. After subsequent washing with PSS-B and equilibrating for 30 minutes, aorta was incubated with KB-R7943 for 20 minutes, followed by the second cumulative concentration-response curves of phenylephrine (1 nM – 10 µM). In another aortic ring obtained from the same animal, the vehicle dimethyl sulfoxide (DMSO, 0.1%), instead of KB-R7943 was administered. The experiments were conducted on both endothelium denuded and intact vessels.
Figure 2.4  A typical trace of rat aorta rings

The aorta rings in each channel are taken from separate rings in the same rat. Y-axis indicates the constriction measured in grams. The elapsed time on the experimental day is shown on X-axis (h:m:s). Panel A: response to phenylephrine (1 µM) followed by acetylcholine (1 µM) in endothelium denuded aorta. Panel B: response to phenylephrine (1 µM) followed by acetylcholine (1 µM) in endothelium intact aorta.
2.2.4 The effect of KB-R7943 on the vascular responsiveness to phenylephrine in the absence of extracellular Ca\textsuperscript{2+} in rat aortic rings

After tissue viability and removal of endothelial cells had been assessed, the tissues were allowed to equilibrate for a further 45 minutes. Following these 45 minutes, the bathing solution was replaced with Ca\textsuperscript{2+} free PSS-C (see 2.2.2.3). After 5 minutes, the first cumulative concentration-response curve to phenylephrine (10 nM –10 µM) was performed. Once responses had reached a maximum the bathing solution was repeatedly replaced with fresh drug free, Ca\textsuperscript{2+} containing PSS-B until the baseline tension had been re-established. The tissues were again allowed to equilibrate for 45 minutes after which time the tissues were treated with the Ca\textsuperscript{2+} free PSS-C for 5 minutes and then the second cumulative concentration-response curve of phenylephrine (10 nM –10 µM) was conducted.

2.2.5 The effect of KB-R7943 on Bay K8644-induced constriction in rat aortic rings

Bay K8644, the L-type Ca\textsuperscript{2+} channel opener, was freshly made in ethanol as a stock solution (1 mM), kept on ice and further diluted with PSS. The experiment was carried out under light-protective environment: with no direct light exposure, the organ baths were wrapped with aluminum foil (Asano \textit{et al.}, 1986; Wanstall \textit{et al.}, 1989).

See further in 2.3.3, Bay K8644 can acts as a L-type Ca\textsuperscript{2+} channel activator only under depolarized or partially depolarized conditions (Asano \textit{et al.}, 1999; Bechem \textit{et al.}, 1987;
Ferrante et al., 1989; Schramm et al., 1983). Preliminary experiments in normal PSS-A produced variable constriction to Bay K8644. Therefore, K⁺-enhanced PSS-D (see 2.2.2.4) was used throughout the experimental period. PSS-D had the same composition with normal PSS, except equimolar substitution of 10 mM NaCl with 10 mM KCl, the total KCl concentration = 15.73 mM. Under the environment of K⁺-enhanced solution, because the endothelium-denuded aortae were partially constricted, the testing of 0.5-gram tension increment to 1 μM phenylephrine was not used.

The cumulative phenylephrine concentration-effect curve was conducted. Following 20 minutes pretreatment of either vehicle or KB-R7943 (1 μM or 10 μM), Bay K8644 concentration-response curve was performed (10 nM -1 μM) in a cumulative manner.

2.2.6 The effect of KB-R7943 on high K⁺-induced constriction in rat aortic rings

PSS-E containing 80 mM KCl was prepared by replacing NaCl with equimolar KCl (see 2.2.2.5). The aorta bathing solution was replaced twice with this high (80 mM) K⁺ solution. After the high K⁺-induced constriction reaches its peak; the tissue was washed and re-equilibrated. To examine the effects of KB-R7943 on contraction elicited by a depolarizing concentration of K⁺, the endothelium-denuded rat aortic rings were preincubated with KB-R7943 (1 μM or 10 μM), and then challenged with 80 mM KCl the second time for 1h. All values were normalized as the percentage of the maximum constriction from the 1st round of 80 mM KCl treatment.
2.2.7 The effect of KB-R7943 on CPA-induced constriction in rat aortic rings

To assess the effect of KB-R7943 on constriction caused by release of Ca$^{2+}$ from intracellular stores, we studied the effect of KB-R7943 (1 µM or 10 µM) on constriction induced by cyclopiazonic acid (CPA), a sarcoplasmic reticulum (SR) Ca$^{2+}$ ATPas inhibitor in Ca$^{2+}$-containing solution.

A preliminary phenylephrine concentration-response curve (0.01 µM-10 µM) was carried out, after which vessels were washed with PSS-A and re-equilibrated. Then the tissue was incubated with either vehicle or KB-R7943 (1 µM) or KB-R7943 (10 µM) for 20 min, followed by the addition of CPA (10 µM) for 2h.

2.2.8 The effect of KB-R7943 on low-Na$^+$ induced constriction in rat aortic rings

To estimate the capacity of KB-R7943 to inhibit the forward mode of NCX, the extracellular Na$^+$ was withdrawn. Lower Na$^+$ PSS-F was obtained by replacing 118 mM NaCl with isosmolar amounts of Choline Chloride (see 2.2.2.6), the final concentration of [Na$^+$] was 25 mM. Following 20-minute pretreatment of either vehicle or KB-R7943 (10 µM), vessel was changed to 25 mM Na$^+$ PSS-F, and observed for 30 minutes.

2.2.9 The effect of KB-R7943 on ouabain-induced constriction in rat aortic rings

In some experiments in endothelium denuded aorta, constriction was induced by ouabain (100 µM). KB-R7943 (1 µM) was administered 20 minutes before the application of
Ouabain.

2.2.10 Drugs and materials

The following drugs were used: L- Phenylephrine Hydrochloride, Acetylcholine Chloride, (S) (-) Bay K8644 and 9-(2-tetrahydrofuryl) adenine (SQ 22536) from Sigma (St Louis, USA), Cyclopiazonic acid (CPA) from RBI (Frederick, MD, USA), Verapamil-HCl from BIOMOL (Plymouth Meeting, USA), 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl] isothiourea mesylate (KB-R7943 mesylate), from TOCRIS (Ellisville, USA), 2-[4-[(2,5-difluorophenyl)methoxy]-5-ethoxyaniline (SEA0400), a gift from Taisho Pharmaceutical Co., Ltd. (Saitama, Japan). Phenylephrine and acetylcholine were dissolved in distilled water, and the remaining drugs were dissolved in DMSO at 10 mM as a stock solution, and further diluted in PSS prior to use. The final concentration of vehicle was no more than 0.1% (volume/volume). Buffer salts and chemicals were obtained from Sigma (St Louis, USA) unless otherwise stated, include: NaCl, NaHCO₃, KCl, KH₂PO₄, MgSO₄·7H₂O, D-(†)Glucose, CaCl₂·2H₂O, L-ascorbic acid, Na₂EDTA, EGTA, Choline Chloride.

2.2.11 Data analysis

All data were collected by a computerized data acquisition system (MacLab). Results are
expressed as mean ± standard error of the mean, and were considered different when P values were less than 0.05. Multiple comparisons were determined using analysis of variance in the GbStat package (version 7.0, Dynamic Microsystems, Inc., Silver Spring, MD, USA). One ring obtained from one animal was used for each experiment type, and therefore the number of experiments (n value) indicates the number of animals. Each aorta generated multiple rings (normally 4) to minimize the number of animals used.

The number of animals used is not relevant except for reporting purposes for animal welfare. In all cases the number of different rings in each experimental group is recorded with the data in the thesis.
2.3 RESULTS

2.3.1 The effect of KB-R7943 on the vascular responsiveness to phenylephrine in the presence of extracellular Ca\textsuperscript{2+} in rat aortic rings

In rat denuded aortic rings, two concentration-response curves to phenylephrine (1 nM-10 µM) were performed. When KB-R7943 (10 µM) was present for the second curve, the phenylephrine constriction was significantly reduced (Figure 2.5), compared to vehicle controls. A similar effect was observed in endothelium-intact aortic rings (Figure 2.6).

2.3.2 The effect of KB-R7943 on the vascular responsiveness to phenylephrine in the absence of extracellular Ca\textsuperscript{2+} in rat aortic rings

In Ca\textsuperscript{2+}-free PSS with EGTA (10 mM), the phenylephrine-induced constriction was significantly smaller than that in the presence of Ca\textsuperscript{2+}, see table 2.4. The transient contractions induced by phenylephrine in the absence of Ca\textsuperscript{2+} was not significantly affected by the application of KB-R7943 (10 µM) in both endothelium-denuded (Figure 2.7) and intact vessels (Figure 2.8).
Figure 2.5  Effect of KB-R7943 (10 µM) on phenylephrine concentration-response curve in endothelium-denuded rat aortic rings

Two concentration-response curves to phenylephrine (1 nM-10 µM) were performed, and the responses in curve 2 were expressed as a percentage of the maximum in curve 1. Each point demonstrates mean ± s.e.mean. The absolute value of maximum constriction was shown in table 2.4. KB-R7943 (10 µM, n=7) or control (DMSO, 0.1%, n=5) was present for curve 2. * represents a significant difference between curves (CON and KBR), P<0.05, two way analysis of variance with repeated measures.
<table>
<thead>
<tr>
<th>Experimental solution</th>
<th>Endothelium Denuded(g)</th>
<th>n</th>
<th>Endothelium Intact(g)</th>
<th>n</th>
<th>Constrictor</th>
<th>Figure</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSS-A</td>
<td>0.99 ± 0.11</td>
<td>6</td>
<td></td>
<td></td>
<td>Phenylephrine</td>
<td>2.11</td>
</tr>
<tr>
<td>PSS-B</td>
<td>1.32 ± 0.08</td>
<td>5</td>
<td>1.02 ± 0.23</td>
<td>5</td>
<td>Phenylephrine</td>
<td>2.5; 2.6</td>
</tr>
<tr>
<td>PSS-C</td>
<td>0.20 ± 0.03</td>
<td>5</td>
<td>0.27 ± 0.05</td>
<td>5</td>
<td>Phenylephrine</td>
<td>2.7; 2.8</td>
</tr>
<tr>
<td>PSS-D</td>
<td>1.11 ± 0.08</td>
<td>7</td>
<td></td>
<td></td>
<td>Bay K8644</td>
<td>2.9</td>
</tr>
<tr>
<td>PSS-E</td>
<td>3.02 ± 0.16</td>
<td>7</td>
<td></td>
<td></td>
<td>80 mM K⁺</td>
<td>2.10</td>
</tr>
</tbody>
</table>

Table 2.4 Raw data of maximal constriction to vasoconstrictors in control group

In PSS-B and PSS-C, both endothelium denuded and intact vessels were tested. In PSS-D, PSS-E, PSS-A, only endothelium denuded vessels were tested. The above raw data were the values in the presence of vehicle (0.1% volume/volume) instead of KB-R7943. Maximum constriction was achieved respectively during first phenylephrine concentration-response curve (1 nM – 10 µM) in PSS-B and PSS-C; phenylephrine concentration-response curve (10 nM – 10 µM) in PSS-D; 80 mM KCl treatment for 20 minute in PSS-E; and phenylephrine concentration-response curve (10 nM -10 µM) in PSS-A. See sections 2.2.2.1 - 6 for PSS composition.
Figure 2.6   The effect of KB-R7943 on phenylephrine concentration-response curve in endothelium-intact rat aortic rings

Two concentration-response curves to phenylephrine (1 nM-10 µM) were performed, and the responses in curve 2 were expressed as a percentage of the maximum in curve 1. Each point represents mean ± s. e. mean. The absolute value of maximum constriction was shown in table 2.4. KB-R7943 (10 µM, n=7) or control (DMSO, 0.1%, n=5) were present for curve 2. * represents a significant difference between curves (CON and KBR), P<0.05, two way anova with repeated measures.
Figure 2.7  The effect of KB-R7943 on phenylephrine concentration-response curve in the absence of extracellular calcium in endothelium-denuded rat aortic rings.

Two concentration-response curves to phenylephrine (10 nM -10 μM) were performed, and the responses in curve 2 were expressed as a percentage of the maximum in curve 1. Each point represents mean ± s. e. mean. The absolute value of maximum constriction was shown in table 2.4. KB-R7943 (10 μM, n=8) or control (DMSO, 0.1%, n=5) was present for curve 2. There were no significant differences between curves (CON and KBR) (P>0.05), two way analysis of variance.
Figure 2.8  The effect of KB-R7943 on phenylephrine concentration-response curve in
the absence of extracellular calcium in endothelium-intact rat aortic rings

Two concentration-response curves to phenylephrine (10 nM -10 µM) were performed, and
the responses in curve 2 were expressed as a percentage of the maximum in curve 1. Each
point represents mean ± s. e. mean. The absolute value of maximum constriction was shown
in table 2.4. KB-R7943 (10 µM, n=5) or control (DMSO, 0.1%, n=5) was present for curve
2. There were no significant differences between CON and KBR (P>0.05), two way analysis
of variance.
2.3.3 The effect of KB-R7943 on Bay K8644 vasoconstriction in rat aortic rings

In order to evaluate the involvement of L-type calcium channels in response to KB-R7943, constriction was evoked using the L-type Ca\(^{2+}\) channel opener: Bay K8644. In preliminary experiments, Bay K8644 produced an inconstant constriction, therefore, further experiments were conducted with a K\(^+\) enhanced PSS-D (2.2.2.4) to partially depolarize the aorta (as has been previously reported) (Asano et al., 1999; Bechem et al., 1987; Ferrante et al., 1989; Schramm et al., 1983). Cumulative addition of Bay K8644 (10 nM to 1 \(\mu\)M) in denuded aortic rings induced an increase in contractile force, see table 2.4 for absolute grams. KB-R7943 (10 \(\mu\)M) significantly antagonized the effect of Bay K8644 (Figure 2.9). However, KB-R7943 (1 \(\mu\)M) had no significant effect (Figure 2.9).

2.3.4 The effect of KB-R7943 on K\(^+\) vasoconstriction in rat aortic rings

KCl (80 mM) was used to constrict endothelium denuded rat aortic rings. The absolute constriction in grams is shown in table 2.4. KB-R7943 (10 \(\mu\)M, Figure 2.10) significantly inhibited the KCl depolarization-elicited constriction, whereas KB-R7943 (1 \(\mu\)M, Figure 2.10) had no significant effect.
Figure 2.9  The effect of KB-R7943 on Bay K8644 -induced constrictions in endothelium-denuded rat aortic rings

The concentration-response curve to phenylephrine (10 nM-10 µM) was performed. Then the responses of Bay K8644 were examined. Constriction was expressed as a percentage of the maximum in the curve of phenylephrine. Each point represents mean ± s. e. mean. KB-R7943 (1 µM, n=7 or 10 µM, n=7) or control (DMSO, 0.1%, n=7) was present for the curve of Bay K8644. * represents a significant difference between curves (CON and KBR 10), P<0.05, two way anova with repeated measures.
Figure 2.10 The effect of KB-R7943 on 80 mM KCl-induced constrictions in endothelium-denuded rat aortic rings

Constriction in the second application of KCl was expressed as a percentage of the initial KCl-induced constriction. Each point represents mean ± s. e. mean. The absolute value of maximum constriction was shown in table 2.4. KB-R7943 (1 µM, n=5; or 10 µM, n=7) or control (DMSO, 0.1%, n=5) was present for each curve. * represents a significant difference between curves (CON and KBR 10), P<0.05, two way anova with repeated measures.
Figure 2.11 The effect of KB-R7943 on CPA-induced constrictions in endothelium-denuded rat aortic rings

The concentration response curve to phenylephrine was performed then a response to cyclopiazonic acid (CPA) was measured. Constriction to CPA was expressed as a percentage of the maximum in curve 2 to phenylephrine. Each point represents mean ± s. e. mean. The absolute value of maximum constriction was shown in table 2.4. KB-R7943 (1 µM, n=6; or 10 µM, n=6) or control (DMSO, 0.1%, n=6) was present for each curve. * represents a significant difference between curves (CON and KBR 10), P<0.05, two way anova with repeated measures.
2.3.5 The effect of KB-R7943 on CPA-induced constriction in rat aortic rings

In endothelium-denuded rat aortic rings, the SERCA inhibitor CPA (10 μM) incubated in PSS containing Ca^{2+} produced a pronounced constriction (see table 2.4, n=6, P<0.01). Pretreatment with KB-R7943 (1 μM) did not influence the CPA-induced constriction (Figure 2.11), whereas, at higher concentration, KB-R7943 (10 μM) markedly attenuated the response (Figure 2.11).

2.3.6 The effect of KB-R7943 on low-Na^{+} induced constriction in rat aortic rings

In endothelium denuded rat aortic rings reducing [Na^{+}]_{o} from 143 mM to 25 mM induced immediate constriction, see (Table 2.4) for the absolute value of constriction. The maximum responses were seen within 8 minutes. At the concentration of 10 μM, KB-R7943 significantly inhibited the low-Na^{+} induced effects (Figure 2.12).

2.3.7 The effect of KB-R7943 on ouabain-induced constriction in rat aortic rings

Ouabain (100 μM) elicited a long-lasting constriction in endothelium-denuded rat aortic rings (Figure 2.13). KB-R7943 (1 μM) showed significant inhibitory effect on ouabain-induced constriction (Figure 2.13).
Figure 2.12 The effect of KB-R7943 on low Na\(^+\)-induced constrictions in endothelium-denuded rat aortic rings

In each ring there was one cycle of constriction to low [Na\(^+\)]. Each point represents mean ± s.e. mean. The number of experiments ranged from 5 to 11 different rings in each group. * represents a significant difference between CON and KBR, P<0.05, two way anova with repeated measures.
Figure 2.13  The effect of KB-R7943 on ouabain-induced constrictions in endothelium-denuded rat aortic rings

In each ring there was one cycle of constriction to ouabain. Each point represents mean ± s.e. mean. The number of experiments ranged from 4 to 5 different rings in each group. * represents a significant difference between CON and KBR, P<0.05 two way analysis of variance.
2.4 DISCUSSION

In view of conflicting data about selectivity for NCX, preliminary experiments were conducted to characterize the actions of the NCX inhibitor KB-R7943 in rat aortic rings. The aim was to elicit constriction by a variety of mechanisms known to involve multiple Ca^{2+} mechanisms and examine two concentrations of KB-R7943: 10 μM where non-selective actions have been suggested and 1 μM that in the literature appears selective (see Introduction 2.1.7).

Phenylephrine is a relatively selective α_{1}-adrenoceptor agonist (Guimaraes et al., 2001; Nishimaru et al., 2001; Toma et al., 1995). It is well established that α_{1}-adrenoceptors induce biphasic constriction in rat aortic rings with an initial phasic constriction followed by sustained tonic constriction (Tanaka et al., 2000). The initial triggering underlying the phasic constriction is the agonist-receptor binding and the following production of inositol 1,4,5-trisphosphate (IP₃) after cleavage of PIP₂ by phospholipase C, which activates the internal calcium release from sarcoplasmic reticulum (See reviews Dimopoulos et al., 2007; Williamson et al., 1985). The concomitant production of DAG may activate PKC and subsequent protein kinase C signaling cascades to maintain constriction. The inhibition of myosin light chain phosphatase (MLCP) and the increased Ca^{2+} sensitivity are currently thought to play as important role as the Ca^{2+}–dependent activation of myosin light chain kinase (MLCK) in the sustained tonic contraction (refer to 1.1.4) (See reviews Dimopoulos et al., 2007; Lincoln, 2007; Murthy, 2006).
In the present study we examined the steady phase constriction produced by phenylephrine which contains all components of the $\alpha_1$-adrenoceptor signaling. In the present study the NCX inhibitor KB-R7943 (10 $\mu$M) inhibited the constriction to phenylephrine in rat aortic rings with or without endothelium. This could indicate either that KB-R7943 was affecting some of the phenylephrine signaling or that NCX was involved in the $\alpha_1$-adrenoceptor response. This latter observation is perhaps unlikely since under normal conditions NCX should be mediating Ca$^{2+}$ exit from the smooth muscle (Schweda et al., 2001) as it normally operates in forward mode and so inhibition would have expected to enhance constriction. On the face of it at this concentration of 10 $\mu$M it is possible that KB-R7943 is non-selective for NCX. Indeed in acutely isolated rat hippocampal neurons, guinea-pig ventricular myocytes, guinea pig single cardiac ventricular cells, KB-R7943 has been reported to inhibit multiple calcium-related mechanism (Sobolevsky et al., 1999; Tanaka et al., 2002; Watano et al., 1996). It should be noted that KB-R7943 inhibited phenylephrine constriction also in the absence of endothelium in the present study. Thus endothelial release of vasoactive substances is not involved.

In order to isolate further the effect of KB-R7943, phenylephrine constriction was carried out in the absence of extracellular Ca$^{2+}$, this should highlight the effects to the intracellular signaling pathways involving Ca$^{2+}$ release from internal stores. In rat aortic rings, stimulation of the $\alpha_1$-adrenoceptors by phenylephrine induced a transient constriction in Ca$^{2+}$-free solution, this has previously been shown to involve a transient increase in [Ca$^{2+}$]$_i$ due to Ca$^{2+}$ release from intracellular Ca$^{2+}$ pools by the activation of IP$_3$ receptor on sarcoplasmic reticulum (Carafoli, 1987; Iwata et al., 2001; Marin et al., 1999; Tanaka et al., 2000). The
phenylephrine-induced transient increase in muscle tension was not affected by KB-R7943 (10 μM). This result suggests that 10 μM KB-R7943 may have little or no effect on the signal transduction of the α1-adrenoceptor IP3-induced Ca2+ release mechanism. Indeed other workers have found no effect on components of this system at this concentration or higher in cultured astrocytes (Matsuda et al., 2001). This may suggest an effect of KB-R7943 on Ca2+ entry in terms of α1-adrenoceptor constriction.

Another way to look at internal Ca2+ release mechanisms is to use cyclopiazonic acid (CPA). CPA is an indole tetrameric acid mycotoxin, was first produced from Penicillium Cyclopium Westling (Holzapfel, 1968). CPA can inhibit the ATP-dependent Ca2+ pumps of the sarcoplasmic reticulum (SR) or endoplasmic reticulum (ER) without affecting ATP-driven Ca2+ transport in plasma membrane (see 1.1.3.2). Treatment with CPA depletes stored [Ca2+] in the SR and increases cytosolic [Ca2+], a net effect presumably due to an endogenous Ca2+ leak while SR calcium reuptake is blocked (Seidler et al., 1989). It has been widely used in physiological studies since it can mimic the Ca2+-mobilization action of an agonist, an effect that is independent of both inositol phosphate production and stimulation of protein kinase C. In endothelium denuded rat aortic rings, KB-R7943 (10 μM) depressed CPA-stimulated constriction. Depletion of intracellular calcium stores in the SR/ER could activate Ca2+ influx into the cell through store-operated Ca2+ channels (SOC) (refer to 1.3). Our results suggest that KB-R7943, at high concentration (10 μM), may influence SOC activation caused by the Ca2+ store depletion, as reported previously (Arakawa et al., 2000). However, in our hands KB-R7943 at a lower concentration (1 μM) had no such effect suggesting that this concentration may be a useful tool.
Ca\textsuperscript{2+} influx through L-type Ca\textsuperscript{2+} channels was studied directly using Bay K8644, a dihydropyridine derivative, which activates the channel (Asano et al., 1986; Cohen et al., 1997; Ferrante et al., 1989; Tsien et al., 1986). The S and R enantiomers of Bay K8644 exert the opposing effect on the voltage sensitive slow calcium channel. In the present study, the S enantiomer was used.

It has been previously shown that Bay K8644 did not induce a constriction in resting condition of normal physiological saline solution unless the K\textsuperscript{+} concentration in the bathing solution was increased to 15 mM and tissue was partly depolarized (Asano et al., 1999; Schramm et al., 1983). Membrane depolarization increases Ca\textsuperscript{2+} influx by opening the L-type Ca\textsuperscript{2+} channel to elevate [Ca\textsuperscript{2+}], and finally induces constriction (Karaki et al., 1984; Preuss et al., 1985; Yu et al., 1991). In the current study, we observed comparatively less responses of Bay K8644 in normal PSS than 15.73 mM K\textsuperscript{+}-depolarizing PSS (data not shown) and so adopted the protocol in all experiments that 15.73 mM K\textsuperscript{+}-depolarizing PSS was used.

In the present experiments in endothelium denuded rat aortic rings, KB-R7943 (10 \textmu M) decreased the Bay K8644-evoked constriction but KB-R7943 had no effect at 1 \textmu M. This agrees with the study in cardiac ventricular cells of guinea pig which suggests that KB-R7943 inhibits L-type Ca\textsuperscript{2+} channels at high concentrations (\textgeq 7 \mu M) (Watano et al., 1996). But the current data suggest that at 1 \mu M this may not be an issue.

The preceding data discussed the non-NCX actions of KB-R7943 but it is also important to determine whether KB-R7943 can inhibit NCX mediated events. In endothelium denuded
rat aortic rings, lowering extracellular [Na⁺] to 25 mM induced a vasoconstriction. This constriction has been shown by many workers to be due to Ca²⁺ entry via NCX (Bova et al., 1990; Kim et al., 1999; Reuter et al., 1973; Schweda et al., 2001). KB-R7943 (10 μM) inhibited the vasoconstriction. Further in the next chapter 3.3.1 we demonstrated that KB-R7943 (1 and 10 μM) inhibited constriction of rat aortic rings induced by low Na⁺.

Another means of activating NCX is to inhibit the smooth muscle Na/K-ATPase that reverses NCX action by causing a build up of intracellular Na⁺ and causing the inflow of Ca²⁺. This in part explains the constrictions to ouabain (Ashida et al., 1987; Espinosa-Tanguma et al., 2004) although other mechanisms are also involved, such as sympathetic nerve activation (Karaki et al., 1977). In the present study, ouabain constriction in endothelium denuded rat aortic rings was partially inhibited by KB-R7943 (1 μM). The effectiveness of 1 μM suggests that KB-R7943 may be a useful tool.

The present research provided extensive investigation data of KB-R7943 in rat aortic rings. Our data showed that 10 μM KB-R7943 inhibited smooth muscle constriction by inhibiting calcium entry through L-type Ca²⁺ channel. Likewise, 10 μM KB-R7943 may affect the capacitative Ca²⁺ entry pathway. Nevertheless, KB-R7943, at the concentration that inhibited NCX (1 μM), did not affect the function of L-type Ca²⁺ channel, store-operated channel, and α adrenergic receptor. In conclusion, KB-R7943 can be used as a selective inhibitor of NCX at the concentration of 1 μM in rat aortic rings. The aorta was used as a simple model of vascular smooth muscle and it is assumed that the results are translatable to other tissues. Indeed in all previous studies with KB-R7943 reports of non selective actions occur in
concentrations in excess of this (see Table 2.2).
CHAPTER 3 THE EXISTENCE AND FUNCTION OF THE Na⁺/Ca²⁺ EXCHANGER IN RAT AORTAE

3.1 INTRODUCTION

3.1.1 Vascular endothelium

The walls of arteries and veins are composed of three distinct layers: tunica intima (the innermost layer), tunica media (the middle layer) and tunica adventitia (the outermost layer) (see Figure 3.1) (Shepro et al., 1974; Tortora et al., 1981).

The tunica intima is composed of endothelium underlain by a subendothelial layer of loose connective tissue, and the internal elastic lamina at its outer surface. The monolayer of endothelium lines the lumen of all vessels and separates the blood vessel from continuously flowing blood.

The tunica media essentially comprises spirally arranged smooth muscle cells and sheets of elastin. The outer border is marked by external elastic lamina. This is the bulkiest layer in arteries, rich in vasomotor fibers that can cause vasoconstriction and vasodilation.

The tunica adventitia is composed of a mixture of elastic and collagenic fibers; the latter protects the blood vessel from overexpansion and also contains axons of the vasomotor fibers.
Figure 3.1 The structure of a muscular artery.

The illustration shows the structure of a typical arterial wall which consists of three layers: tunica intima, tunica media and tunica adventitia.
The vascular endothelium lines the blood vessel lumen and is composed of a monolayer of endothelial cells. Like other eukaryotic cells in the human body, endothelial cells have a nucleus, cytoplasm and all the organelles inside it, enclosed by a double-layer phospholipid membrane. There is a variety of contractile proteins in the cytoplasm including actin, myosin, tropomyosin, and α-actin. Some specialized structures made of contractile proteins comprise cortical web, the junction-associated actin filament system and the striated myofibril-like filament bundles or stress fibers (Esper et al., 2006).

There is broad agreement that the vascular endothelium plays a pivotal role in the basic and dynamic control of circulation (Loscalzo et al., 1995). Sir John Vane named vascular endothelium ‘the maestro of blood circulation’ (Gryglewski, 2005).

Located on the front line of blood vessel, endothelium acts as a sensor to chemical and physical stimuli. The delicate role of endothelium in circulatory control relies on its ability to release substances in response to the physiological stimuli (Esper et al., 2006; Verma et al., 2003). One of the most common stimuli is shear stress, which is produced by the increased blood velocity. Endothelium responds with nitric oxide (NO) release and subsequent vasodilatation (refer to 3.1.2.1 and 3.1.2.2).

Endothelial cells can release a variety of chemical substances. Among them, the most prominent vasoconstrictors are endothelin and thromboxane A₂, the most prominent vasodilators are nitric oxide, endothelium-derived hyperpolarizing factor (EDHF) and prostacyclin (PGI₂), (see reviews Bredt et al., 1994; Denninger et al., 1999). Under normal physiological condition, there is a balance of endothelium-derived vasoactive substances
(Spieker et al., 2005; Verma et al., 2003). However, the endothelium predominantly releases substances to maintain the underlying smooth muscle in a relaxed state.

When the endothelium is impaired (which is called “endothelial dysfunction”), the pathological states occur (Rikitake et al., 2005). For example, lipids and leukocytes invade the endothelium causing an inflammatory response, platelets aggregation, smooth muscle thrombogenesis or vascular occlusion. (See review Esper et al., 2006). Jones et al reported that endothelium release of NO maintains basal vascular tone, vasodilator reserve and resistance distribution, thereby endothelium dysfunction might cause hypertension (Jones et al., 1995). Apart from physical damage, endothelial cell dysfunction can manifest itself by impairment of release of the various chemical mediators and defects in endothelium-induced NO-mediated vasodilation have been shown in the condition of hypertension, diabetic angiopathies, reperfusion injury, hypercholesterolaemia and atherosclerosis (Dinerman et al., 1993; Gryglewski, 2005; Semenza, 2005; Shelkovnikov et al., 2004; Spieker et al., 2005), (see review Loscalzo et al., 1995; Ruehlmann et al., 2000).

3.1.2 Nitric oxide (NO)

What was originally described as endothelium-derived relaxing factor (EDRF) is now identified to be nitric oxide (NO) (Gryglewski, 2005).
3.1.2.1 Production

L-arginine, an endogenous amino acid, was discovered by Schultze in 1886. It attracts intense attention as the only substrate for the production of NO (Akopova et al., 2002; Wileman et al., 2003), Arginine has other functions in ammonia detoxification, the urea cycle, the formation of active enzyme centers, and the precursor of proteins, ornithine, urea and creatinine (Cylwik et al., 2005).

The initial stimulus for endogenous NO release comes from the deformation of endothelium by mechanical force such as shear stress or stretch, or the activation of endothelial receptor by agonist such as acetylcholine or bradykinin (see 3.1.2.2.2). Upon stimulation, which is generally due to the elevation of intracellular Ca$^{2+}$, nitric oxide synthase (NOS) catalyses the formation of NO from arginine (Binko et al., 1998; Molin et al., 2004). The chemical reaction is shown in Figure 3.2. NOS oxidizes the guanidine group of L-arginine in a process that consumes five electrons and gives rise to NO with stoichiometric formation of L-citrulline (see Figure 3.2).

3.1.2.2 Nitric oxide synthase (NOS)

(1) Isoforms of NOS

There are three known NOS isoforms: one inducible NOS and two constitutive ones. All of them contain heme, are dioxygenases and NADPH-dependent. NOS cofactors include
Figure 3.2  Chemical reaction for the production of nitric oxide

One molecule of arginine is oxidized by NOS producing one molecule of nitric oxide and one molecule of citrulline.
NADPH, flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN) (Bredt et al., 1994; Martinez-Lemus et al., 2005).

Constitutive NOS comprises two kinds: neuronal (nNOS) and endothelial (eNOS) NOS, (see review Semenza, 2005). nNOS is mainly found in neurological tissue and encode by NOS-I. NOS-III encodes for eNOS that exists mainly in vascular endothelium and smooth muscle. Inducible NOS (iNOS) is expressed by inflammatory processes especially in macrophages, neutrophils and endothelial cells, encode by human NOS-II gene (Esper et al., 2006). Both constitutive NOS and inducible NOS are found in endothelial cells.

Whilst constitutive NOS (eNOS, nNOS) are Ca^{2+}/calmodulin dependent, iNOS is not. Constitutive NOS is activated by the augmented level of intracellular calcium induced by agonists (acetylcholine, bradikinin etc) producing NO for a short period. Inducible NOS is immunologically synthesized by cells after exposure to bacterial endotoxin (such as lipopolysaccharide) or pro-inflammatory cytokines and produces large quantities of NO for a longer period (Jones et al., 1995; Loscalzo et al., 1995; Muller et al., 1998; Ruehlmann et al., 2000; Wileman et al., 2003).

(2) Regulation of NOS

As a lipid-soluble and unstable gas, NO can not be stored thus biosynthetic regulation is important for NO. NOS is one of the most regulated enzymes. NOS enzymes can be discriminated by their regulation by calcium. In blood vessels,
acetylcholine binds to muscarinic receptors on endothelial cells to generate Ca\(^{2+}\), which stimulates NOS. Thus, the production of NO is under the control of calcium-regulated NOS (Bredt et al., 1994).

In the cardiovascular system, the constitutively expressed enzyme: endothelial nitric oxide synthase (eNOS) controls the production of NO. And the activity of eNOS is inactivated by a structural protein: caveolin-1 in a specialized area of the membrane: caveolin. Upon stimulation of endothelial receptors or deformation of the endothelium by sheer stress from blood flow, eNOS can be activated via an enhanced level of intracellular [Ca\(^{2+}\)] and/or protein kinase stimulation (Bryan et al., 2005). When intracellular Ca\(^{2+}\) increases, Ca\(^{2+}\) binds to calmodulin, Ca\(^{2+}\)/calmodulin complex dissociates the NOS-caveolin complex and activates eNOS (Schneider et al., 2002b).

3.1.2.3 NO donors

NO is soluble in water (2 – 3 mM), but the solutions of authentic NO are unstable and difficult to handle. As a result, chemical compounds that can generate NO in situ are desirable for researchers. Many nitrogen-oxygen-bonded compounds can be decomposed, oxidized or reduced to produce reactive nitrogen species. Diversity of NO donors has been developed including organic nitrates (e.g. nitroglycerine), organic nitrates (e.g. glyceryl trinitrate), metal-NO complexes (e.g. sodium nitroprusside), and nitrosothiols (e.g. SNAP) etc (Mulsch et al., 2001; Wang et al., 2002a). NO donors are used as NO delivery agents in disease states that are related to impaired expression or activity of endothelial NO (Li et al.,
They all produce vasodilatation.

Sodium nitroprusside (SNP) is a well-documented compound used as a NO carrier (Amoroso et al., 2000; Asano et al., 1995; Dainty et al., 1990; Perez-Vizcaíno et al., 1999; van der Zypp et al., 1998). Its structure is depicted in Figure 3.3. Although it is extremely photosensitive, it can be kept stable for many years in a dry and photo-protective environment. Its therapeutic properties are related to NO release which occurs via its redox conversion to the one or two electron-reduced forms. The requirement for light or reductant to the transition from SNP to NO is clear, although the exact mechanism remains obscure (Smith et al., 2001; Wolak et al., 2003). It is a potent, rapid and efficient hypotensive agent being used clinically to reduce blood pressure (Smith et al., 2001; Wang et al., 2002a; Wolak et al., 2003). However, its undesired side effect at high concentration can cause cellular toxicity via cyanide release (Wang et al., 2002a).

3.1.2.4 Inhibitors

As discussed in 1.1.2.1, L-arginine is the substrate for the formation of NO. L-\(\text{N}^\omega\)-substituted arginines are the substrate analogs acting as competitive NOS inhibitors. L-\(\text{N}^\omega\) nitroarginine (L-NNA) is the most potent known inhibitor of the nNOS and eNOS (\(K_i = 200-500\) nM), and L-\(\text{N}^\omega\) aminoarginine (L-NAA) is the most potent blocker of the iNOS (\(K_i = 1-5\) \(\mu\)M), (see review Bredt et al., 1994). In the present study, L-\(\text{N}^\omega\)-nitroarginine methyl ester (L-NAME) was used as the competitive inhibitor of eNOS (Mendizabal et al., 2000; Molin et al., 2004; Muller et al., 1998; Schuh et al., 2003).
Figure 3.3 Structure of SNP

The molecular formula of SNP is: $\text{Na}_2[\text{Fe(CN)}_5\text{NO}]$
3.1.2.5  The biological functions of NO

NO has been found to be a key signal agent playing a central role in the nervous, immune, and cardiovascular systems. Its wide spectrum of biological functions comprise smooth muscle relaxation, blood pressure regulation, neurotransmission, antithrombogenic, antiproliferative, leukocyte-adhesion inhibiting effects, and influences myocardial contractility (Denninger et al., 1999; Esper et al., 2006; Feelisch et al., 1999; Ignarro, 2002; Li et al., 2000; Spieker et al., 2005).

The dysfunction in the NO pathway (such as decreased NO production and increased degradation of NO) is related to vascular diseases: hypertension, diabetic angiopathy, hypercholesterolaemia and atherosclerosis, (see review Li et al., 2000).

Soluble guanylate cyclase is the principal target for NO. Guanylate cyclase can be classified as two isoenzymes: membrane-bound (particular) and cytosolic (soluble). The two proteins are quite distinct with different activation mechanisms. Whilst pGC is predominantly expressed in intestinal mucosa and retina, sGC in platelet, most tissues such as vascular smooth muscle contain both types. The main mode of action of NO is mediated via the stimulation of soluble guanylyl cyclase (sGC), which catalyses conversion of guanosine triphosphate (GTP) to cyclic guanosine-3', 5'–monophosphate (cGMP) (Denninger et al., 1999; Munzel et al., 2005; Spieker et al., 2005). The key target enzyme of NO is soluble guanylate cyclase (sGC) (Lau et al., 2004; Munzel et al., 2005). Both NO gas and NO released from NO donors were shown to activate sGC by binding to iron in the heme (Miki
et al., 1977; Schultz et al., 1977). Soluble GC family consists of isoforms GC-\(\alpha_{1-3}\)β\(_{1-3}\).

Mammalian particulate GC family consists of GC-A to GC-G, and retGC (Denninger et al., 1999). Unlike sGC, pGC is activated with agents such as enterotoxin (Guerrant et al., 1980), atrial natriuretic factor (ANF) (Waldman et al., 1984a; Winquist et al., 1984) and hemin (Waldman et al., 1984b). It is not NO reactive.

Actions of cGMP

Vasodilators (such as acetylcholine, bradykinin, or histamine) act at receptors on endothelial cells causing an influx of calcium to activate eNOS. The newly synthesized NO diffuses into adjacent smooth muscle cells where it activates GC and subsequently enhances the concentration of cGMP. The key second messenger (cGMP) has three major targets: cGMP-dependent protein kinases, cGMP-regulated phosphodiesterases (PDE\(_n\)), and cGMP-gated ion channels (See reviews Birschmann et al., 2004; Lincoln et al., 1993).

cGMP-dependent protein kinase is protein kinase G (PKG) (Lohmann et al., 1997). PKG is a homodimer that dissociation is not involved during activation. There are two isotypes of PKG that have been reported in mammalian tissues: type I and type II (Browner et al., 2004; Keef et al., 2001; Lincoln et al., 1993; Schroder et al., 2003). Type I PKG exists in a large amount in vascular smooth muscle cell, and includes two isoforms: type 1α and type 1β (Browner et al., 2004; Lincoln et al., 1993). Both type 1α and type 1β co-exist in rat aorta (Browner et al., 2004; Lincoln et al., 2001; Sekhar et al., 1992). Type II PKG exists mainly in intestine, kidney and brain (Lincoln et al., 1993; Lohmann et al., 1997).
Both cGMP and cAMP can activate PKG in VSMC (Pelligrino et al., 1998). PKG exerts its biological effect via the phosphorylation of serine and threonine residues on target proteins (Graves et al., 1999; Pelligrino et al., 1998). Although the exact mechanism by which cGMP induces vasodilatation via PKG is still not well understood, the following pathways are proposed: (1) acting on various ion transport systems, such as stimulating plasma membrane Ca\(^2+\) pump in rat aorta (Furukawa et al., 1988) and the sarcoplasmic reticulum Ca\(^2+\) pump in rat aorta (Twort et al., 1988), stimulating NCX in rat astrocyte (Asano et al., 1995), (see also review Lincoln et al., 1993). (2) altering muscle contractility, such as the phosphorylation of myosin kinase in bovine aorta (Fukuta et al., 1997; Hathaway et al., 1985). (3) reducing Ca\(^2+\) sensitivity of contractile system, such as reported in rat mesenteric artery (See reviews Nishimura, 2006; Nishimura et al., 1989).

The degradation of cGMP (and cAMP) is catalyzed by phosphodiesterases (PDEs) via the hydrolysis of the 3’-phosphodiester bond of cAMP and cGMP to yield AMP and GMP respectively (Beavo et al., 1990; Degerman et al., 1997; Houslay et al., 1997; Lo et al., 2005). In mammals, the PDE superfamily includes at least 10 isoforms: PDE1-PDE10. PDE5, PDE6 and PDE9 hydrolyze preferentially the cGMP rather than cAMP. PDE3 exhibits affinity to both cGMP and cAMP (Degerman et al., 1997).

The guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) was also used to block the cGMP-dependent effect of NO (Perez-Vizcaino et al., 1999; Shelkovnikov et al., 2004; Tsang et al., 2003).

In addition to the cGMP-mediated action, NO has been reported to have direct effects on
vascular reactivity without requiring cGMP (Feelisch et al., 1999; Trottier et al., 1998). For example, NO was reported to activate directly tetrabutylammonium (TBA)-sensitive K\(^+\) channels in rat aorta (Muller et al., 1998); and activate calcium-dependent potassium channels (BK\(_{Ca}\)) in vascular smooth muscle (Bolotina et al., 1994).

NO has an unpaired electron in its outer electron shell, and therefore possesses the radical character. As a free radical, NO can easily react with other members in the free radical family, such as superoxide anion (OO\(^-\) or O\(_2\)\(^-\)). O\(_2\)\(^-\) can be produced under oxidative stress in the vascular wall, such as ischaemia-reoxygenation. O\(_2\)\(^-\) quickly inactivates NO and gives rise to peroxynitrite (ONOO\(^-\)), chemical reaction is described below.

\[
\text{O}_2^- + \text{NO} = \text{ONOO}^- 
\]

ONOO\(^-\) is a potent oxidant and nitrating agent that can destroy proteins, lipids, and nucleic acids, trigger apoptosis of cell (Chakraborti et al., 1999), (see also review Dowell et al., 1997). Accelerated breakdown of NO and the formation of O\(_2\)\(^-\) occurs in atherosclerosis in rat aorta (Minor et al., 1990). Owing to the central role of NO in the regulation of the vascular system, the decomposition of NO by OO\(^-\) could have tremendous pathological effects in the vascular system (Bredt et al., 1994; Li et al., 2000; Maxwell, 2002).

3.1.3 Prostacyclin

Prostacyclin can be produced by endothelial cells. Prostacyclin was discovered in 1976 by a group of scientists while searching for a drug producing thromboxane A\(_2\) (TXA\(_2\)) from
prostaglandin endoperoxides (PGG₂ or PGH₂) (Gryglewski et al., 1976; Moncada et al., 1976). The common precursor for prostanoids is arachidonic acid (AA). The chemical reaction is as below (see Figure 3.4).

Upon stimulation of hormones (such as arginine vasopressin), cytosolic lipases (e.g. phospholipase A₂, phospholipase C, phospholipase D) catalyze the biosynthesis of arachidonic acid from plasma membrane phospholipids. Arachidonic acid has two metabolic fates. It is either converted into leukotrienes by lipoxygenases or by cyclooxygenase-1 and cyclooxygenase-2 (COX1/2) into prostaglandin G₂ (PGG₂) and further into prostaglandin H₂ (PGH₂). PGH₂ is the common precursor for a series of prostanoids (PGs): PGD₂, prostacyclin (PGL₂), PGE₂, PGF₂α and thromboxane (TXA₂) which are formed by specific synthases. Prostanoids exert their effects through G protein-linked receptors, (see reviews Hanoune et al., 1997; Smith, 1992).

Endothelial COX-2/PGIS generates vasoprotective prostacyclin (PGI₂) causing platelet inhibition, vasodilation, and anti-inflammation. In distinct contrast to the former, COX-1/TXAS produces vasotoxic thromboxane (TXA₂) inducing platelet aggregation and vasoconstriction (Becker, 2005; Cheng et al., 2002). Nonsteroidal antiinflammatory drugs (NSAIDs), for example indomethacin and aspirin indiscriminately inhibit COX1 and COX2 (Perez-Vizcaíno et al., 1999; Vane et al., 1987). The inhibition of COX2 renders its therapeutic effects whereas the inhibition of COX1 provides the side effect of gastrototoxicity and bleeding (see review McAdam et al., 1999).

The key target of prostacyclin is prostacyclin receptor which activates adenylate cyclase by a
G-protein dependent process (Hanoune et al., 1997; Schwede et al., 2000; Wedel et al., 1997). Adenylate cyclase (ACs) catalyzes the transition of adenosine triphosphate (ATP) to cyclic adenosine-3', 5'-monophosphate (cAMP).

Similar to cGMP, the level of cAMP is controlled not only by its synthesis via adenylate cyclase, also by its breakdown via PDEs as well (Bielefeldt, 1999; Lo et al., 2005). Amongst the 10 members in PDE superfamily, PDE4, PDE7, and PDE8 are specific for cAMP. cGMP-stimulated PDE2 and cGMP-inhibited PDE3 render the crosstalk between two kinds of cyclic nucleotides.

The target protein for cAMP is protein kinase A (PKA). PKA is a tetramer, including two catalytic subunits and two regulatory subunits. cAMP can act on the regulatory subunits and dissociate them causing the activation of PKA (Pelligrino et al., 1998). Both isoforms of PKA (PKA-I and PKA-II) are expressed in vascular smooth muscle. PKA exerts its effect via the phosphorylation of serine and threonine residues on target proteins (Graves et al., 1999; Pelligrino et al., 1998). cAMP produces a vasodilatation, and cAMP-producing drugs such as β-adrenergic hormones and forskolin presumably induce vasodilation through PKA by decreasing [Ca^{2+}], as well as sensitivity of the contractile apparatus to Ca^{2+} (Abe et al., 1989; Nishimura et al., 1989). cAMP/PKA was reported to decrease vasoconstriction via activating Ca^{2+}-activated K^{+} channels (Sadoshima et al., 1988), stimulating Ca^{2+} uptake into the intracellular stores (Mueller et al., 1979), and increasing sarcolemmal Ca^{2+} pump activity (Bulbring et al., 1987). Similar to cGMP, cAMP was reported to alter muscle contractility, such as the phosphorylation of myosin kinase in bovine aorta (Hathaway et al., 1985).
Figure 3.4  Biosynthesis of prostanoids

Phospholipases catalyzes the hydrolysis of phospholipid to form arachidonic acid. Under inflammatory condition, arachidonic acid is converted into leukotrienes. Under physiological condition, COX converts arachidonic acid into endoperoxides (PGG$_2$ or PGH$_2$). Finally, PGH$_2$ is transformed into TXA$_2$, PGI$_2$, PGF$_{2\alpha}$, PGD$_2$, and PGE$_2$ respectively by TXA-synthase, PGI$_2$-synthase, PGF-synthase, PGD-synthase, and PGE-synthase (Cheng et al., 2002; McAdam et al., 1999).
3.1.4 EDHF

EDHF is a residual endothelium vasorelaxation after blockade of NOS and COX. It is due to the membrane hyperpolarization of the smooth muscle cells (Waldron et al., 1999).

After it was found in the late 1980s and early 1990s, the mechanism of EDHF remains a mystery even now. Its complexity arises from the mechanisms of hyperpolarizing the membrane. EDHF may be a metabolite of arachidonic acid formed through activation of the enzyme of cytochrome P450 in some tissues such as bovine and porcine coronary arteries (Hecker et al., 1994). Bryan has suggested the following requirement for the definition of EDHF (Bryan et al., 2005): (1) requires endothelium; (2) is distinct from both endothelium-derived nitric oxide or COX metabolites; (3) dilates by hyperpolarizing the vascular smooth muscle; and (4) involves potassium channel activation, most often calcium-activated potassium channels (KCa) (Chen et al., 1989).

As a vasodilator, the effect of NO is of primary importance in systemic vasculature, and EDHF in smaller arteries and arterioles (Fukuta et al., 1997; Garland et al., 1995; Ogura et al., 2004). The vasodilation from EDHF might be up regulated when NO pathway is suppressed, such as in the stress states (e.g. hypertension) (Perez-Vizcaíno et al., 1999). This protective response helps maintain the homeostasis under those pathological conditions (see review Bryan et al., 2005).
3.1.5 Aims of the study

Given the important role of the vascular endothelium in modulating vascular smooth muscle contractility, the aim of the present study was to investigate whether the operation of NCX could be affected by the endothelium. NCX does have phosphorylation sites on its intracellular loop for both PKG and PKA (Caroni et al., 1983; DiPolo et al., 1987c; He et al., 1998; Iwamoto et al., 1995; Mene et al., 1993; Yamanaka et al., 2003) and these cyclic nucleotides could therefore have an important role.

It is likely that NCX normally operates to transport Ca\(^{2+}\) out of cells; however, this function is difficult to investigate in functional blood vessel studies. In the present study NCX operation was studied in reverse mode after lowering extracellular Na\(^+\) from 144.18 mM to 1.18 mM (Kim et al., 1999; Rebolledo et al., 2006; Schweda et al., 2001). Whilst this is not the normal physiological operation, it is relevant to various pathological states such as ischaemia (Schweda et al., 2001) and importantly the vasoconstriction produced is relatively easy to measure.

The specific questions that were asked using isolated rat aorta were:

- Does the endothelium modulate NCX mediated constriction?
- What endothelial factors are involved?
- What is the mechanism of action?

In the present study, NO/cGMP and prostacyclin/cAMP pathways were investigated in rat aortic rings to clarify the possible role for NCX modulation.
Figure 3.4a  NO/cGMP pathway
3.2 METHODS

3.2.1 Tissue preparation for functional studies

Male Sprague Dawley rats (5-7 weeks old, weighing 200-350 g) were anaesthetized with Thiopentone sodium (100 mg/kg, i.p. Sigma, USA) and then killed by decapitation. The thoracic aorta was removed, placed in a cold physiological salt solution (PSSA, see 3.2.3.1), cleaned of surrounding fat and connective tissue and cut into equal-size ring segments (4mm in length) with scissors. Two thin stainless-steel hooks were inserted through the lumen of aorta ring. The lower hook was connected to a tissue holder and the upper to an isometric force Grass FT03 displacement transducer. Each ring was suspended in an organ bath containing 2 ml of physiological salt solution. Tissues were washed thoroughly by replacing the physiological salt solution repeatedly and were then allowed to equilibrate for a period of 60 minutes under 2 g of resting tension.

For the study of NCX operating in reverse mode, the extracellular $[\text{Na}^+]$ was reduced from 144.18 mM to 1.18 mM. Low Na$^+$ PSS (PSSB, see 3.2.3.2) was obtained by substituting choline chloride for NaCl, bubbled with 100% $\text{O}_2$ and the pH was adjusted to 7.4 with KOH. In the control group, the normal PSS (PSSA, see 3.2.3.1) was bubbled with 100% $\text{O}_2$ and the pH was adjusted to 7.4 with NaOH (Horiguchi et al., 2001).

Drugs and reagents were added directly to the bathing fluids using a micro syringe, and mixed thoroughly by repeated syringing with the bath solution and were allowed to reach a
steady concentration in the incubation fluid within the tissue chamber. Baseline tone was readjusted when necessary.

In some cases, the endothelium was removed by gentle rubbing of the luminal surface with a plastic rod.

3.2.2 Tissue viability and endothelium-denudation checking

Following the 60-minute equilibration period, the viability of the tissues was assessed. Tissues, which failed to produce 0.5-g increase in tension to phenylephrine (1 µM), were rejected. Successful removal of the endothelial cells was confirmed by the inability of acetylcholine (1 µM) to induce relaxation in the presence of phenylephrine (see Figure 3.5). The tissue bathing solution was then replaced repeatedly with fresh drug free PSS until a stable baseline tension was achieved. The tension was re-adjusted to 2 g.

3.2.3 Composition of physiological salt solution (PSS) (in mM):

3.2.3.1 Normal physiological salt solution for NCX operating on reverse mode (PSSA):

NaCl 143; KCl 4.7; NaH₂PO₄ 1.18; MgSO₄·7H₂O 1.17; CaCl₂·2H₂O 2.5; glucose 11; and N-2-hydroxy-ethylpiperazine-N-2-ethylsulphonic acid (HEPES) 5.
Figure 3.5 The typical traces of rat aortic rings

The aortic rings in each channel are taken from separate rings in the same rat. Y-axis indicates the constriction measured in grams. The elapsed time on the experimental day is shown on X-axis (h:m:s). Panel A: response to phenylephrine (1 μM) followed by acetylcholine (1 μM) in endothelium denuded aorta. Panel B: response to phenylephrine (1 μM) followed by acetylcholine (1 μM) in endothelium intact aorta.
3.2.3.2 Low Na\(^+\) physiological salt solution for NCX operating on reverse mode (PSSB):

Choline chloride 143; KCl 4.7; Na\(_2\)HPO\(_4\) 1.18; MgSO\(_4\)·7H\(_2\)O 1.17; CaCl\(_2\)·2H\(_2\)O 2.5; glucose 11; and HEPES 5.

3.2.4 Experimental protocols

3.2.4.1 Low Na\(^+\) constriction in rat aortic rings

In this series of experiments, external [Na\(^+\)] was lowered to 1.18 mM which is below the intracellular concentration, thereby turning the direction of calcium transport through NCX to an inward direction (Calcium Entry Mode or Reverse Mode) (Kim et al., 1999; Schweda et al., 2001). Extracellular [Na\(^+\)] was replaced with isomolar choline chloride.

Low [Na\(^+\)]\(_o\) may exert its effect by changing the neurotransmitter release from the perivascular nerve endings. Therefore, the same experiment was conducted in the presence of adrenergic neuron blocker: guanethidine. Guanethidine was reported to be an effective adrenergic neuron transmission blocker in rat aorta at the concentration of 3 \(\mu\)M (Toma et al., 1995)(Berry et al., 1992). Aortic rings were pretreated for 30 minutes with guanethidine (3 \(\mu\)M) before the manipulation of lowering [Na\(^+\)]\(_o\).
3.2.4.2 The effect of ODQ on low Na\(^+\) constriction in rat aortic rings

The soluble guanylate cyclase inhibitor: ODQ was used to determine whether nitric oxide-linked processes might be involved in the modulation of NCX. In both endothelium denuded and intact vessels, ODQ (1 \(\mu\)M) was added for 10 minutes before the manipulation of lowering [Na\(^+\)]\(_o\).

3.2.4.3 The effect of L-NAME on low Na\(^+\) constriction in rat aortic rings

The nitric oxide synthase inhibitor: L-NAME was used to determine whether NO-linked processes might be involved in the endothelial modulation of NCX. L-NAME (50 \(\mu\)M) was added to the rings for 20 minutes before the manipulation of lowering [Na\(^+\)]\(_o\).

3.2.4.4 The effect of SQ 22536 on low Na\(^+\) constriction in rat aortic rings

In this set of experiments, the involvement of cAMP pathway was evaluated. SQ 22536, the adenylate cyclase blocker was used as a pharmacological tool to this end (Lo et al., 2005). In both endothelium denuded and intact vessels, the aortic rings were incubated with SQ 22536 (100 \(\mu\)M) for 30 minutes before reducing [Na\(^+\)]\(_o\). At the concentration of 100 \(\mu\)M, SQ 22536 was reported to be an effective inhibitor of adenylate cyclase in rat aorta (Lo et al., 2005; Morello et al., 2006; Wu et al., 2005).
3.2.4.5 The effect of Indomethacin on low Na$^+$ constriction in rat aortic rings

In order to ascertain if there is any alternative pathway of endothelial modulation of NCX apart from NO, the production of prostacyclin was inhibited using the COX inhibitor: indomethacin. In the endothelium intact vessels, the aorta strips were pretreated with indomethacin (10 μM) for 30 minutes prior to the withdrawal of [Na$^+$]o. At the concentration of 10 μM, indomethacin was reported to be an effective inhibitor of COX in rat aorta (Molin et al., 2004)(Ashraf et al., 2004; Rapoport et al., 2000; Sofola et al., 2003). In vehicle group, 0.1% Na$_2$CO$_3$ (v/v) was used.

3.2.4.6 The relaxant effect of Sodium Nitroprusside (SNP) in rat aortic rings after constriction to U46619 or low Na$^+$

In an attempt to gather direct evidence of the involvement of nitric oxide in the operation of NCX, the NO donor SNP was studied. These experiments were designed to determine whether the method of preconstriction altered the ability of SNP to relax endothelium denuded rat aortic rings.

Rat denuded aortic rings were exposed to different types of pre-constriction. The thromboxane A$_2$ agonist U46619 (0.1 μM), or Na$^+$ PSS (1.18 mM) was used to induce vasoconstriction. In control group, vehicle (0.1% Ethanol) was used. After the tension reached its peak, SNP (30 nM) was applied to elicit vasodilatation.
3.2.4.7 The relaxant effect of SNP in rat aortic rings after constriction to 80 mM KCl or low Na⁺

In a separate series of experiments, the denuded aortic rings were pre-constricted with either KCl (80 mM) or low Na⁺, and then challenged with SNP (30 nM). At the even lower concentration of 60 mM, high K⁺ was shown to effectively induce membrane depolarization and vasoconstriction in rat aorta (Iwata et al., 2001; O'Donnell et al., 1987). In control group, vehicle (0.1% DMSO) was used.

3.2.5 Drugs and materials

The following drugs were used: L-phenylephrine hydrochloride, acetylcholine chloride, 9-(2-tetrahydrofuryl) adenine (SQ 22536), indomethacin, guanethidine monosulfate, sodium nitroprusside dihydrate (SNP), Nω-nitro-L-arginine methyl ester (L-NAME) from Sigma (St Louis, USA), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) from Cayman Chemical (MI, USA), KB-R7943 (2-2-[4-(4-nitrobenzyloxy) phenyl] ethyl isothioureac methanesulphonate), from TOCRIS (Ellisville, USA), 9,11-Dideoxy-9α, 11α-methanoepoxy prostaglandin F₂α (U46619) from BIOMOL (Plymouth Meeting, USA). Phenylephrine and acetylcholine were dissolved in distilled water, and the remaining drugs were dissolved in DMSO at 10 mM as a stock solution, and further diluted in PSS prior to use. The final concentration of vehicle was no more than 0.1% (v/v). Buffer salts and chemicals were obtained from Sigma (St Louis, USA) unless otherwise stated, include: NaCl, NaHCO₃, KCl, KH₂PO₄, MgSO₄·7H₂O, D(-)Glucose, CaCl₂·2H₂O, EGTA, HEPES, Choline Chloride.
3.2.6 Data analysis

Results are expressed as mean ± standard error of the mean, and were considered different when P<0.05. Multiple comparisons were determined using ANOVA in GbStat package (version 7.0, Dynamic Microsystems, Inc., Silver Spring, MD, USA).

3.3 RESULTS

3.3.1 Constriction in rat aortic rings induced by low Na⁺

In endothelium denuded rat aortic rings, lowering extracellular [Na⁺] to 1.18 mM induced immediate constriction and spontaneous constrictor activity (vessel motion), as seen in the original traces (Figure 3.6). Group data are shown in Figure 3.7. The low Na⁺ did not produce constriction after the employment of NCX inhibitor KB-R7943, at the concentration of 10 μM (Figure 3.7) and 1 μM (Figure 3.8).

The adrenergic neuron blocker guanethidine (3 μM) showed no effect on low-Na⁺ induced constriction in endothelium denuded rat aortic rings (Figure 3.7) indicating that sympathetic nerves were not involved.

In endothelium intact vessels, lowering [Na⁺]o did not constrict the aortic rings significantly
3.3.2 The effect of ODQ on constriction in rat aortic rings induced by low Na$^{+}$

In endothelium intact rat aortic rings, the guanylate cyclase inhibitor ODQ (1 μM) greatly amplified the vasoconstriction to lowering extracellular Na$^{+}$, but had no effect on the low Na$^{+}$ constriction when the endothelium was removed (Figure 3.10). The typical trace was shown in Figure 3.11.
Figure 3.6  The representative trace in the experiment of low-Na\(^+\) induced effect

The endothelium denuded aortic ring in each channel was taken from separate ring in the same rat. Y-axis indicates the constriction measured in gram, and X-axis indicates the elapsed time on the experimental day (h:m:s). Panel A: response to vehicle (0.1% DMSO). Panel B: response to \([\text{Na}^+]_o\) lowering from 144.18 mM to 1.18 mM.
Figure 3.7  Effect of KB-R7943 (10 μM) and guanethidine (3 μM) on constriction in endothelium denuded rat aortic rings induced by lowering [Na⁺]₀ from 144.18 mM (CON) to 1.18 mM (low Na⁺).

In each ring there was one cycle of constriction to low [Na⁺]. Either vehicle or drug (KB-R7943 or guanethidine) was present before the constriction. Mean and s.e. mean are shown in the columns. The number of experiments ranged from 6 to 8 different rings in each group.* represents a significant difference from respective constriction before lowering of Na⁺ (CON), P<0.05 Student’s t-test. # represents that guanethidine had no significant effect on low Na⁺ constriction, P>0.05, two way analysis of variance.
Figure 3.8  Effect of KB-R7943 (1 μM) on constriction in endothelium denuded rat aortic rings induced by lowering \([\text{Na}^+]_o\) from 144.18 mM (CON) to 1.18 mM (low Na\(^+\))

In this series of experiments, lower concentration of KB-R7943 (1 μM) was used. In each ring there was one cycle of constriction to low \([\text{Na}^+]\). Mean and s.e. mean are shown in the columns. The number of experiments was 6 different rings in each group. * represents a significant difference from respective constriction before lowering of Na\(^+\) (CON), P<0.05 Student’s t-test. # represents significant inhibition by KB-R7943 (1 μM), P<0.05, two way analysis of variance.
The influence of the endothelium on constriction induced by lowering $[\text{Na}^+]_o$ from 144.18 mM (control) to 1.18 mM (low Na$^+$) in rat aortic rings. In each ring there was one cycle of constriction to low [Na$^+$]. Mean and s.e. mean are shown in the columns. The number of experiments ranged from 5 to 9 different rings in each group. * represents a significant difference from respective constriction before lowering of Na$^+$ (CON), $P<0.05$ Student’s t-test.
Figure 3.10  Effect of ODQ on constriction in rat aortic rings induced by lowering [Na⁺]o from 144.18 mM (control) to 1.18 mM (low Na⁺)

In each ring there was one cycle of constriction to low [Na⁺]. Mean and s.e. mean are shown in the columns. The number of experiments ranged from 5 to 8 different rings in each group.

* represents a significant difference from respective constriction before lowering of Na⁺ (CON), P<0.05 Student’s t-test.
The aortic rings in each channel are taken from separate rings in the same rat. Y-axis in each channel represents the constriction of aortic rings measured in grams. X-axis represents the elapsed time of the day (h:m:s). Comment line indicates where the treatments started. Panel A: response to lowering extracellular Na\(^+\) in the presence of SQ 22536 (100 μM) in endothelium intact aortic rings; Panel B: response to lowering extracellular Na\(^+\) alone in endothelium intact aortic rings; Panel C: response to lowering extracellular Na\(^+\) in the presence of ODQ (1 μM) in endothelium intact aortic rings.
3.3.3 The effect of L-NAME on constriction in rat aortic rings induced by low Na$^+$

In endothelium intact rat aortic rings, L-NAME (50 μM) greatly amplified the vasoconstriction to lowering extracellular Na$^+$, it had no effect in endothelium denuded aortic rings (Figure 3.12). The typical trace was shown in Figure 3.13.

3.3.4 The effect of SQ 22536 on constriction in rat aortic rings induced by low Na$^+$

The adenylate cyclase blocker: SQ 22536 (100 μM) showed no significant effect on low-Na$^+$ induced vasoconstriction in either endothelium denuded or intact aortic rings (Figure 3.14). The typical trace was shown in Figure 3.11.

3.3.5 The effect of indomethacin on constriction in rat aortic rings induced by low Na$^+$

Indomethacin (10 μM), the COX inhibitor, had no effect on low Na$^+$-induced vasoconstriction in endothelium intact aortic rings (Figure 3.15). The typical trace was shown in Figure 3.13.

3.3.6 The effect of Sodium Nitroprusside (SNP) in rat aortic rings after preconstriction with low Na$^+$ or U46619

Endothelium denuded rat aortic rings were preconstricted to the same extent with different means: low Na$^+$ (1.18 mM), or the thromboxane A$_2$ agonist U46619 (0.1 μM). The absolute values of maximum constriction were shown in table 3.1. There was no significant difference
between the pre-constriction induced by distinct means (P>0.05, Student’s t-test). SNP (30 nM) was added in each of these conditions and produced a vasorelaxation. The vasorelaxation in low Na\(^+\) constriction was significantly greater than that in U46619 (Figure 3.16).

3.3.7 The effect of SNP in rat aortic rings after preconstriction with low Na\(^+\) or high K\(^+\)

In another series of experiments, endothelium denuded rat aortic rings were preconstricted to the same extent with either low Na\(^+\) (1.18 mM) or high K\(^+\) (80 mM). The absolute values of maximum constriction were shown in Table 3.2. There was no significant difference between the pre-constriction induced by distinct means (P>0.05, Student’s t-test). SNP (30 nM) was added in each of these conditions and produced a vasorelaxation. The vasorelaxation in low Na\(^+\) constriction was significantly greater than that in high K\(^+\) (Figure 3.17).
Table 3.1 The maximum constriction induced by different vasoconstrictors in rat aortic rings (in grams)

<table>
<thead>
<tr>
<th>Name of vasoconstrictor</th>
<th>Constriction (gram)</th>
</tr>
</thead>
<tbody>
<tr>
<td>low Na(^+) (1.18 mM)</td>
<td>1.19 ± 0.18</td>
</tr>
<tr>
<td>U46619 (0.1 μM)</td>
<td>1.32 ± 0.19</td>
</tr>
</tbody>
</table>

Table 3.2 The maximum constriction induced by low Na\(^+\) or high K\(^+\) in rat aortic rings (in grams)

<table>
<thead>
<tr>
<th>Name of vasoconstrictor</th>
<th>Constriction (gram)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low Na(^+) (1.18 mM)</td>
<td>0.83 ± 0.12</td>
</tr>
<tr>
<td>High K(^+) (80 mM)</td>
<td>1.09 ± 0.10</td>
</tr>
</tbody>
</table>
Figure 3.12 Effect of L-NAME on constriction in endothelium intact rat aortic rings induced by lowering $[\text{Na}^+]_o$ from 144.18 mM (control) to 1.18 mM (low Na$^+$).

In each ring there was one cycle of constriction to low $[\text{Na}^+]$. Mean and s.e. mean are shown in the columns. The number of experiments ranged from 5 to 9 different rings in each group.

* represents a significant difference from respective constriction before lowering of Na$^+$ (CON), $P<0.05$ Student’s t-test.
Figure 3.13  The representative trace in the experiment of L-NAME and Indomethacin on low Na\(^+\)-induced effect in endothelium intact rat aortic rings

The aortic rings in different channels are taken from separate rings in the same rat. Y-axis in each channel represents the constriction of aortic rings measured in grams. X-axis represents the elapsed time of the day (h:m:s). Comment line indicates where those treatments started. Panel A and D are the traces for the treatment of lowering Na\(^+\) in the presence of L-NAME (50 µM). Panel B is the trace for the treatment of lowering Na\(^+\) in the presence of indomethacin (10 µM). Panel C is the trace for the treatment of indomethacin (10 µM) alone.
Figure 3.14  Effect of SQ 22536 on constriction in rat aortic rings induced by lowering [Na\(^+\)]\(_{o}\) from 144.18 mM (control) to 1.18 mM (low Na\(^+\))

In each ring there was one cycle of constriction to low [Na\(^+\)]. Mean and s.e. mean are shown in the columns. The number of experiments ranged from 6 to 7 different rings in each group. * represents a significant difference from respective constriction before lowering of Na\(^+\) (CON), P<0.05 Student’s t-test.
Figure 3.15  Effect of Indomethacin (10 μM) on constriction in endothelium intact rat aortic rings induced by lowering $[\text{Na}^+]_o$ from 144.18 mM (control) to 1.18 mM (low Na$^+$). In each ring there was one cycle of constriction to low $[\text{Na}^+]$. Mean and s.e. mean are shown in the columns. The number of experiments ranged from 6 to 9 different rings in each group. There was no significant difference from respective constriction before lowering of Na$^+$ (CON), $P>0.05$ Student’s t-test.
Figure 3.16 The relaxation effect of SNP (30 nM) in endothelium denuded rat aortic rings preconstricted with U46619 or low Na\(^+\).

In each ring there was one cycle of constriction to low [Na\(^+\)]. SNP was added after the constriction had plateaued. Mean and s.e. mean are shown in the columns. The number of experiments ranged from 4 to 6 different rings in each group. * represents a significant difference of SNP-induced vasorelaxation in low Na\(^+\) (1.18 mM) from that in U46619 (0.1 \(\mu\)M), P<0.05 Student’s t-test.
Figure 3.17  The relaxation effect of SNP (30 nM) in endothelium denuded rat aortic rings preconstricted with high K⁺ or low Na⁺.

In each ring there was one cycle of constriction to low [Na⁺]. SNP (30 nM) was added after the constriction had plateaued. Mean and s.e. mean are shown in the columns. The number of experiments was 5 different rings in each group. In control group (CON), SNP relaxation in the presence of vehicle (0.1% DMSO) instead of constrictor was used. * represents a significant difference of SNP-induced vasorelaxation in low Na⁺ (1.18 mM) from that in high K⁺ (80 mM), P<0.05 Student’s t-test.
3.4 DISCUSSION

The existence of NCX has been shown in a variety of vascular smooth muscle cells, such as in cultured rat aortic VSMC (Juhaszova et al., 1994; Nakasaki et al., 1993; Wakimoto et al., 2000; Zhu et al., 1994), in rat mesenteric artery myocytes (Slodzinski et al., 1998b; Slodzinski et al., 1995), in myocytes of rat mesenteric artery (Juhaszova et al., 1994), in VSMC of both normal and hypertensive rats (Taniguchi et al., 2004), in rat pulmonary artery VSMC (Wang et al., 2000), in cultured rat cortical neurons, astrocytes, aortic endothelial, aortic VSMC (Quednau et al., 1997), in either bovine or porcine smooth muscle sarcolemmal membrane vesicle (Slaughter et al., 1989) and in cultured human pulmonary artery VSMC (Zhang et al., 2005b).

The present study was to investigate the role of the vascular endothelium in modulating vasoconstriction mediated by the Na⁺/Ca²⁺ exchanger (NCX). In endothelium denuded rat aorta, lowering extracellular [Na⁺] from 144.18 mM to 1.18 mM induced an immediate constriction. Other studies have also demonstrated a constriction induced by lowering Na⁺ such as in rat thoracic aorta and bovine tail artery (Ashida et al., 1987), in human umbilical artery smooth muscle cells (Rebolledo et al., 2006), in rat isolated skeletal muscle small arteries and mesenteric small arteries (Horiguchi et al., 2001), in guinea-pig aortic strips (Bova et al., 1988), in guinea pig tracheae (Espinosa-Tanguma et al., 2003), in canine coronary artery (Maseki et al., 1990), in rabbit aortic strip and pulmonary artery (Reuter et al., 1973), and in single myocyte of rabbit middle cerebral artery (Kim et al., 1999). The constriction is most likely due to the inflow of Ca²⁺ through NCX as reducing the Na⁺ gradient across the membrane makes the exchanger operate in reverse mode (Horiguchi et
al., 2001; Schweda et al., 2001; Takai et al., 2004). Indeed in the present study the constriction was blocked by the NCX inhibitor KB-R7943 (1 μM and 10 μM). A key feature is the effectiveness of KB-R7943 at 1 μM, since at this concentration other non-NCX actions appear minimal (see chapter 2.1.7). Other studies have also shown that low Na⁺ vasoconstriction is blocked by NCX inhibitors such as in rat cremaster arterioles (present studies Chapter 4.3.3), human umbilical artery smooth muscle cells (Rebolledo et al., 2006), rat carotid arterial myocyte (Takai et al., 2004), single myocyte of rabbit middle cerebral artery (Kim et al., 1999) rat intrapulmonary arteries (Becker et al., 2007) and rat renal resistance artery (Schweda et al., 2001).

As NCX is also present in neurons, other workers have suggested alternate mechanisms of vasoconstriction such as the stimulation of sympathetic nerve endings in rabbit aorta (Karaki et al., 1977). Nevertheless in the present protocol the adrenergic neuron blocking drug guanethidine had no effect on low Na⁺ constriction excluding this possibility. This agrees with the observation that low Na⁺ vasoconstriction was maintained in the presence of the α adrenoceptor blocker phentolamine in rat aorta (Ashida et al., 1987).

A key question is what role the vascular endothelium may play in this low Na⁺ induced constriction. It has been well established that the vascular endothelium by releasing nitric oxide has an important buffering effect on vasoconstrictor agents (see reviews Bredt et al., 1994; Gryglewski, 2005). In the present study in endothelium intact rat aorta, lowering extracellular [Na⁺] from 144.18 mM to 1.18 mM induced a far smaller constriction than when the endothelium was removed. To the best of my knowledge, this is the first report on the different vasoconstriction effect induced by Na⁺ removal between endothelium-denuded
and intact vessels.

The constriction in the presence of the endothelium due to low Na\(^+\) was nonsignificant (0.20 gram). In early studies constriction was reported as significant, e.g. 0.11 gram in rat aorta (n=2) and 0.45 gram (n=4) in bovine tail artery (Ashida et al., 1987), but the sample numbers were small. In most of those studies, endothelium status was not mentioned or assessed and it may be that the endothelium was not entirely intact, namely, the endothelium may be partially damaged during tissue preparation. Indeed, endothelium damage is often encountered in functional studies and this was the discrepancy of the diversity of endothelium dependent vasorelaxation (Meininger et al., 1992). The other possibility is that the role of endothelium varies between tissues.

One explanation for the lack of constriction to NCX is that the release of vasodilator substances from the endothelium inhibits NCX operation in the smooth muscle. Endothelium exerts its regulatory effect on vessel contractility through the release of intermediate substances. Among these, the best identified are prostacyclin, nitric oxide and endothelium-derived hyperpolarizing factor, and this is discussed in depth in section 3.1.1. Some of these were examined in the present study.

It is known that prostacyclin can be released from endothelial cells upon stimulation by hormones (such as arginine vasopressin), (see reviews Bredt et al., 1994; Denninger et al., 1999). Prostacyclin activates G-protein linked prostacyclin receptors (Wedel et al., 1997) on vascular smooth muscle and these receptors stimulate adenylate cyclase to produce cAMP which in turn activates PKA (Cheng et al., 2002; McAdam et al., 1999). There are identified
sites (on the intracellular loop) for the regulation of PKA phosphorylation of NCX (see review Blaustein et al., 1999), along with some conflicting reports regarding the modulation of NCX by cAMP/PKA pathway, e.g. the up-regulatory effect (He et al., 1998; Perchenet et al., 2000; Yamanaka et al., 2003), and down-regulatory effect (Mene et al., 1993). However in the present study the adenylate cyclase inhibitor: SQ 22536 (Lo et al., 2005) did not affect low Na⁺ vasoconstriction in endothelium intact rat aorta. Prostacyclin is synthesized from membrane phospholipids by the action of PLA₂ to release arachidonic acid which is converted to prostacyclin by the sequential action of cyclooxygenase and prostacyclin synthase (Hanoune et al., 1997; Smith, 1992). Indomethacin (Molin et al., 2004) blocks both isoforms of COX (COX1, COX2) (Perez-Vizcaino et al., 1999; Vane et al., 1987) and in the present study had no effect on low Na⁺ constriction in endothelium intact rat aorta. These results rule out the involvement of prostacyclin in endothelial modulation of low Na⁺ vasoconstriction.

The most prominent endothelium-dependent vasodilator is nitric oxide which is formed by nitric oxide synthase and released by the vascular endothelium to activate soluble guanylate cyclase in the vascular smooth muscle (Bredt et al., 1994; Denninger et al., 1999). The synthesis and release of nitric oxide from endothelium is due to activation of nitric oxide synthase after being stimulated by a rise in intracellular calcium (Binko et al., 1998; Molin et al., 2004). In the present study two sets of results confirmed that this mediator was in fact involved in the diminished low Na⁺-induced vasoconstriction when the endothelium was present. Firstly, ODQ, a soluble guanylate cyclase inhibitor (Shelkovnikov et al., 2004), potentiated the low-Na⁺ induced constriction in aortas with intact endothelium to a level similar to that observed in endothelium denuded rat aorta. In the absence of endothelium the
low Na\(^+\) constriction was not affected by ODQ. This indicates that soluble guanylate cyclase was involved in the response and suggests that NO release was involved. This was confirmed when the nitric oxide synthase inhibitor L-NAME also enhanced the low-Na\(^+\) induced constriction in aortas with intact endothelium. To my knowledge this is the first report of nitric oxide inhibition of NCX mediated vasoconstriction.

There are several mechanisms whereby this could occur. The first is that NCX may be present in vascular endothelium. Indeed this has been shown in a variety of endothelial cells (refer to 1.4.8.2). Therefore, lowering Na\(^+\) may release nitric oxide from the endothelium as inflow of Ca\(^{2+}\) has been shown to increase nitric oxide production and release in endothelial cells, such as in rat aortic endothelial cells (Ogura et al., 2004; Schneider et al., 2002a), in porcine aortic endothelial cells (Teubl et al., 1999), in cardiac microvascular endothelial cells (Kaye et al., 1999), in rat intestinal microvasculature endothelial cells (Zani et al., 2005), in human pulmonary arterial endothelial cells (Mizuno et al., 2002) and in pig coronary arterial endothelial cells (Szewczyk et al., 2007). Indeed, NCX appears involved in this response as the NO release due to intracellular Na\(^+\) loading is blocked by KB-R7943 (Schneider et al., 2002a). Thus low Na\(^+\) constriction may be inhibited by the concomitant NO release.

The other possibility that could occur is simply that the normal basal physiological release of nitric oxide from the endothelium could inhibit NCX operation. It is well established that there is a basal release of nitric oxide from the endothelium (see reviews Bredt et al., 1994; Li et al., 2000; Maxwell, 2002). The question is whether NO could somehow antagonize low Na\(^+\) constriction. To test this hypothesis, the endothelium was removed and nitric oxide
added to the system in the form of sodium nitroprusside. In these experiments the ability to relax endothelium denuded rat aortic rings was compared after preconstriction by a variety of means: U46619 a thromboxane A2 mimetic which operates through the phospholipase C/ IP₃ signaling pathway (Streefkerk et al., 2002), high K⁺ which depolarized smooth muscle and opens voltage dependent Ca²⁺ channels (Mendizabal et al., 2000; O'Donnell et al., 1987) as well as the low Na⁺ vasoconstriction via the NCX mechanism. Each constrictor mechanism produced an equivalent constriction, however SNP produced a more marked vasorelaxation in the low Na⁺ induced constriction than with the other agents. This observation is unique and has several important consequences. It suggests that NCX operation is uniquely sensitive to NO or that NCX downstream vasoconstrictor mechanism is different to the other methods. If this latter case is true then there must be some spatial separation in the signaling since all means of vasoconstriction work through an elevation in intracellular Ca²⁺. High K⁺ might inhibit EDHF and contribute to the reduced extent of vasodilation of SNP. However, the intracellular IP₃ mediated process (U46619) gave the same results as the Ca²⁺ channel opening mechanism (high K⁺). The most likely explanation is that NCX is tightly controlled by NO pathways compared to the other mechanisms, which is consistent with other reports (Amoroso et al., 2000; Asano et al., 1995; Azatian et al., 1998; Nagano et al., 2005).

The question arises how this could occur? As introduced in 1.4.7.2, NCX can be regulated by direct phosphorylation. NCX was shown to be augmented by cGMP in nonvascular smooth muscle cells, such as in cultured rat astrocytes and neuronal preparations (Asano et al., 1995), in guinea-pig ventricular myocytes (Hinde et al., 1999), and in squid axons and heart cells (Caroni et al., 1983; DiPolo et al., 1987b). In rat aorta, cGMP was also shown to augment the activity of NCX, e.g. in cultured rat aortic vascular smooth muscle cell
(Furukawa et al., 1991). A similar result (Iwamoto et al., 1995) demonstrated that cGMP activates the activity of NCX through the PKC-dependent protein phosphorylation in quiescent and growth factor-stimulated cultured rat aortic vascular smooth muscle cells. These results suggest that an enhanced cGMP mediated phosphorylation of NCX can not account for SNP inhibition of the NCX constriction, as it has a stimulating instead of inhibiting effect and leading to a constriction not relaxation. Therefore another explanation must be found.

NCX can be modulated indirectly through Na⁺/K⁺ ATPase. Both Na⁺ pump and NCX localize to plasma membrane overlying junctional SR in smooth muscle cells, refer to 1.4.8.1 and see review (Blaustein et al., 1999). This noteworthy colocalization discloses some clue of the interaction of these two plasma membrane transporters (Juhaszova et al., 1996)(Pittner et al., 2006). As introduced early in chapter 1.4.5, 2.4 and further in chapter 4.4, NCX-mediated Ca²⁺ influx can be increased by an enhanced Na⁺ gradient through Na⁺/K⁺ ATPase inhibition or extracellular Na⁺ removal. cGMP through PKG can stimulate Na⁺/K⁺ ATPase (Eva et al., 2006; Tagaya et al., 2001). Thereby the Na⁺ gradient is decreased, and the forward mode of NCX is activated to extrude Ca²⁺ and decrease the intracellular Ca²⁺ level. The process is shown in Figure 3.18 (Nishimura, 2006). Our hypothesis is corroborated by the result in mouse aorta stating that Na⁺/K⁺ ATPase inhibits endothelium-dependent relaxation by activating NCX (Kim et al., 2005).

Taking this result back to the present study where the endothelium effectively dampened low Na⁺ vasoconstriction, an important component could be an effect of the NO indirectly via Na⁺/K⁺ ATPase. This implies a tonic role for NO on the Na⁺/K⁺ ATPase inhibiting NCX
operations.

An alternative explanation is that the endothelium effectively dampened low Na\(^+\) vasoconstriction through decreasing the sensitivity of the contractile process to Ca\(^{2+}\). As discussed earlier in this chapter 3.1.2.5, cGMP can reduce the Ca\(^{2+}\) sensitivity, and therefore, the subsequent vasoconstriction induced by NCX would be decreased (See reviews Nishimura, 2006; Nishimura et al., 1989)(Somlyo et al., 2003).

Since both ODQ and NAME amplified the effect of low Na\(^+\) vasoconstriction to the same extent as endothelium removal in the present study, it suggests that NO is the sole endothelial modulator of NCX operations and that EDHF is not involved.

Our result is supported by (Teubl et al., 1999) demonstrating that membrane depolarization did not play a role in the activation of eNOS. This is also consistent with the knowledge that EDHF-induced vasodilatation is not as prominent in aorta as in microvessels, its importance increases with decreasing diameter of vessels (Fukuta et al., 1997; Ogura et al., 2004).

3.4.1 Conclusion

Our results suggest that endothelium modulates the NCX operation via the guanylate cyclase, not the adenylate cyclase system; the release of nitric oxide, not prostacyclin is involved. Thus NCX may be another mechanism by which the endothelium-derived nitric oxide modulates contractility in vascular smooth muscle.
Figure 3.18 Model for the activation of NCX by the cGMP through Na\(^+\)/K\(^+\) ATPase
CHAPTER 4 THE ROLE OF Na⁺/Ca²⁺ EXCHANGER ON MYOGENIC RESPONSE IN MICROVESSEL

4.1 INTRODUCTION

4.1.1 Definition of myogenic response

The phenomena of myogenic response were first observed by Bayliss in 1902 (Bayliss, 1902). Myogenic response refers to the acute reaction of a blood vessel wall in response to transmural pressure changing. Generally speaking, vessels constrict in response to increased intraluminal pressure and dilate in response to decreased intraluminal pressure (see review Meininger et al., 1992).

4.1.2 Features of myogenic response

One of the main features of myogenic response (or myogenic tone, basal tone, or spontaneous tone, or intrinsic tone), is that it is inherent, and independent of external neural, metabolic, and hormonal influences. It is noted that endothelial cells are not required for normal myogenic response (see review Davis et al., 1999). A typical myogenic response is shown in Figure 4.1.

The strength and importance of the myogenic response is inversely related to the diameter of vessel (see review Davis et al., 1999). In general, small vessels possess a relatively stronger myogenic response compared to large vessels. Furthermore, in different vascular beds, the
strength of the myogenic response is different. For example, myogenic response in rat small mesenteric vessels was smaller than that in a similar sized cerebral (Osol et al., 1991) or skeletal muscle vessels (Watanabe et al., 1993).

The myogenic tone represents a sustained, partial smooth muscle contraction. The physiological significance of the mechanism is that it allows the vessel to further contract/dilate to maintain an adequate blood perfusion to the tissue. For example in reduced pressure conditions, tissue perfusion would be expected to decrease but myogenic relaxation compensates for this and maintains perfusion. In raised pressure conditions, tissue perfusion would be expected to increase and the myogenic constriction would attenuate this to maintain balance.

4.1.3 Isometric versus isobaric studies

The most commonly used experimental approaches in the investigation of vascular contractions or constrictions are: isometric and isobaric. Under isometric conditions, the diameter is held constant. As such, when the smooth muscle contracts, the diameter is kept constant and tension in the vessel wall increases. The studies on aortic rings using the organ bath techniques in chapter 2 and chapter 3 are good examples of isometric method (Angus et al., 2000).

Under isobaric conditions the equivalent transmural pressure is held constant. This is accomplished by sealing the vessel at one end and maintaining a constant internal pressure
Figure 4.1 The prototypical graph for isobaric constriction

With in vitro isobaric vessel preparation, the above prototypical graph shows a two-phase myogenic response (see review Davis et al., 1999). A step increase produced experimentally in intraluminal pressure induces an initial, passive vessel distension, which is followed by two phases of constriction: transient and sustained. After releasing the pressure step experimentally, the arteriole transiently constricts, and then dilates (Zou et al., 1995).
Figure 4.2 The pressure-diameter relationship in both active and passive condition.

Curve A indicates the pressure-diameter relationship in active condition in which vessel transiently dilates and then constricts in response to the increment of transmural pressure (Zou et al., 1995). This is the myogenic response when Ca\(^2+\) is present. Curve B indicates the pressure-diameter relationship in passive condition in which vessel progressively dilates in response to the increment of transmural pressure until a maximum diameter (passive tone) reaches (Zou et al., 1995). This is when Ca\(^2+\) is absent and the myogenic response eliminated. It represents pressure-induced passive distention (See review Davis et al., 1999).
using a gravity pressure head. What is measured in this case is a change in vessel diameter. The present study using cremaster arteriole is a typical example of isobaric method (Murphy et al., 2002a; Potocnik et al., 2000; Schiffrin et al., 1997).

4.1.4 Active versus passive condition for the studies of myogenic response

In comparison to the normal active condition in the presence of external Ca\textsuperscript{2+} (Figure 4.1), passive state refers to the condition in the absence of external Ca\textsuperscript{2+} in the bath solution. As depicted in Figure 4.2, the pressure-diameter relationship in the active condition is different from that in the passive condition. Without Ca\textsuperscript{2+}, vessel progressively dilates as the pressure increases (see reviews Davis et al., 1999; Hill et al., 2006). This indicates that the influence of Ca\textsuperscript{2+} is fundamental to myogenic tone.

4.1.5 Regulation of blood flow

There are three major types of blood vessels: artery, capillary, and vein. Arteries and veins act as conduits for blood. Arteries carry blood away from the heart to peripheral tissues, and form smaller and smaller branches. As the smallest division of arteries, arterioles feed into the capillary beds of tissues. They are often called resistance arteries due to their responsibility for a large proportion of peripheral vascular resistance, as small change in arteriolar diameter can affect pressure and blood flow (see review Thomas et al., 2004). Blood flow into the capillary beds is determined largely by arteriole diameter, which varies in response to changing neural stimuli and local chemical influences. The interweaving networks of the microscopic capillaries are the place where exchanges between the blood
and tissue cells take place.

The equation below depicts the relationship between blood flow, blood pressure and resistance. If the difference in blood pressure increases, blood flow speeds up and vice versa. In contrast, if the resistance increases, the blood flow decreases and vice versa.

\[
\text{Blood flow (F)} = \frac{\text{difference in blood pressure (}\Delta P\text{)}}{\text{peripheral resistance (R)}}
\]

There is a rich investigation with respect to the regulation of blood flow, and this is well-documented in the exercising skeletal muscle. Multiple signaling pathways including nervous system, blood-borne metabolites and autacoids feedback system, and myogenic response interact to regulate short-term blood flow (see reviews Dora et al., 2005; Segal, 2005). The long-term control of blood flow (e.g. in hypertensive condition) is beyond the scope of present discussion.

4.1.5.1 The autonomic regulation

There are rich nerve supplies surrounding the arteries, arterioles and their terminal branches. The autonomic regulation of blood flow in skeletal muscle is dominated by sympathetic nervous system irrespective of at rest or during exercise. Sympathetic effect is stimulatory through the release of norepinephrine in most tissues except the skeletal muscle (Gantt et al., 1996; Marieb et al., 1998; Shepro et al., 1974). Norepinephrine is a potent agonist of \(\alpha\) adrenoreceptor and has little effect on \(\beta_2\) adrenoreceptor; therefore it constricts all resistance vessels, increases the peripheral vascular resistance and reduces the blood flow (see book
Gantt et al., 1996; Marieb et al., 1998; Shepro et al., 1974) and (see review Thomas et al., 2004). Exceptionally, the sympathetic efferents (vasomotor fibers) in skeletal muscles may release acetylcholine instead of norepinephrine to induce vasodilation (Gantt et al., 1996; Marieb et al., 1998; Shepro et al., 1974). Furthermore, there is a parallel activation of both somatic motor and autonomic nervous system during exercise, the former causes the contractions of skeletal muscle which activate the local vasodilator signals and cause the relaxation of blood vessel and a local hyperemia (relaxation) (see review Thomas et al., 2004). At rest, sympathetic vasoconstriction renders the vessel a tonic state; During exercise, the sympathetic vasoconstriction is mainly in the proximal arterioles and feed arteries maintaining the systemic arterial blood pressure and restricting the functional hyperemia (see reviews Segal, 2005; Thomas et al., 2004). Without such protective sympathetic vasoconstriction, it would be unable to maintain adequate blood flow to the vital organs (such as brain) during strenuous exercise. By contrast, in the distal arterioles, sympathetic and local mechanism inducing vasodilation prevail to meet the metabolic needs and facilitate the oxygen supplies (see reviews Segal, 2005; Thomas et al., 2004).

4.1.5.2 Auto regulation

Autoregulation refers to the automatic adjustment of blood flow to each tissue in proportion to its requirements at any point in time. Organs regulate their own blood flow by varying the resistance of their arterioles, which is accomplished by modifying the diameter of local arterioles feeding the capillaries. It has been proposed that auto regulation is achieved through several mechanisms: metabolic control and myogenic control (see review Schubert et al., 1999) and (Gantt et al., 1996; Marieb et al., 1998; Shepro et al., 1974).
The metabolic control is based on the balance between the oxygen delivery and metabolic demand of the tissue. For example, during exercise the reduced oxygen, pH, increased CO₂ and lactate are the common metabolic stimuli which can cause the immediate vasodilation of the arteriole so the blood flow to local area increases. The possible local vasodilators for metabolic control include: adenosine, NO, prostacyclin and endothelial-derived hyperpolarization factor (EDHF) (see reviews Boushel, 2003; Ito et al., 1995). Other chemicals that are also involved in the short-term control of blood flow include the atrial natriuretic peptide (ANP), angiotensin II, antidiuretic hormone (ADH), they can be produced by the atria of heart, kidneys, and hypothalamus respectively in response to the inadequate tissue perfusion (Gantt et al., 1996; Marieb et al., 1998; Shepro et al., 1974). Apart from the above mentioned chemicals, some inflammatory substances such as histamine and kinins are involved as well.

Myogenic control is discussed further in 4.1.6.

4.1.6 Physiological significance of myogenic response

It is well defined that the stimulus for myogenic response is vessel wall tension (Schubert et al., 1999). Large conduit vessels cannot change their diameter substantially to offset the alteration in hemodynamic forces. In contrast, small arteries respond directly to increased intravascular pressure with increased tone (see Figure 4.1), to resist the stretch. Decreased intravascular pressure in small arteries, on the other hand, induces vasodilation and enhancement of blood flow to the tissue. The myogenic control relies exclusively on smooth muscle cells in the vessel wall, not nerves, endothelium or any other factors (refer to 4.1.2).
This myogenic control keeps the blood supply to tissues fairly constant in spite of variations in systemic pressure (see review Dora et al., 2005). Although the exact mechanism is still unknown, the myogenic response of small arteries and arterioles has been shown to be critically important in the auto regulation of local blood flow, the setting of basal peripheral vascular resistance, and the regulation of capillary hydrostatic pressure (see reviews Davis et al., 1999; Hill et al., 2001). Owing to the specific impact of myogenic response on vascular smooth muscle cell, it has fueled interest in its implication in pathological conditions, such as hypertension and diabetic vascular complications (see reviews Izzard et al., 2005; Paulson et al., 1990; Wiernsperger, 2001).

4.1.7 Cellular mechanism underlying myogenic response

All cells live in a changeable environment where they are subject to physical forces, such as pressure, shear-stress, flow, stretch, and compression. As we know, myogenic response is the ability of vessel wall to adjust its diameter in response to the change of wall tension. This occurs by a variety of signalling processes. The cascade of signaling processes may starts on the extracellular matrix, and then is transmitted to cell surface triggering ion channel events and the ensuing membrane depolarization (Hill et al., 2006; Potocnik et al., 2004). The first step or requirement is that the change of wall tension needs to be sensed or detected by the vessel (mechanosensitivity) which is followed by the conversion of sensing signal into cellular signal (mechanotransduction) (see review Schubert et al., 1999). There exist several hypotheses about the sensor element which detects the stimulus of pressure alteration, coupled to calcium mobilization, and ultimately initiates the myogenic response (see reviews Davis et al., 2001; Koller, 2002).
Evidence suggests that stretch-activated channels (SACs) on vascular smooth muscle or endothelium play an important role in the mechanosensitivity and mechanotransduction (see reviews Kalapesi et al., 2005; Meininger et al., 1992). The characterized non-selective cation stretch-activated channels (NSCCs), with a relative permeability of $K^+ \geq Na^+ > Ca^{2+}$ (see reviews Davis et al., 1992; Kalapesi et al., 2005), are reported to be the likely candidates (see reviews Albert et al., 2002b; Davis et al., 1992; Hill et al., 2001; Kirber et al., 1988; Meininger et al., 1992), as reported for guinea-pig urinary bladder myocytes (Wellner et al., 1994), vascular smooth muscle cells dissociated from toad stomach (Kirber et al., 1988), dispersed vascular smooth muscle cells from porcine coronary artery (Davis et al., 1992), and guinea-pig mesenteric arterial cells (Setoguchi et al., 1997). It is assumed that inward current flowing through stretch-activated channels would depolarize the cell membrane, and activate VGCC (Wellner et al., 1994) (see also review Hill et al., 2006).

Apart from the ionic channels aforementioned, there are other potential mechanosensors reported. Membrane-bound enzymes including phospholipase A$_2$, phospholipase C, and tyrosine kinases were reported to be involved in mechanosensitivity (see review Kalapesi et al., 2005), and cytoskeletal proteins such as actin and intermediate filaments, by virtue of their support to cell membrane and the linkage to extracellular matrix, are viewed as potential mechanosensors (see review Kalapesi et al., 2005).

Integrins, refer to a family of membrane-spanning glycoproteins. There is a growing evidence showing that they may be important elements in the mechanosensitivity and mechanotransduction of myogenic response, due to their linkage to both extracellular matrix and cytoskeleton inside vascular smooth muscle cell and their ability to initiate cellular
responses to stretch (see reviews Davis et al., 2001; Kalapesi et al., 2005). For example, an integrin-specific peptide was shown to directly inhibit the myogenic tone in injured rat cremaster arteriole (Mogford et al., 1996).

The generation of myogenic constriction:

There is consensus that sustained increase of intracellular calcium level is necessary for the occurrence and maintenance of myogenic response once it is initiated. As described in chapter 1, multiple mechanisms contribute to the calcium homeostasis, such as voltage-operated calcium channels, store-operated calcium channels, calcium pump, and the Na⁺/Ca²⁺ exchanger (refer to 1.1).

According to the prevailing thought, myogenic contraction is initiated by the depolarization of vascular smooth muscle cell which then allows Ca²⁺ entry through voltage-operated calcium channel (Davis et al., 1999) This is buttressed by solid evidence (Brayden et al., 1992; Harder, 1984; Knot et al., 1995; Kotecha et al., 2005; Potocnik et al., 2000), which indicate that the pressure-induced electrical signaling mediated Ca²⁺ influx. Furthermore, blockers of L-type voltage-operated Ca²⁺ channels, including nifedipine, verapamil and nicardipine, have been shown to greatly reduce myogenic responses (Knot et al., 1995; Potocnik et al., 2000; Wesselman et al., 1996). But there may be other mechanisms involved.

Interestingly, the contribution of IP₃ and subsequent calcium release from intracellular stores during myogenic responses has attracted strong attention (Nakayama, 1982; Nakayama et al., 1986). Some authors argue that repetitive SR Ca²⁺ release might be linked with the
aforementioned ionic event (L-type voltage-operated Ca\(^{2+}\) channels) contributing to the initial Ca\(^{2+}\) rise in myogenic response, or even predominating over in some cases (Lee et al., 2001; Meininger et al., 1992; Zhang et al., 2002).

As introduced in 1.1.4, the modulation of vasoconstriction can result from changing Ca\(^{2+}\) sensitivity. Likewise, the mechanism of myogenic constriction could involve Ca\(^{2+}\) sensitivity. Decreased Ca\(^{2+}\) sensitivity via the inhibition of Rho kinase resulting in decreased myogenic tone has been reported in rat cerebral artery (Lagaud et al., 2002)(Jarajapu et al., 2005), in porcine coronary artery (Randriamboavonjy et al., 2003), in rat ophthalmic artery (Ito et al., 2007), and in rat basilar artery (Ahn et al., 2007). However, there was a controversy over the contribution of protein kinase C pathway, another factor affecting Ca\(^{2+}\) sensitivity to myogenic response. It was reported to play a minimal role in the myogenic response in rat ophthalmic artery (Ito et al., 2007), in rat cerebral artery (Jarajapu et al., 2005) and in small porcine coronary artery (Randriamboavonjy et al., 2003). In contrast, it was shown to play a role in the myogenic response in rabbit facial vein (Laher et al., 1989), in rat cerebral artery (Lagaud et al., 2002), and in rat cremaster arteriole (Bakker et al., 1999; Hill et al., 1990a).

Ultimately, the increase of the intracellular calcium concentration and Ca\(^{2+}\) sensitivity finally activates myosin light chain kinase, which phosphorylates the 20-kDa regulatory myosin light chain leading to smooth muscle constriction (1.2.2).
4.1.8 Aims of the current study

Collectively, with the limitations in experimental techniques and major deficiencies in our knowledge of myogenic signaling pathways, the true cellular mechanism underlying myogenic response remains to be unequivocally established. There are other calcium regulators apart from ion channels that contribute to the calcium homeostasis in vascular smooth muscle cell as discussed in the previous chapter (1.1). NCX has a central role in regulating calcium levels through forward mode and/or reverse mode. Its contribution in myogenic response needs to be intensively investigated.

4.1.8.1 Choice of cremaster arterioles for the present study

Recognizing the regional and longitudinal differences in reactivity in the peripheral vasculature, in particular, that a large proportion of the total vascular resistance resides in microvessels (Henrion, 2005; Thomas et al., 2004), the current study progressed from macrovessels into microvessels. In particular, the myogenic response is more pronounced in microvessel than macrovessel, microvessel is the most appropriate tissue to study.

Due to the complicated interactions among parenchymal cells, vascular smooth muscle cells, endothelial cells, and the autonomic nerves in vivo, effort has been made to develop in vitro preparations to simplify the study of myogenic response of vascular smooth muscle. Among a variety of in vitro preparations, cremaster muscle microvessels have been extensively used since the early report of (Grant, 1964) as a classical model for the study of skeletal muscle microvascular hemodynamics and reactivity. Apart from the studies with cremaster muscle
arterioles in other laboratories, Hill et al developed a successful method for the dissection, cannulation, tone development, and Ca\(^{2+}\) fluorescent imaging of cremaster muscle arterioles in our laboratory in the past studies, and collected much data of cremaster muscle arterioles which were shown in previous publications in our laboratory (Kotecha et al., 2005; Murphy et al., 2002a; Murphy et al., 2002b; Potocnik et al., 2004; Potocnik et al., 2001; Potocnik et al., 2000). Hence, cremaster muscle arteriole is a ready model for the current study.

4.1.8.2 The hypothesis of the present study

In the previous chapters (chapter 2 and chapter 3), it was demonstrated that decreasing extracellular Na\(^+\) leads to Ca\(^{2+}\) entry in rat aorta, indicating pharmacological functionality of NCX in arterial smooth muscle, this is consistent with other reports in many laboratories (Bova et al., 1990; Kim et al., 1999; Reuter et al., 1973). However, the investigation of NCX on vascular smooth muscle cell is mainly conducted in large vessels, much less evidence is available for small resistance vessels (see review Sheu, 1986). It has been reported that myogenic response is entirely dependent upon extracellular calcium (Duling et al., 1981; Meininger et al., 1991; Uchida et al., 1969; Zou et al., 1995; Zou et al., 2000), and the development of the myogenic response probably involves multiple Ca\(^{2+}\) mediated mechanisms (Hill et al., 2001). As one such mechanism, NCX may play a role in the modulation of myogenic response either in participating in Ca\(^{2+}\) entry or by removing intracellular Ca\(^{2+}\) (see reviews Blaustein, 1989; Blaustein et al., 1999; Reuter et al., 1973).

It is unclear from data at present how important NCX activity is for constriction in
microvessels and that was one of the goals of the study. Therefore the goals of the present study in chapter 4 were to examine (1) whether withdrawal extracellular Na$^+$ mediates Ca$^{2+}$ entry and constriction in rat cremaster arterioles, (2) whether NCX is involved in the modulation of arteriolar tone and myogenic constriction. Experiments were conducted using manipulation of extracellular Na$^+$ as well as two inhibitors of NCX: KB-R7943 (Satoh et al., 2000) and SEA0400 (Takahashi et al., 2003).
4.2 METHODS

4.2.1 Animals

The present study used male Sprague-Dawley rats, aged 6-9 weeks weighing 200-350 gram. Before experiments, rats were housed in a dedicated animal facility with a 12:12 h light-dark cycle. During this period, rats were allowed free access to a standard rat chow and drinking water. All procedures were approved by the RMIT Animal Ethics Committee.

4.2.2 Isolation of arteriole preparation

The current applied method comprises optical instruments, fabrication procedures, and dissection methodology as described previously (Angus et al., 2000).

Rats were anaesthetized (100mg kg$^{-1}$ Pentothal, Abbott Australasia), and the right or left cremaster muscle was exteriorized, excised from the animal and placed in a temperature-controlled chamber (4°C) containing dissection buffer (see 4.2.3) (Duling et al., 1981). The dissection was carried out at 4°C, to avoid tissue spasm and vessels becoming unresponsive. Segments of the main intramuscular arteriole (1A) (Hill et al., 1990b) were dissected under a microscope as previously described (Meininger et al., 1991). The cremaster muscle has relatively long unbranched lengths of arterioles making it easy to cut the desirable length of single vessel for the present study. Isolated arterioles were then cannulated at both ends with glass micropipettes (filled with physiological salt solution), secured using 10-0
monofilament suture and mounted in a 5 ml volume tissue chamber. The vessel preparation was positioned on the stage of an inverted microscope (Olympus) and the arterioles continuously superfused (2-4 ml min⁻¹) with a physiological salt solution (PSS) (see 4.2.3). Arteriole segments were gradually pressurized from 50mmHg to 60mmHg, and then to 70 mmHg, and warmed to 33°C-34°C during a 60-min equilibration period. Arteriole with significant leaks or branches was discarded; this was tested by closing a stopcock disconnecting to the perfusion pipette without any diameter decrease during a 30-second leak test period (Duling et al., 1981). Only vessels that exhibited stable spontaneous basal tone (gradually developed tone, around 60% of the initial diameter after 60-min equilibration period) were studied further under isobaric conditions (Figure 4.3). In order to result in optimal myogenic responsiveness, before the development of spontaneous tone, the vessel length was adjusted by increasing segment length such that pressure steps to 120 mmHg did not cause a lateral bowing of the vessel. Diameter of the arterioles was measured with a video camera and displayed on a monitor screen (Angus et al., 2000). Data was recorded and stored using a MacLab A-D system.

To exclude the possible vasorelaxing influence of the endothelium via release of vasodilator autacoids (e.g., nitric oxide and prostacyclin) on myogenic constriction of smooth muscle cells, the current study was conducted on endothelium denuded arterioles. Meininger and Davis criticized the chemical denudation methods (using chemicals e.g. nonionic detergent to disrupt endothelium) for its vascular smooth muscle cell damage effect (Meininger et al., 1992). In order to avoid the possible tissue damage arising from chemical denudation method, in the present study air was passed through the lumen to remove the endothelium mechanically.
4.2.3 Preparation of buffers

4.2.3.1 Dissection Buffer:

Composition (in mM): 3-N-morpholino propanesulphonic acid (MOPS) 3; NaCl 145; KCl 5; CaCl\(_2\) 2.5; MgSO\(_4\) 1; NaH\(_2\)PO\(_4\) 1; EDTA 0.02; pyruvate 2; glucose 5 and 1% albumin.

The pH of dissection buffer was adjusted with 1 N NaOH at room temperature to 7.4, which will make the final pH 7.35 ~ 7.4 when chilled to 4°C.

4.2.3.2 Physiological salt solution (PSS):

Composition (in mM): NaCl 111, NaHCO\(_3\) 25.7, KCl 4.9, CaCl\(_2\) 2.5, MgSO\(_4\) 1.2, KH\(_2\)PO\(_4\) 1.2, glucose 11.5 and 2-N-hydroxyethylpiperazine-N\(^\prime\)-2-ethanesulphonic acid (HEPES) 10.

The pH of PSS was adjusted with 1 N NaOH at room temperature to 7.27, which will make the final pH 7.4 when warmed to 33°C ~ 34°C. Before the experiment, the PSS was bubbled with 95% O\(_2\) and 5% CO\(_2\) for 1 hour.
Figure 4.3  Cremaster arteriole before (A) and after (B) tone development

The same arteriole was shown for A (before tone development) and B (after tone development). Arteriole was gradually pressurized from 50mmHg (20 minutes) to 60mmHg (20 minutes), and then to 70mmHg (20 minutes), and warmed to 33°C ~ 34°C during a 60-min equilibration period. Only vessels that exhibited gradually developed stable spontaneous basal tone (B) which was around 60% of the initial diameter (A) were used. Initial internal diameter for cremaster muscle arteriole ranged from 150 ~ 250 μm (Angus et al., 2000).
4.2.4 Fluorescent Ca\textsuperscript{2+} indicators

Calcium-sensitive indicators have proven to be very useful in monitoring the changes of calcium during smooth muscle contraction. This has stirred an interest in searching for specific indicators of calcium.

A BAPTA derivative: quin2, was synthesized and tested as a fluorescent Ca\textsuperscript{2+} indicator (Morgan et al., 1982; Tsien, 1980). The excitation wavelength (339 nm) of quin2 is so short that it needs to overcome cell autofluorescence. Thereby, the second generation of indicators including fura-2 and indo-1 were developed (DeFeo et al., 1987; Grynkiewicz et al., 1985; Smith et al., 1987; Takeuchi et al., 1989). In spite of the acknowledged advantage of 30-fold brighter fluorescence than quin2, these indicators are not membrane-permeable. To increase permeability, an acetoxyethyl radical is attached to these indicators. After loading smooth muscle cells with the acetoxyethyl esters of these indicators, the acetoxyethyl moiety is cleaved by endogenous esterases and the indicator is trapped in the cell. Therefore, fura 2-acetoxy methylester (fura 2-AM) instead of fura-2 is used as Ca\textsuperscript{2+} indicator (DeFeo et al., 1987; Goldman et al., 1994; Smith et al., 1987; Takeuchi et al., 1989).

The present study used the hydrolysis of the membrane-permeant acetoxyethyl ester of fura-2 for calcium imaging (Meininger et al., 1991; Paltauf-Dobrzynska et al., 2000; Pritchard et al., 1986; Yamanaka et al., 2003).
4.2.5 Imaging of Ca$^{2+}$

To measure the changes in $[\text{Ca}^{2+}]_i$, vessels were loaded (60 min, room temperature) with the calcium indicator: 2 μM fura 2-AM (Molecular Probes, Eugene, U.S.A.) in PSS containing 0.2% DMSO and 0.01% pluronic acid (Molecular Probes, Eugene, U.S.A.) (Goldman et al., 1994; Paltauf-Doburzynska et al., 2000; Yamanaka et al., 2003). During the 60-min incubation time, sample illumination was prevented, with no direct light exposure and the aluminum foil covering the monitor. After loading, the vessels regained the spontaneous tone during 30-min wash period. Digital imaging of Ca$^{2+}$ indicator fluorescence in arterioles at excitation wavelengths of 340, and 380 nm were performed using a spinning filter wheel operating at 20Hz with emission detection at 510 nm. Simultaneous transillumination with wavelengths greater than 610 nm provided a non-fluorescent image which enabled measurement of internal arteriolar diameter while fluorescence intensities were measured. A beam splitter directed the high wavelength image to a charge-coupled device (CCD) camera. This procedure did not interfere with measurements of Ca$^{2+}$-related fluorescence. Fluorescent image intensities were expressed as the 340:380 nm ratio to allow quantitative estimates of changes in arteriolar wall $[\text{Ca}^{2+}]_i$. Details of these procedures have been presented in previous publications (Potocnik et al., 2000; Zou et al., 1995).

4.2.6 Drugs and materials

KB-R7943 2-2-[4-(4-nitrobenzyloxy) phenyl] ethyl isothiourea methanesulphonate), from TOCRIS (Ellisville, USA), was made as a 10 mM stock solution in DMSO, and further diluted in PSS prior to use. SEA0400: 2-[4-[(2,5-difluorophenyl) methoxy] phenoxy]-5-
ethoxyaniline, (Taisho Pharmaceutical Co., Ltd, Japan), was dissolved in DMSO at 10 mM as a stock solution and diluted before using. The final concentration of vehicle was no more than 0.1% (volume/volume). Buffer salts and chemicals were obtained from Sigma (St Louis, USA) unless otherwise stated, include: NaCl, NaHCO₃, KCl, KH₂PO₄, MgSO₄·7H₂O, D-(-)Glucose, CaCl₂·2H₂O, EGTA, HEPES, Choline Chloride.

4.2.7 Statistical analysis

Data are expressed as mean ± standard error of the mean, and were considered different when P<0.05. Statistical tests were performed using GbStat package (version 7.0, Dynamic Microsystems, Inc., Silver Spring, MD, USA).
4.3 RESULTS

4.3.1 Relationship of pressure and calcium level

This is one of the standard preliminary experiments to show the technique works and demonstrate some basic features of myogenic response. To define the relationship between intraluminal pressure and intracellular calcium concentration in isolated cremaster muscle arterioles exhibiting myogenic tone, step-wise increase of pressure was conducted. Steady-state [Ca$^{2+}$], as measured by the 340/380 nm fluorescence ratio (R$_{340/380}$), was found to increase with increasing intraluminal pressure from 30, 50, 70 to 120 mmHg consecutively (Figure 4.4). Similar results have been published in previous studies (Potocnik et al., 2004; Potocnik et al., 2001; Potocnik et al., 2000; Spurrell et al., 2003; Spurrell et al., 2000; Zou et al., 1995). Time control experiments were conducted in the previous studies in our laboratory (Potocnik et al., 2000; Spurrell et al., 2000; Zou et al., 1995), data showed that the pressure-induced change in calcium level is not a function of time.

4.3.2 Pressure-diameter relationships under passive conditions

This set of standard preliminary experiments was to show the pressure-diameter relationship under passive conditions, in which arterioles were superfused with PSS containing 2 mM EGTA and 0 Ca$^{2+}$. The cremaster muscle arterioles responded passively to increasing intraluminal pressure in the absence of Ca$^{2+}$ (please refer to 4.1.4 and Figure 4.2). Additionally, to decide whether vessel diameter was affected by the incubation of fluorescent
dye: fura 2-AM (2 μM), fura 2 loaded arteriolar diameter responses to increasing intraluminal pressure from 30, 50, 70 to 120 mmHg were measured in the absence of extracellular Ca^{2+}. No measurable difference in the vessel diameters in the absence and presence of fura 2-AM loading was detected (Figure 4.5).

4.3.3 Na⁺ lowering experiment

In order to identify the functional presence of NCX, we examined the arteriolar response to lowering extracellular Na⁺. This was explored in cremaster muscle arterioles by removing [Na⁺]₀ abruptly. The extracellular Na⁺ was reduced stepwise from 137 to 75 and then 25 mM. The Na⁺ was replaced with choline to maintain osmolarity. Reducing extracellular Na⁺ resulted in a graded decrease in arteriolar diameter with a maximal reduction of 83.9 ± 5.0% at 25 mM Na⁺ (Figure 4.6). When the NCX inhibitors KB-R7943 (1 μM) and SEA0400 (1 μM) were present, they did not affect arteriolar diameter at 137 mM Na⁺, but at 25 mM Na⁺ they significantly increased arteriolar diameter (Figure 4.6) indicating that the vasoconstriction at 25 mM Na⁺ was inhibited by NCX inhibitors.

4.3.4 The effect of NCX on myogenic response

To further study the physiological effect of NCX in cremaster arterioles, we tested the role of NCX in the myogenic response.

Myogenic responses were induced by an intra-lumen pressure increase from 70 mmHg to
120 mmHg. The diameter was significantly decreased to 88.3 ± 2.4% of initial (n=5, Student’s t-test, P<0.05). The pressure-induced constriction was prevented by NCX inhibitor: 1 μM SEA0400 (Fig. 4.7), indicating that NCX was involved. In fact the pressure increment resulted in vasodilatation (Fig. 4.7).

Simultaneously, we observed the digital fluorescence video imaging of arterial wall [Ca$^{2+}$] change (Figure 4.8). In fura 2-AM loaded arterioles the increases in lumen pressure from 70 mmHg to 120 mmHg was accompanied by a rise in intracellular [Ca$^{2+}$] to 115.8 ± 3.3% of initial (n=5, Student’s t-test, P<0.05), and this rise was attenuated by SEA0400 (1 μM) to 106.8 ± 2.0% of initial (n=5, Student’s t-test, P<0.05).
Figure 4.4  The effect of intraluminal pressure on \([\text{Ca}^{2+}]_i\) levels expressed as the 340/380 nm fluorescent ratio (R\(_{340/380}\)) in rat cremaster arterioles

Steady-state \(\text{Ca}^{2+}\) value were obtained within a 5-min period. \(\text{Ca}^{2+}\) ratio (R\(_{340/380}\)) was expressed as a percentage of the control (basal diameter at 70 mmHg). The number of experiments equaled 8 arterioles in each group (n=8). Mean and s.e. mean are shown in the columns. * represents a significant difference from the 70 mmHg (P < 0.05, one-way analysis of variance followed by Dunnett’s test).
Figure 4.5  Pressure-diameter relationships obtained under passive (0 mM Ca\(^{2+}\) - 2 mM EGTA superfusate) conditions in rat cremaster arterioles

Data were shown for fura and nonfura rat cremaster arterioles. Diameter was presented as a percentage of respective diameter at 70 mmHg (control). The number of experiments ranged from 4 to 8 arterioles in each group. Mean and s.e. mean are shown in the columns. There were no significant differences between groups: fura 2 loaded and non-fura 2 loaded arterioles (P>0.05, Student’s t-test).
Figure 4.6  The effect of lowering extracellular Na\(^+\) on vessel diameter in rat cremaster arterioles

The isolated arterioles were pressurized to 70 mmHg and myogenic tone allowed to develop during a 60- min equilibration period. Subsequently the extracellular Na\(^+\) was reduced from 137 mM to 75 and then 25 mM by replacing the physiological salt solution until a new baseline diameter reached. Diameter was presented as a percentage of respective control at normal [Na\(^+\)] (137 mM) before the addition of either vehicle (DMSO 0.1%, n=6) or KB-R7943 (1 \(\mu\)M, n=5) or SEA0400 (1 \(\mu\)M, n=5). Each point represents mean and s.e. mean. The number of experiments ranged from 5 to 6 arterioles in each group. * represents a significant difference at the 25 mM [Na\(^+\)] between vehicle and SEA0400 (P<0.05, Student’s t-test), and between vehicle and KB-R7943 (P<0.05, post hoc bonferroni test after two way analysis of variance).
Figure 4.7  The effect of SEA0400 on myogenic response in rat cremaster arterioles

The intraluminal pressure was stepwisely increased from 70 mmHg to 120 mmHg to induce myogenic response in the presence of either vehicle (DMSO, 0.1%, n=5) or SEA0400 (SEA0400, 1 μM, n=5). Diameter was expressed as a percentage of respective control at 70 mmHg (5 mins. after pressure changing). Each point represents means and s.e. means. The number of experiments equalled 5 arterioles in each group. * signifies a significant difference between 70 and 120 (P <0.05, paired t-test).
Figure 4.8 The effect of SEA0400 on calcium level in rat cremaster arterioles

Isolated single vessels were loaded with acetoxymethyl ester form of fura-2 (2 μM, 60min, at room temperature). Ca$^{2+}$ ratio (R$_{340/380}$) was expressed as a percentage of the respective control at 70 mmHg. In each experiment, either vehicle (DMSO, 0.1%) or SEA0400 (1 μM) was present. Each point represents means and s.e. means. The number of experiments equalled 5 arterioles in each group. * signifies a significant change of Ca$^{2+}$ ratio after pressure increase (P<0.05, paired t-test). # represents significant differences between groups: vehicle and SEA0400 at 120 mmHg (P <0.05, paired t-test).
4.4 DISCUSSION

4.4.1 Myogenic response in cremaster muscle arteriole

The exact cellular signalling mechanism underlying the arteriolar myogenic response remains unclear, however, it is a widely accepted fact about arteriolar myogenic response through which arteriole constricts being accompanied by an overall increase of [Ca$^{2+}$], when the transmural pressure is enhanced (see reviews Davis et al., 1999; Hill et al., 2006; Hill et al., 2001; Meininger et al., 1992). This was demonstrated in the present study using the calcium indicator fura 2-AM. In the present study in endothelium-denuded rat cremaster arterioles, the intracellular [Ca$^{2+}$] increased with increasing intraluminal pressure from 30, 50, 70, to 120 mmHg consecutively (see Figure 4.4). The similar results were previously shown in cremaster muscle arteriole (Potocnik et al., 2000; Zou et al., 1995) and in rat cerebral artery (Jaggar, 2001).

As introduced in section 4.1.4, extracellular Ca$^{2+}$ is fundamental to myogenic response. This was further confirmed by the abolishment of myogenic constriction in the absence of extracellular Ca$^{2+}$, in which arteriole dilated progressively with increasing intraluminal pressure from 30, 50, 70, to 120 mmHg consecutively (see Figure 4.5). This is consistent with previous results in cremaster muscle arteriole (Potocnik et al., 2001; Spurrell et al., 2000; Zou et al., 1995). We also showed that there was no influence of Ca$^{2+}$ indicator: fura-2 AM in this case due to the abolishment of myogenic constriction irrespective of fura-2 loading in passive condition (see Figure 4.5), which is consistent with the result by (Potocnik
et al., 2001).

4.4.2 Functional studies of NCX in cremaster muscle arteriole

In the studies of NCX in smooth muscle, the most used functional response has been the observation of tension in response to alterations in Na⁺ electrochemical gradient (ΔυNa) (Blaustein, 1989; Friedman et al., 1959), since decrease extracellular Na⁺ will cause NCX to expel intracellular Na⁺ in exchange for extracellular Ca²⁺ and therefore increase intracellular Ca²⁺. Increasing intracellular Na⁺ will also alter the Na⁺ gradient to allow Ca²⁺ to enter. The history of functional studies of NCX can be tracked back to 1957, when a endeavor was made to clarify the role of Na⁺ in regulating the tension in vascular smooth muscle cells (Leonard, 1957). It has been demonstrated that tonic contraction (now known to be through NCX) could be induced by Na⁺ pump inhibition (increase intracellular Na⁺), either by K⁺ removal (Broekaert et al., 1973; Chin et al., 1993; Goldman et al., 1994; Karaki et al., 1977; Pritchard et al., 1986) or addition of cardiac glycosides (Leonard, 1957; Maseki et al., 1990; Ozaki et al., 1979; Wakimoto et al., 2000). Alternative way to cause Ca²⁺ entry and vasoconstriction through NCX is to reduce extracellular Na⁺ (Ashida et al., 1987; Bondarenko, 2004; Bova et al., 1988; Chau et al., 2003; Goldman et al., 1994; Hinke et al., 1962; Kim et al., 1999; Maseki et al., 1990; Nazer et al., 1998; Pritchard et al., 1986; Rebolledo et al., 2006; Reuter et al., 1973; Tang et al., 2000; Tsang et al., 2003). The low Na⁺-induced vasoconstriction was previously reported in rat aortic rings in chapter 2 and chapter 3.
Consistent with the above studies, in the present study the low Na\(^+\)-induced vasoconstriction after reducing extracellular Na\(^+\) from 137 to 75, and then to 25 mM was apparent in cremaster muscle arteriole (Figure 4.6) indicating the functionality of NCX (refer to 3.4), this was confirmed by the inhibition from NCX inhibitors: SEA0400 (1 \(\mu\)M) and KB-R7943 (1 \(\mu\)M).

In the present study, reduced Na\(^+\) electrochemical gradient induced constriction. In other systems this has been shown to be due to an enhanced level of intracellular calcium, such as in cultured aortic vascular smooth muscle cell (Zhu et al., 1994) and (Poburko et al., 2006), in human umbilical arterial vascular smooth muscle cell (Rebolledo et al., 2006), in rat aortic and mesenteric arterial vascular smooth muscle cell (Bova et al., 1990), in rabbit aortic and pulmonary arterial vascular smooth muscle cell (Reuter et al., 1973), in rat pulmonary arterial vascular smooth muscle cell (Wang et al., 2000), and in rat carotid arterial myocytes (Takai et al., 2004).

The question arises as to how reducing extracellular Na\(^+\) can elevate intracellular Ca\(^{2+}\)? The involvement of voltage-gated Ca\(^{2+}\) channels in low Na\(^+\)-induced vasoconstriction has been discussed in 3.4. The results from (Ozaki et al., 1979), (Ashida et al., 1987), (Goldman et al., 1994) and (Sekine et al., 1984) suggested that this possibility could be ruled out, because of the observation of contractions induced by reduced Na\(^+\) electrochemical gradient in the presence of Ca\(^{2+}\) channel blockers (e.g. verapamil). Also, studies indicated that there were no significant change of membrane potential after [Na\(^+\)]\(_o\) lowering from 143 mM to 1.2 mM in rat mesenteric artery and femoral artery (Horiguchi et al., 2001), and in rabbit aorta and pulmonary artery (Reuter et al., 1973). Thereby, it seems unlikely that low Na\(^+\)-induced
vasoconstriction is through voltage-gated Ca\textsuperscript{2+} channels.

Another possibility is that low [Na\textsuperscript{+}]\textsubscript{o} exerted its effect via the inhibition of Na\textsuperscript{+}/H\textsuperscript{+} exchanger (NHE) by the production of acidosis. If this were true, the opposite effect would have been expected. Removal of extracellular Na\textsuperscript{+} would induce the accumulation of intracellular proton through the down-regulated Na\textsuperscript{+}/H\textsuperscript{+} exchanger. Decreased pH\textsubscript{i} is related to the decrease of intracellular calcium concentration and vasodilation instead of constriction (Aalkjaer et al., 1997; Chen et al., 1995; Saesue et al., 2004). This contradicts with our result. Further the NCX inhibitors SEA0400 (1 μM) and KB-R7943 (1 μM) do not affect the NHE activity at the concentration used that appears selective for NCX (refer to 2.1.6 and 2.1.7).

In chapter 1 the general formula for NCX operation (refer to 1.4.5) showed that the activity and direction of NCX was dependent on the concentration gradient for Na\textsuperscript{+} (Blaustein et al., 1999). It normally operates in forward mode. Only in conditions with intracellular sodium concentrations exceeding [Na\textsuperscript{+}]\textsubscript{o}, NCX operates in reverse-mode (Ca\textsuperscript{2+} entry mode). However, tonic arterioles pressurized to physiological levels (70 mmHg) are in an active state with membrane potential level at a less negative level which was reported to be around -40 mV in cremaster arteriole (Harder, 1984; Potocnik et al., 2000; Smeda et al., 1987). As introduced in 1.4.4, NCX is electrogenic owing to the coupling ratio of 3 Na\textsuperscript{+} : 1 Ca\textsuperscript{2+}. On the forward mode of NCX an inward current flows into the cell, whilst an outward current exists on the reverse mode. The electrogenic property of NCX was postulated to favour Ca\textsuperscript{2+} entry mode (reverse mode) when membrane potential is more positive. In addition, this myogenic tone is also thought to involve Na\textsuperscript{+} influx through stretch-activated channel (see review Hill
et al., 2001). Thereby, an increase in the local subsarcolemmal [Na$^+$]$_i$ would be expected while the overall [Na$^+$]$_i$ may remain unchanged. If the intracellular [Na$^+$] was elevated then the extracellular [Na$^-$] concentrations required to switch from forward mode operation to reverse mode would be less than in normal inactivated cells. Thus two mechanisms in arterioles may favour reverse mode NCX operation.

In the present study, lowering extracellular Na$^+$ generated constriction. It may be that these extracellular Na$^+$ reductions were sufficient to switch the exchanger to operate in reverse mode even though in other tissues a far lower extracellular [Na$^+$] was required before the change in direction occurred. Indeed the NCX inhibitors: SEA0400 (1 μM) and KB-R7943 (1 μM) reduced the constriction produced by low Na$^+$ (25 mM). Consistent with these results, Saesue et al demonstrated that SEA0400 effectively inhibited the activity of NCX in rat cerebral arteriole (Sausue et al., 2004), and Ren et al reported that KB-R7943 (30 μM) blocked NCX function in rabbit afferent arteriole (Ren et al., 2003). Furthermore, Zani et al showed that KB-R7943 (50 μM) blocked NCX activity in rat intestinal microvasculature (Zani et al., 2005). In the present study chapter 2, it is suggested that KB-R7943 is selective only at concentrations below 1 μM in vascular smooth muscle so the present study provides better evidence than the literature for the involvement of NCX. With regard to SEA0400, available literature suggests that it is more selective than KB-R7943 for NCX (refer to 2.1.6) and at 1 μM it has been shown to be selective for NCX in cultured rat aortic smooth muscle cell and mesenteric small artery (Matchkov et al., 2007), and cerebral arteriole (Sausue et al., 2004). One issue arising from this experiment is that NCX inhibitors did not affect the initial basal tone under normal Na$^+$ condition (Figure 4.6). As introduced in chapter 1 (1.4.7.1),
NCX can only be turned on when the intracellular Ca\(^{2+}\) binds to the Ca\(^{2+}\) regulatory site, and at normal resting [Ca\(^{2+}\)], NCX is only partially activated. Furthermore, there were a increase in the subsarcolemmal [Na\(^{+}\)]; in pressurized arteriole, the activity of NCX was depressed. Hence, before the manipulation of Na\(^{+}\) removal the inhibitory effect of NCX inhibitors (SEA0400 and KB-R7943) on the partially-activated NCX, if any, would be expected to be negligible.

To sum up, the presence of functional NCX in pressurized cremaster arteriole was confirmed by the constriction in response to removal of [Na\(^{+}\)]\(_o\). The hypothesis promoted in the present study is that tonic vessels have elevated local intracellular Na\(^{+}\) and this depressed the NCX at normal extracellular Na\(^{+}\) levels and induces it to operate on reverse mode when extracellular Na\(^{+}\) is lowered.

4.4.3 The study of NCX on myogenic response (myogenic tone)

In line with the result shown in Figure 4.4 (refer to 4.4.1), the data in control group in Figure 4.7 demonstrated a myogenic vasoconstriction and the accompanying increase of intracellular Ca\(^{2+}\) level (Figure 4.8) after increasing the transmural pressure from 70 to 120 mmHg in cremaster muscle arteriole.

It is well established that myogenic response requires an extracellular Ca\(^{2+}\) supply (Jaggar, 2001; Potocnik \textit{et al.}, 2001). Removal of extracellular Ca\(^{2+}\), the vessel exhibits a state of passive distention because of the abolishment of tone (as illustrated in Figure 4.2). In order to generate myogenic response, the [Ca\(^{2+}\)]\(_i\) must be increased and maintained above the contraction threshold for long periods of time (Zhang \textit{et al.}, 2002).

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The question arises as to how the Ca\textsuperscript{2+} elevation occurs. There are competing theories regarding the mechanism of the elevation of Ca\textsuperscript{2+} during myogenic constriction (see 4.1.7). There is convincing body of evidence showing that L-type calcium channel (LTCC) is a major contributor of Ca\textsuperscript{2+} entry in tonically activated vascular smooth muscle as nifedipine reduced myogenic constriction (Davis et al., 1999; Horiguchi et al., 2001; Jaggar, 2001; Wesselman et al., 1996). The pressure enhancement-induced membrane depolarization could lead to Ca\textsuperscript{2+} influx via L-type calcium channel and the subsequent myogenic constriction (Harder, 1984; Knot et al., 1998). However, Blaustein and co-workers (Zhang et al., 2002) opened a new look at the generation of myogenic tone. They suggested that store-operated Ca\textsuperscript{2+} channel was the key elements in intact rat mesenteric artery. While much of the trigger Ca\textsuperscript{2+} may enter the cells via L-type calcium channel (Hagiwara et al., 1967), store-operated Ca\textsuperscript{2+} channels may also contribute to the overall increase of intracellular Ca\textsuperscript{2+} during myogenic response.

The contribution of NCX to the Ca\textsuperscript{2+} entry in this case needs to be clarified. As introduced earlier (refer to 4.1.7.1), stretch-activated channels (SAC), namely the non-selective cation channel (NSCC) with a permeability to Na\textsuperscript{+} play a key role in detecting stimulus and initiate myogenic response (see reviews Hill et al., 2006; Hill et al., 2001). Na\textsuperscript{+} entry through this pathway is postulated to cause the accumulation of intracellular Na\textsuperscript{+} and then reduce the Na\textsuperscript{+} electrochemical gradient (\(\Delta\bar{u}_{Na}\)) and as suggested in the present study activating reverse mode NCX.

Under normal physiological condition, NCX is assumed to operate in forward mode to mediate Ca\textsuperscript{2+} exit (see 1.4.5). However, after the increase of transmural pressure and the
activation of L-type calcium channel and/or store-operated Ca\(^{2+}\) channels, it may be in an active condition. As introduced in 1.4.4, NCX is electrogenic owing to the coupling ratio of 3 Na\(^+\): 1 Ca\(^{2+}\). On the forward mode of NCX an inward current flows into the cell, whilst an outward current exists on the reverse mode. The electrogenic property of NCX was postulated to favour Ca\(^{2+}\) entry mode (reverse mode) at a more positive membrane potential after the depolarization of cell.

Furthermore, evidence showed a Na\(^+\) entry pathway mediated by store-operated Ca\(^{2+}\) channel in cultured rat mesenteric artery (Arnon et al., 2000), and rabbit inferior vena cava (Lee et al., 2001). Likewise, Na\(^+\) entry through store-operated Ca\(^{2+}\) channel is postulated to reduce the Na\(^+\) electrochemical gradient (\(\Delta \bar{u}_{Na}\)) activating reverse mode NCX (see also 1.3.2).

Collectively, NCX (in reverse mode) is postulated to be activated through multiple pathways during increasing transmural pressure and play a role in the elevation of intracellular Ca\(^{2+}\) during myogenic vasoconstriction. The schematic diagram illustrating the cellular mechanism underlie myogenic response is depicted in Figure 4.9. The current data fitted nicely with our hypothesis and confirmed that part of the elevation in [Ca\(^{2+}\)]\(_i\) is through NCX as the NCX inhibitor SEA0400 decreased the elevation in intracellular Ca\(^{2+}\) level after a pressure increasing from 70 to 120 mmHg by about 50% (Figure 4.8). The corresponding functional change replaced a myogenic constriction with a myogenic relaxation (Figure 4.7). This suggests that the pressure increase may have also activated underlying vasodilator pathways which are normally obscure, in addition to activating a constriction or that passive distension occurs. With respect to the contribution of NCX to myogenic tone, there are gaps in the current knowledge and scarce evidence has been presented. Schweda et al
demonstrated that the extracellular Na\(^+\) removal increased the renal vascular resistance in rat indicating the functional role of NCX in the regulation of renal vascular resistance (Schweda et al., 2001), Horiguchi et al demonstrated that the extracellular Na\(^+\) removal enhanced the myogenic response in rat mesenteric and skeletal muscle small arteries (Horiguchi et al., 2001), Similarly, the extracellular Na\(^+\) removal enhanced the myogenic response in rabbit urethra smooth muscle strips which was inhibited by NCX inhibitor SEA0400 (Bradley et al., 2006). Fang Y et al reported that NCX was the potential underlying mechanism for hypoxia-depressed myogenic constriction in CK1.4 cells (Fang et al., 1997); likewise, Lydrup et al reported that NCX might be involved in cyanide-induced myogenic reduction. Bychkov et al reported that NCX affected myogenic relaxation indirectly via spontaneous transient outward potassium current (STOC) in human coronary artery (Blaustein et al., 2006; Bychkov et al., 1997). And further more, the involvement of NCX in the regulation of myogenic tone and blood pressure was discussed at length in the review papers by {Reuter, 1973 #639; Zhang et al., 2005a; Zwadlo et al., 2006). Consistent with the aforementioned, our result adds more evidence to the existing knowledge. However, further studies are required in order to unequivocally establish the exact mechanism of myogenic response.

4.4.4 Conclusion

Previous data has shown for cremaster arterioles a role for VGCC (De Clerck et al., 1989; Hill et al., 1994; Potocnik et al., 2000), SOC (Potocnik et al., 2001) and SAC (Sun et al., 1995) in the myogenic response. In conclusion, the generation and maintenance of myogenic response in cremaster arteriolar include multiple mechanisms. The underlying pathways may involve stretch-activated channel, voltage-gated calcium channel, and store-operated Ca\(^{2+}\).
channel. The contribution from NCX through the reverse mode needs to be especially considered in cremaster arterioles, and this may also apply to other small arteries.
The increase of transmural pressure initiated the mechanosensitivity and mechanotransduction process via the activation of stretch-activated channel (SACs), non-selective cation channels (NSCCs) in particular, which mediated Na\(^+\) entry. Na\(^+\) entry had multiple effects: 1. caused the depolarization of cell membrane and the following opening of L-type calcium channel (LTCCs) which mediated Ca\(^{2+}\) flux; 2. activated the reverse mode NCX which mediated Ca\(^{2+}\) flux. Also, depolarization activated the reverse mode NCX. In parallel with the aforementioned mechanisms, the increase of transmural pressure activated...
store-operated Ca\textsuperscript{2+} channels (SOCs) releasing Ca\textsuperscript{2+} from sarcoplasmic reticulum, and activated the reverse mode NCX.
Appendix

Conference communications arising from the thesis

J. Zhao, S Rajanayagam & H Majewski. Modulation by nitric oxide donor on Na\(^+\)/Ca\(^{2+}\) exchanger in rat isolated aorta. *XVth World Congress of Pharmacology, Poster Abstract P110059*

J. Zhao & H Majewski. Modulation of smooth muscle Na\(^+\)/Ca\(^{2+}\) exchanger in rat isolated aorta by endothelium. *JAPSA/ASCEPT annual scientific meeting, Poster Abstract 1-5*

J. Zhao & H Majewski. Modulation of the Na\(^+\)/Ca\(^{2+}\) exchange in rat isolated aorta. *1st International Conference on Frontiers in Vascular Medicine, Poster Abstract P46*

J. Zhao, S Rajanayagam & H Majewski. KB-R7943 a Na\(^+\)/Ca\(^{2+}\) exchange inhibitor inhibits multiple modes of vasoconstriction in rat isolated aorta. *ASCEPT 2004 Melbourne Regional Meeting,*

J. Zhao, T V Murphy, M A Hill, H Majewski. The Na\(^+\)/Ca\(^{2+}\) exchanger contributes to the regulation of myogenic tone in rat arterioles. *Clin Exp Physiol Pharmacol, 31, Supplement 1, A192, 2004*

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