FUNCTIONAL SIGNIFICANCE OF SODIUM CALCIUM EXCHANGE IN ARTERIOLAR MYOGENIC TONE

BY

HEMA RAINA
(MBBS, MD)

A thesis submitted in fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY

School of Medical Sciences
RMIT University
Melbourne, Australia
June 2006
DEDICATION

To my family

-To always be there, where I wanted them the most-

In the field of observation, chance favors only the prepared mind.

*Louis Pasteur*
DECLARATION

I, the candidate, Hema Raina, 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.

Signature: --------------------
Name: --------------------
Date: --------------------
ACKNOWLEDGEMENTS

This is tough; whom shall I thank as I don’t have any friends left after 4 years of my PhD. As of my family, they are tired of asking me the same question over and over again; when will I finish? Finally phew……..I can see the end.

I’m going to limit myself to people who more or less directly helped with my thesis. I would like to express my sincere thanks to a number of people, who have helped, encouraged and inspired me along the way, in all the different ways they could.

My supervisor, Professor Michael Andrew Hill, for his ideas and patience throughout my PhD. Thanks for giving me freedom to work and grow in the lab despite numerous mistakes I made in that G1 (growing phase) of my PhD. His constant reminder “Hema think like a researcher” has finally made one out of me.

I would like to express my gratitude to my supervisor Professor Chris Triggle, for being supportive and encouraging and to take me under his wings when I needed it most.

Without whom it was not possible; my parents, Raksha Raina and Surrinder Raina, for nurturing my passion for science and to let me do whatever it takes to fulfill that, and to have faith in me and finally not nagging me to get married. My wonderful Brother Avinash Raina, thank you for years of immense support, love and kindness, and also to tolerate my cranky behaviour many a times. To his lovely wife Shibu Raina for saving me from cooking in the later part of my thesis writing.

A special thanks to Dr. Tim Murphy whose tolerant approach to my constant nagging and interruptions to his work; so much needed and appreciated during the initial period of my training.

Sincere thanks are given to Dr Neela Kotecha, who passed away during the course of this thesis, for assistance with the sharp glass electrode measurements of smooth muscle membrane potential.

I would like to thank Dr. Andrew Carey who helped in learning PCR. Dr Simon Potocnik thanks you for your constant presence in the lab and helping me when ever it
was required. I am grateful to Dr Hong Ding who provided invaluable discussions and for her soothing presence in the lab. I am especially thankful to Dr Karen Anderson for getting me out of the problems I used to get caught in, and to be so generous with me and definitely to teach me Australian ways. Thanks to Dr Ruth Hannan for helping me with Datapad Prism and to listen to all my sane and insane arguments and rantings at the letter stages of my thesis writing.

Thanks to Dr Vikas Kumar whose constant encouragement and perseverance has helped immensely, who taught me that distance doesn’t matter when it come to keeping friendships. Thanks to Dr Ashok Jaryal whose casual intelligence puts me to shame. He always said that it is more important to read between the lines than to read the actual lines. Thank you Nidhi Gupta, you have been just like a younger sister, a friend and a sink to me.

I would like to thank Yogita Kaul, Rakhi Kaul, Natasha Kaul and all the other friends at RMIT for helping me stay sane and not feel homesick and lonely. Thanks to all my fellow students and Post Doctoral researchers for their assistance and making the lab environment friendly. To all my friends I express my deepest appreciation. I thank RMIT International for providing me with the financial assistance and giving me the most needed extension of my scholarship. While I believe that all of those mentioned have contributed to an improved final thesis, none is, of course, responsible for remaining weaknesses. The errors and inconsistencies if any remain my own. My apologies if I have inadvertently omitted anyone to whom acknowledgement is due. I also thank two anonymous referees; I hope they'll see the improvements their comments brought.

Thank you all
**TABLE OF CONTENTS**

DEDICATION............................................................................................................................. II  
DECLARATION ........................................................................................................................ III  
ACKNOWLEDGEMENTS......................................................................................................... IV  
TABLE OF CONTENTS ............................................................................................................ VI  
PUBLICATIONS / ABSTRACTS .............................................................................................. XII  
HONORS / AWARDS............................................................................................................... XII  
LIST OF TABLES.................................................................................................................... XIII  
LIST OF FIGURES.................................................................................................................. XIV  
LIST OF ABBREVIATIONS................................................................................................... XVIII

**SUMMARY**.......................................................................................................................... 1

**CHAPTER 1** ........................................................................................................................ 7

**LITERATURE REVIEW** .................................................................................................... 7

*The Myogenic response and the biochemistry of smooth muscle contraction* .......... 9

*Historical perspectives* .................................................................................................... 11

*Blood flow dynamics* ....................................................................................................... 17

*Physiological significance of myogenic response* .......................................................... 18

*Membrane events, depolarization and ion channels* ....................................................... 20

1. Potassium channels ........................................................................................................ 23
   i. Inward rectifier K⁺ channels (K_{IR}) ........................................................................... 23
   ii. Voltage-dependent K⁺ channels (K_v) ....................................................................... 24
   iii. ATP-dependent K⁺ channels (K_{ATP}) .................................................................. 25
   iv. Ca^{2+}-dependent K⁺ channels (K_{Ca}) ................................................................. 25
### GENERAL METHODS

- **Animals**
- **Isolated arteriole dissection and preparation**
- **Measurement of membrane potential**
- **Intracellular Ca\(^{2+}\) measurements**
- **Dye loading and preparation**
- **Western blotting**
- **Arteriolar RP**
- **NCX Oligonucleotides**
- **Drugs and Chemicals**
- **Statistical and data analysis**

### CHAPTER 3

**FUNCTIONAL SIGNIFICANCE OF THE SODIUM CALCIUM EXCHANGER AND RELEVANCE TO MYOGENIC TONE**

- **INTRODUCTION**
- **MATERIALS AND METHODS**
  - **Relationship of intra luminal pressure and arteriolar diameter**
  - **Effect of extracellular manipulation of Na\(^+\) on vessel diameter and [Ca\(^{2+}\)]\(_{cyt}\)**
  - **Effect of NCX inhibitors on vessel diameter**
  - **Effect of NCX inhibitor KB-R7943 and VGCC blocker nifedipine on vessel diameter and \(E_m\) of cremaster arterioles**
  - **Effect of decreased extracellular [Na\(^+\)] on acute myogenic reactivity and MI in cremaster arteriole**
RESULTS ................................................................................................................... 89

Effects of extracellular $[\text{Na}^+]$ on arteriolar diameter and $[\text{Ca}^{2+}]_{\text{cyt}}$ of cremaster arterioles. ................................................................................................................................. 89

Concentration-dependent effects of NCX inhibitors on the diameter of cremaster arterioles. ........................................................................................................ 90

Effects of the VGCC blocker, nifedipine, on the diameter of cremaster arterioles during decreasing extracellular $[\text{Na}^+]$. ......................................................................................... 91

Effects of NCX inhibitor KB-R7943 and VGCC blocker nifedipine on arteriolar diameter and membrane potential ........................................................................ 92

Effect of decreasing extracellular $[\text{Na}^+]$ on acute myogenic reactivity and MI in cremaster arterioles. .......................................................................................... 92

DISCUSSION ........................................................................................................... 118

CHAPTER 4 ................................................................................................................. 123

IDENTIFICATION OF THE SODIUM CALCIUM EXCHANGER IN THE ARTERIOLAR WALL USING CELLULAR APPROACHES ................................................................. 123

INTRODUCTION ........................................................................................................ 124

MATERIALS AND METHODS ................................................................................ 128

Western blotting ................................................................................................... 128

Reverse-transcriptase polymerase chain reaction (PCR) ...................................... 131

mRNA preparation and cDNA synthesis ................................................................ 131

Real-time RT-PCR ............................................................................................... 131

RESULTS ................................................................................................................. 135

Identification of the NCX protein by Western blotting ........................................ 135

Detection of NCX isoforms by real-time PCR ....................................................... 136
CHAPTER 5................................................................................................................. 151

EFFECT OF ANTISENSE OLIGONUCLEOTIDES DIRECTED AT THE SODIUM CALCIUM EXCHANGER ON MYOGENIC REACTIVITY ........................................ 151

INTRODUCTION ...................................................................................................... 152

MATERIALS AND METHODS .................................................................................. 157

Effects of time and temperature on myogenic responsiveness of isolated arterioles .......................................................................................................................... 157

i. Pressure-diameter response curve ................................................................. 157

ii. Pressure step ............................................................................................... 158

iii. Dose-response curve .................................................................................. 158

iv. Passive pressure response curve ................................................................ 159

Effects of RP on the myogenic response of the vessel ........................................ 159

Efficacy of RP as demonstrated by PKI peptide loading into arteriolar smooth muscle ................................................................................................................. 159

Effects of NCX knockdown on the myogenic response of the vessel............. 160

Effects of NCX knockdown on protein expression .......................................... 160

RESULTS ................................................................................................................. 162

Effects of time and temperature on the myogenic responsiveness of cremaster arterioles. ............................................................................................................. 162

Effects of RP on the myogenic response of cremaster arterioles...................... 163

Use of a different experimental model to check the efficacy of RP by loading peptides into in-situ arteriolar smooth muscle. ................................................... 164
Effects of oligonucleotide-induced NCX knockdown on myogenic responsiveness and protein expression in cremaster arteriole. ......................................................165

DISCUSSION ........................................................................................................... 186

CHAPTER 6 ................................................................................................................. 192

GENERAL DISCUSSION AND FUTURE DIRECTIONS .............................................192

BIBLIOGRAPHY .......................................................................................................... 203


F. Colin Courtice Award at the 12th Australian and New Zealand Microcirculatory society conference at Terrigal, NSW, Australia, Oct 2005. This award is given for the best presentation by an investigator with less than 5 years postdoctoral experience.

Young investigators award at the 8th international symposium on resistance arteries held in Angers, France, June 2004.
LIST OF TABLES

Table 2-1. Solution 1 77
Table 2-2. Solution 2 77
Table 2-3. Solution 3 78
Table 2-4. Solution 4 78

Table 3-1. Myogenic indice of endothelium-intact and endothelium-denuded vessels in experimental group. 116
Table 3-2. Myogenic indice of endothelium-intact and endothelium-denuded vessels in control group. 116

Table 4-1. Primer sequences 134

Table 5-1. Effects of a pressure step on arteriolar diameter of rat cremaster arterioles, 24 hours after sense and antisense treatment. 181
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fig 1-1</td>
<td>Examples of myogenic behavior in arterioles.</td>
<td>13</td>
</tr>
<tr>
<td>Fig 1-2</td>
<td>Schematic of the possible operative principles for myogenic responses</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>in the VSM cells.</td>
<td></td>
</tr>
<tr>
<td>Fig 1-3</td>
<td>Stretch of smooth muscle cell leads to increase in $[Ca^{2+}]_{cyt}$</td>
<td>20</td>
</tr>
<tr>
<td>Fig 1-4</td>
<td>Stretch activation of $Ca^{2+}$ channels leading to depolarization and VSM</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>contraction.</td>
<td></td>
</tr>
<tr>
<td>Fig 1-5</td>
<td>Regulation of $[Ca]_{cyt}$</td>
<td>32</td>
</tr>
<tr>
<td>Fig 1-6</td>
<td>Topological model of the NCX</td>
<td>49</td>
</tr>
<tr>
<td>Fig 2-1</td>
<td>Video microscopy system for study of cannulated microvessels.</td>
<td>65</td>
</tr>
<tr>
<td>Fig 2-2</td>
<td>$Ca^{2+}$ measurement and imaging system.</td>
<td>67</td>
</tr>
<tr>
<td>Fig 2-3</td>
<td>Isolated and cannulated cremaster muscle arterioles with and without</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>tone at 70 mmHg.</td>
<td></td>
</tr>
<tr>
<td>Fig 3-1</td>
<td>Pressure-diameter curve for rat cremaster arterioles.</td>
<td>94</td>
</tr>
<tr>
<td>Fig 3-2</td>
<td>Effect of extracellular $[Na^+]$ on arteriolar diameter of rat cremaster</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>arterioles.</td>
<td></td>
</tr>
<tr>
<td>Fig 3-3</td>
<td>Effects of extracellular $[Na^+]$ on $[Ca^{2+}]_{cyt}$ in rat cremaster</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>arterioles.</td>
<td></td>
</tr>
<tr>
<td>Fig 3-4</td>
<td>Dose-response curve for NCX inhibitor KB-R7943 ($10^{-7}$-$3\times10^{-4}$ M)</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>on the diameter of de-endothelialized arterioles.</td>
<td></td>
</tr>
<tr>
<td>Fig 3-5</td>
<td>Dose-response curve for NCX inhibitor SEA0400 ($10^{-9}$-$3\times10^{-6}$ M)</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>on the diameter of de-endothelialized arterioles.</td>
<td></td>
</tr>
<tr>
<td>Fig 3-6</td>
<td>Effects of NCX inhibitor, KB-R7943 ($10^{-5}$M) on the diameter of the de-</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>endothelialized cremaster arterioles in decreasing extracellular $[Na^+]$.</td>
<td></td>
</tr>
</tbody>
</table>
Fig 3-7. Effects of NCX inhibitor, KB-R7943 (10^{-5}M) on the diameter of the de-endothelialized cremaster arterioles in 137 mM extracellular [Na^+].

Fig 3-8. Effects of NCX inhibitor, SEA0400 (10^{-6}M) on the diameter of de-endothelialized cremaster arterioles in decreasing extracellular [Na^+].

Fig 3-9. Effects of NCX inhibitor, SEA0400 (10^{-6}M) on the diameter of the de-endothelialized cremaster arterioles in 137 mM extracellular [Na^+].

Fig 3-10. Effects of Nifedipine (10^{-6}M) on the diameter of de-endothelialized cremaster arterioles in decreasing extracellular [Na^+].

Fig 3-11. Effects of Nifedipine (10^{-6}M) on the diameter of the de-endothelialized cremaster arterioles in 137 mM extracellular [Na^+].

Fig 3-12. Effects of NCX inhibition on arteriolar diameter (3\times10^{-5}M of KB-R7943).

Fig 3-13. Effects of NCX inhibition on arteriolar smooth muscle E_m (3\times10^{-5}M of KB-R7943).

Fig 3-14. Effect of nifedipine (10^{-6}M) on arteriolar diameter.

Fig 3-15. Effects of nifedipine (10^{-6}M) on arteriolar smooth muscle E_m.

Fig 3-16. Effect of decreasing extracellular [Na^+] on acute myogenic reactivity in endothelium-intact cremaster arterioles.

Fig 3-17. Effect of time on myogenic reactivity in endothelium-intact cremaster arterioles.

Fig 3-18. Effect of decreasing extracellular [Na^+] on acute myogenic reactivity in endothelium-denuded cremaster arterioles.

Fig 3-19. Effect of time on myogenic reactivity in endothelium-denuded cremaster arterioles.

Fig 3-20. Effect of decreasing extracellular [Na^+] on acute myogenic index in...
endothelium-intact and denuded cremaster arterioles.

Fig 4-1. Detection of NCX protein in rat cremaster arterioles by immunoblotting.  139
Fig 4-2. Real-time RT PCR showing detection of NCX1 (Fig 4-2a), 2 (Fig 4-2b) and 3 (Fig 4-2c).  143
Fig 4-3. NCX amplification graph of actual data using Sybr Green.  144
Fig 4-4. Melting curve analysis for NCX to determine the specificity of amplified PCR product.  145
Fig 4-5. Derivative melt curve for NCX to determine the specificity of amplified PCR product.  146
Fig 4-6. Autoradiogram of NCX cDNA products of PCR.  147

Fig 5-1. Effects of intra luminal pressure on arteriolar diameter of rat cremaster arterioles, after 24 hours.  168
Fig 5-2. Effects of a pressure step on arteriolar diameter of rat cremaster arterioles, after 24 hours.  168
Fig 5-3. Concentration-response curve for phenylephrine ($10^{-9}$-$10^{-5}$M) in rat cremaster arterioles, after 24 hours.  170
Fig 5-4. Effects of intra luminal pressure on passive arteriolar diameter of rat cremaster arterioles, after 24 hours.  170
Fig 5-5. Effects of intra luminal pressure on arteriolar diameter of rat cremaster arterioles, 24 hours after RP.  172
Fig 5-6. Effects of a pressure step on arteriolar diameter of rat cremaster arterioles, 24 hours after RP.  174
Fig 5-7. Concentration-response curve of phenylephrine ($10^{-9}$-$10^{-5}$M) in rat cremaster arterioles, after 24 hours.  174
cremaster arterioles, 24 hours after RP.

Fig 5-8. Effects of intra luminal pressure on passive arteriolar diameter of rat cremaster arterioles, after 24 hours.

Fig 5-9. Concentration-response curve for Forskolin ($10^{-9}$-10$^{-5}$M) of rat cremaster arterioles after RP with 10$^{-4}$M cell permeable PKI.

Fig 5-10. Concentration-response curve for Forskolin ($10^{-9}$-10$^{-5}$M) of rat cremaster arterioles after RP with 10$^{-4}$M cell impermeable PKI.

Fig 5-11. Effects of intra luminal pressure on arteriolar diameter of rat cremaster arterioles, 24 hours after antisense and sense ODN treatment.

Fig 5-12. Effects of an acute pressure step on arteriolar diameter of rat cremaster arterioles 24 hours after antisense and sense ODNs.

Fig 5-13. Concentration-response curve of phenylephrine ($10^{-9}$-10$^{-5}$M) in rat cremaster arterioles, 24 hours after antisense and sense ODNs treatment.

Fig 5-14. Effects of intra luminal pressure on passive arteriolar diameter of rat cremaster arterioles, 24 hours after antisense and sense ODNs treatment.

Fig 5-15. Effect of pressure step on MI in arterioles treated with antisense and sense ODNs.

Fig 5-16. Western blot comparing antisense treatment on NCX protein expression in rat cremaster arterioles to sense ODN treatment.

Fig 5-17. Effects of NCX knockdown on protein expression in rat cremaster arterioles, 24 hours after ODN treatment.

Fig 6-1. Myogenic signalling pathway for NCX.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM</td>
<td>Acetomethyl ester</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BK</td>
<td>Large conductance potassium channel</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees centigrade</td>
</tr>
<tr>
<td>[Ca^{2+}]</td>
<td>Calcium concentration</td>
</tr>
<tr>
<td>[Ca^{2+}]_{cyt}</td>
<td>Cytosolic Ca^{2+} concentration</td>
</tr>
<tr>
<td>[Ca^{2+}]_{o}</td>
<td>Extracellular Ca^{2+} concentration</td>
</tr>
<tr>
<td>CAM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>CLSM</td>
<td>Confocal laser scanning microscopy</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>CICR</td>
<td>Ca^{2+} induced Ca^{2+} release</td>
</tr>
<tr>
<td>Cl_{Ca}</td>
<td>Ca^{2+} activated chloride channel</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DRC</td>
<td>Dose-response curve</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>I_{Ca}</td>
<td>Ca^{2+} current</td>
</tr>
<tr>
<td>IP_{3}</td>
<td>Inositol triphosphate</td>
</tr>
<tr>
<td>IP_{3}R</td>
<td>Inositol triphosphate receptor</td>
</tr>
<tr>
<td>IICR</td>
<td>IP_{3} induced Ca^{2+} release</td>
</tr>
<tr>
<td>I_{Na/Ca}</td>
<td>NCX current</td>
</tr>
<tr>
<td>E_{m}</td>
<td>Membrane potential</td>
</tr>
<tr>
<td>K_{ATP}</td>
<td>ATP dependent K^{+} channel</td>
</tr>
<tr>
<td>K_{Ca}</td>
<td>Ca^{2+} dependent K^{+} channel</td>
</tr>
<tr>
<td>K_{IR}</td>
<td>Inward rectifier potassium channel</td>
</tr>
<tr>
<td>K_{v}</td>
<td>Voltage dependent potassium channel</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MLC_{20}</td>
<td>20 kDa of myosin like chain</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>mM</td>
<td>Millimole</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-N-Morpholino propanesulphonic acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>[Na$^+$]</td>
<td>Sodium concentration</td>
</tr>
<tr>
<td>[Na$^+$]$_{cyt}$</td>
<td>Cytosolic Na$^+$ concentration</td>
</tr>
<tr>
<td>[Na$^+$]$_o$</td>
<td>Extracellular Na$^+$ concentration</td>
</tr>
<tr>
<td>NCX</td>
<td>Na$^+$/Ca$^{2+}$ exchanger</td>
</tr>
<tr>
<td>NSCC</td>
<td>Non selective cation channel</td>
</tr>
<tr>
<td>ODN</td>
<td>Oligonucleotide</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethanesulphonyl fluoride</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PIP$_2$</td>
<td>Phosphoinositol biphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PKI</td>
<td>Protein kinase inhibitor</td>
</tr>
<tr>
<td>PMCA</td>
<td>Plasma membrane Ca$^{2+}$ ATPase</td>
</tr>
<tr>
<td>ROCC</td>
<td>Receptor operated Ca$^{2+}$ channel</td>
</tr>
<tr>
<td>RP</td>
<td>Reverse permeabilization</td>
</tr>
<tr>
<td>RyR</td>
<td>Ryanodine receptor</td>
</tr>
<tr>
<td>SACC</td>
<td>Stretch-activated Ca$^{2+}$ channel</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SERCA</td>
<td>Sarco(endo) plasmic reticulum Ca$^{2+}$ ATPase</td>
</tr>
<tr>
<td>SOCC</td>
<td>Store-operated Ca$^{2+}$ channel</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffer solution</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris buffer solution with tween</td>
</tr>
<tr>
<td>TES</td>
<td>N-Tris [hydroxymethyl] methyl-2-amino-ethane sulphonic acid</td>
</tr>
<tr>
<td>TRPC</td>
<td>Transient receptor potential channel</td>
</tr>
<tr>
<td>VSM</td>
<td>Vascular smooth muscle</td>
</tr>
<tr>
<td>VGCC</td>
<td>Voltage-gated Ca$^{2+}$ channel</td>
</tr>
<tr>
<td>XIP</td>
<td>Exchanger inhibitory peptide</td>
</tr>
</tbody>
</table>
SUMMARY
The arteriolar myogenic response, as demonstrated by the ability of arterioles to adjust their diameter to a change in intra luminal pressure, is fundamental to the control of vascular resistance and local microvascular blood flow and pressure. Despite this vital physiological role, the exact signalling pathways underlying this mechanically-induced vasomotor response remain uncertain.

The mechanical stimulus provided by an increase in intra luminal pressure activates a series of membrane ion gating mechanisms in arteriolar smooth muscle that regulate membrane potential ($E_m$), calcium ($Ca^{2+}$) entry and intracellular release of $Ca^{2+}$, and ultimately the contractile response. Key in the initiation of this sequence of events is a depolarization of the membrane as a result of opening of non-selective cation channels and, non-selective cation entry; predominately sodium ($Na^+$) under physiological conditions with subsequent opening of L-type voltage-gated $Ca^{2+}$ channels (VGCCs).

The aim of the studies described in this thesis is to examine whether the $Na^+/Ca^{2+}$ exchanger (NCX), in myogenically active arteriolar smooth muscle, functions in the reverse mode to remove $Na^+$ from a putative restricted space (between the plasma membrane and sarcoplasmic reticulum) and supply $Ca^{2+}$ from the extracellular space.

First order arterioles (passive diameter approximately 150 $\mu$m) were micro-dissected from the cremaster muscle of male Sprague-Dawley rats. The arterioles were then cannulated on glass micropipettes and mounted in a custom-designed superfusion chamber, continuously superfused with a physiological solution (Krebs-bicarbonate buffer solution) and maintained at 34 degrees centigrade ($^o$C). The superfusion chamber was placed on the stage of an inverted microscope and pressurized to
appropriate levels by connecting one of the cannulating pipettes to a pressure reservoir. Arterioles were studied under zero flow conditions to prevent shear stress interactions and in some studies de-endothelialized to remove any interference from endothelium. The internal diameter was monitored using video microscopy. Arterioles were required to develop spontaneous tone, without evidence of pressure leaks, to be included in the studies. Subsets of vessels were loaded with the Ca\textsuperscript{2+} fluorophore, Fura-2 (2 µM) for the measurement of changes in smooth muscle global cytosolic Ca\textsuperscript{2+} concentration [Ca\textsuperscript{2+}]\textsubscript{cyt}.

In initial studies the arterioles showed a significant myogenic contraction to an increase in intra luminal pressure and relaxation when the pressure was decreased. Functional significance of the NCX in the setting of arteriolar myogenic tone was established by reducing the extracellular Na\textsuperscript{+} concentration [Na\textsuperscript{+}] from 137 mM to 100, 75 and 25 mM. Reverse mode NCX activity led to an arteriolar constriction and an associated increase in [Ca\textsuperscript{2+}]\textsubscript{cyt} levels. Pharmacological NCX inhibitors, KB-R7943 (10^{-7}-3\times10^{-4}M) and SEA0400 (10^{-9}-3\times10^{-6}M) caused dose-dependent dilatation of the de-endothelialized arterioles. To further test the hypothesis that NCX is involved in the regulation of myogenic tone and thus in changes of arteriolar diameter and [Ca\textsuperscript{2+}]\textsubscript{cyt} levels, a single concentration of the inhibitors KB-R7943 (10^{-5}M) and SEA0400 (10^{-6}M) was used with decreasing extracellular [Na\textsuperscript{+}]. The de-endothelialized cremaster arterioles dilated in response to the pharmacological inhibitors, and the subsequent decrease in the extracellular [Na\textsuperscript{+}] failed to cause vasoconstriction. Additional experiments were conducted with another vasodilator, the L-type voltage-gated Ca\textsuperscript{2+} channel (VGCC) blocker nifedipine (10^{-6}M) to determine whether vasodilatation, per se, prevented the constrictor response to decreasing extracellular [Na\textsuperscript{+}] levels. The cremaster arterioles dilated in response to nifedipine, but vasoconstriction was not attenuated following
decreased extracellular $[\text{Na}^+]$ levels. Thus the vasoconstriction may be attributed to the activity of NCX, in the reverse mode, supporting the NCX involvement in the maintenance of the myogenic tone.

To examine the involvement of NCX in acute myogenic reactivity, vessels were subjected to a pressure step from 50 to 120 mmHg, both in endothelium-intact and endothelium-denuded vessels in decreasing extracellular $[\text{Na}^+]$ levels. There was a significant increase in myogenic reactivity (as shown by increased slope of diameter versus pressure) with increase in the extracellular $[\text{Na}^+]$ from 25 to 137 mM in endothelium-intact and endothelium-denuded vessel experiments. Myogenic index, a measure of myogenic responsiveness of a vessel, was calculated from a formula and was shown to be decreased with a decrease in extracellular $[\text{Na}^+]$, both in endothelium-intact and endothelium-denuded vessels suggesting that NCX is involved in an acute myogenic response.

Additional support for NCX involvement in myogenic tone was obtained from intracellular recordings of $E_m$ made in vascular smooth muscle (VSM) cells in pressurized arteriole preparations by using glass microelectrodes. It was observed that the NCX inhibitor, KB-R7943, caused significant concentration-dependent vasodilatation of cannulated arterioles that was associated with significant membrane hyperpolarization. It is unlikely that these data can be simply explained by a non-specific effect of KB-R7943 on VGCCs, as nifedipine (VGCC blocker) caused vasodilatation but not hyperpolarization of the arterioles.
Western blotting was used to characterize cellular NCX expression in the arteriolar smooth muscle, with aorta being used as positive control. Monoclonal anti NCX antibody (R3F1; Swant) was used and NCX expression was demonstrated by the presence of two bands of approximate molecular masses 120 and 70 kDa, with the band at 70 presumably being a proteolytic fragment. Total mRNAs were extracted from different tissues including, liver, cremaster vessels, aorta, heart, and skeletal muscle. Real-time Polymerase chain reaction (PCR) identified the three exchanger isoforms NCX1, NCX2 and NCX3 and their relative abundance. NCX1 was shown to be most abundant, with NCX3 being the least abundant of the three isoforms.

The significance of NCX in arteriolar myogenic reactivity was further examined by utilizing a knockdown antisense approach to decrease the level of functional exchanger in the cremaster arterioles. Oligonucleotide (ODN) sequences were introduced into the VSM layer by reverse permeabilization (RP) and stored with Krebs-bicarbonate buffer solution for 24 hours. To validate the loading technique, preliminary experiments were performed with Protein Kinase A Inhibitor (PKI), introduced by RP, and examining vasodilatation to Forskolin (10^{-9}-10^{-5}M). The arterioles loaded with Protein Kinase A Inhibitor (PKI) showed a rightward shift of the dose-response curve to Forskolin as compared to control arterioles. Additional control studies were performed to see the effect of time, temperature and RP technique on the viability of the arterioles. No significant effects of time, temperature and RP on the viability and myogenic reactivity of the vessels were noted within the constraints of the protocols used (e.g. 24 hour treatment with ODNs).
The NCX antisense and sense ODN’s were introduced in the VSM cells of isolated cremaster arterioles by RP and the vessels kept in Krebs-bicarbonate buffer solution at 34° C for 24 hours to reduce the expression of NCX. At the end of 24 hours incubation, vessels were cannulated and functional studies performed on vessels exposed to antisense or sense ODNs. There was a significant reduction (~ 25%) in NCX protein expression which was quantified by Western blotting. While steady-state pressure-diameter relationships did not show a significant difference between antisense-and sense-treated vessels, calculated myogenic index (for a pressure step of 50 to 120 mmHg) showed a significant decrease in the myogenic reactivity of antisense-treated vessels. This latter observation is consistent with an involvement of the NCX in myogenic constriction; however, further experiments need to be performed under more stringent knockdown conditions like increasing ODN concentration and/or increasing ODN exposure time.

In conclusion, the results of this study support the presence of a functional NCX in arteriolar VSM. The cellular approaches utilized have shown the presence of NCX by immunoblotting; that NCX1, as determined by real-time PCR, is the most abundant isoform; and that all three described families of the exchanger are present in the arteriolar wall. Further, NCX appears to be able to function in the reverse mode and also plays a role in the contractile mechanisms regulating arteriolar tone.
CHAPTER 1

LITERATURE REVIEW
Maintenance of appropriate and adequate internal environment is imperative for the optimal function of the tissues. The cardiovascular system has a mammoth task to manage the circulation of blood to the tissue and this feat is achieved by interaction between blood vessels and the local tissue and also its interaction with the autonomic nervous system and a multitude of humoral signals. The arterioles are the final section of the arterial system and act as control elements through which the blood is delivered to the capillary network of the tissue. Changes in lumen diameter of the arterioles determine the blood supply to the different regional circulations. The arterioles are the effector zone of the interplay of local and neurohumoral factors to regulate the blood flow to the tissue. Arterioles typically maintain a sustained intrinsic contraction with this tone occurring in parallel to the contributions provided by extrinsic control affected by the either of sympathetic nervous system or humoral factors. The modulation of this tone allows changes in arteriolar lumen and thence the blood flow (flow being proportional to the fourth power of the radius). An understanding of these mechanisms at organ-tissue, cellular and molecular levels are necessary for a better comprehension of pathophysiology of disorders relating to vascular tone and a better appreciation of the normal functioning of the circulation. Such knowledge will be useful in designing strategies to treat or control these disorders.

The present review of the literature will deal with a brief description of the myogenic response, local control of blood flow, anatomy of the arterioles, the structure of VSMs, excitation-contraction coupling in smooth muscle, and detailed description of the ion channels of the smooth muscles and regulation of intracellular Ca$^{2+}$ with specific reference to involvement of the NCX.
The Myogenic response and the biochemistry of smooth muscle contraction

The myogenic response or mechanism is the term given to the contraction or relaxation of VSM in response to an increase or decrease, respectively, in intra luminal pressure (Johnson 1981). With an increase in intra luminal pressure blood vessels respond by constriction which is preceded by a passive dilatation and conversely any decrease in the intra luminal pressure will lead to a dilatation of the vessel that is preceded by partial collapse. In describing myogenic properties of blood vessels many authors use different terms including myogenic response; stretch activation; pressure-dependent contraction; myogenic tone; basal tone; spontaneous tone and intrinsic tone to perhaps explain the different facets of similar underlying mechanisms.

To understand the processes involved in smooth muscle contraction and hence the myogenic response, it is necessary to briefly review the structure of arterioles. At the periphery of the circulation the diameter of the muscular arteries gradually tapers to a point where the media consists of only 1-2 layers of smooth muscle cells. The term arteriole is the accepted name given to these vessels, however, as the lumen diameter varies depending on the state of contraction and dilatation (Rhodin 1981) it is somewhat futile to give a range of cross- sectional diameter although it can range approximately from 300 µm to 10 µm (Davis, Ferrer et al. 1986). The vascular wall is composed of an internal endothelium (tunica intima), a middle smooth muscle cell layer (tunica media), and outer connective tissue layer (tunica adventitia). The layer of endothelial cells rests on the inner elastic lamina, which contain bundles of elastin fibres. Elastin fibres provide additional mechanical support and are responsible for the passive mechanical properties of the arterioles (Mulvany and Aalkjaer 1990). Tunica media is comprised of 1-2 layers of smooth muscle cells, which are elongated and spindle-shaped but these are
interspersed with collagen fibers. Smooth muscle cells are transversely oriented to the long axis of the blood vessels such that when the smooth muscle contracts, it also causes the lumen of the arteriole to decrease in diameter or constrict. The tunica adventitia is the outermost layer of the vascular wall and is formed mainly by collagen fibers, networks of autonomic (primarily adrenergic) nerve fibers and varicose terminals (Hirst and Edwards 1989).

There is a distinction between the myogenic response and the length-tension relationship, as the latter is seen in all muscles where as the myogenic response is limited to smooth muscle or to some extent cardiac muscle (Johnson 1981). The myogenic response is seen in arteries, venules, veins and lymphatics but it is most prominent in arterioles. Conduit arteries show little or no myogenic tone (Speden 1984; Lombard, Smeda et al. 1986), small arteries show moderate myogenic tone (Osol and Halpern 1985), while arterioles show significant and sustained constrictions in response to increase in intra luminal pressure (Jackson and Duling 1989; Davis and Sikes 1990; Kuo, Chilian et al. 1990; Falcone, Davis et al. 1991). Davis (Davis 1993), in studies of the hamster cheek pouch arteriolar network examined the relative myogenic responsiveness of the vascular branching orders, demonstrating that there is a myogenic responsiveness gradient, with the maximum responsiveness being of the intermediate size arterioles. This study further demonstrated that the maximum tone achieved by each order of vascular branching was at a pressure near, or slightly higher, to its in vivo pressure. Myogenic responsiveness is reported to increase with a decrease in the vessel size in most vascular beds with the exception of cerebral circulation (Osol 1995).
Fig 1 illustrates three examples of myogenic behavior in arterioles. Fig 1a shows that after a step increase in the intra luminal pressure there is a passive dilatation of the arteriole, which is transient and is followed by a constriction. Once the pressure is lowered back to its original level there is a transient collapse followed by a dilatation (Davis and Sikes 1990). This is a normal property of the resistance vessels and which is considered to participate in local autoregulation. Fig 1b shows that, with an increase in the temperature to 37° C (34° C for cremaster arteriole) and at their in vivo intra luminal pressure, arterioles develop some active force also called tone which is around 50% of passive diameter (Kuo, Davis et al. 1988). Fig 1c depicts two graphs, active and passive diameters at the in vivo intra luminal pressure of an arteriole (Davis 1993). The myogenic range is the range of pressures over which the active diameter curve has a less positive slope than the passive diameter curve (Davis and Hill 1999).

Historical perspectives

Myogenic activity was first described by Jones (Jones 1852), in 1852 in the veins of bat wings. He showed that the contractions of the veins were dependent upon the intra luminal pressure within them. However, Sir William Bayliss, in 1902 (Bayliss 1902), is credited with giving the first description of the myogenic response in arterial vessels. He used plethysmography to record large increases in the volume of blood in the hind limbs of dogs after releasing an aortic occlusion. The increase in pressure resulted in vasoconstriction while decreased pressure led to vasodilatation. As Bayliss considered this response to be too rapid to be caused by the accumulation of metabolites, due to the occlusion of the blood flow, he attributed this response to the same mechanism, which was responsible for the constriction of arteries after a rise in the intra luminal pressure. Thus Bayliss suggested that the intra luminal pressure might act as a
Chapter 1

Literature Review

mechanical stimulus to the vascular wall and induce a certain degree of constriction. His studies also advanced the idea that vascular tone is a determinant of intra luminal pressure to a significant portion of the vasculature.

Anrep (Anrep 1912) repeated the experiments performed by Bayliss and extensively challenged the earlier conclusions by suggesting that a local reduction in the intra luminal pressure led to a decrease in blood flow with subsequent accumulation of metabolites, such as lactic acid, which cause vasodilatation. Further, Anrep was unable to demonstrate an active response to an elevation in the intra luminal pressure. Data from other workers also supported the hypothesis that chemical factors might play a role in local regulation (Roy and Graham Brown 1880; Hooker 1911; Hooker 1912). In contrast, while studying isolated arteries Wachholder (Wachholder 1921), observed constriction in the isolated carotid from horse in response to increased intra luminal pressure. Further, Klemensiewicz (Klemensiewicz 1921) while working with the denervated hind limbs of frogs observed that lowering the intra luminal pressure in the blood vessels of the web caused a corresponding vasodilatation where as increasing the intra luminal pressure caused vasoconstriction. Similarly, Hirose and Schilf (Hirose and Schilf 1931), observed vasodilatation in the hind limbs and intestinal arteries of cat, after occlusion of the blood flow for less than 5 seconds. They suggested this period was too short for metabolite accumulation, supporting the Bayliss hypothesis.

Working with cat pial arterioles, Fog in 1937 (Fog 1937), and Forbes (Forbes, Nason et al. 1937), made similar observations that pial arterioles dilated to a decrease in the intra luminal pressure, while constricting to an increase in pressure. As it was already known
Fig 1-1a.

Fig 1-1b.
Fig 1-1c.

Fig 1-7. Examples of myogenic behavior in arterioles.

Fig 1-1a shows the myogenic response of a cannulated arteriole to an increase in intraluminal pressure. There is an increase in the arteriolar diameter after the pressure step. Initially there is a passive dilatation followed by a sustained constriction. This is followed by constriction caused by passive collapse of the vessel due to release of the intraluminal pressure back to the original value. Arteries maintain active constriction as shown by Fig 1-1b, where they spontaneously constricts to ~ 50% of the passive diameter after being cannulated and pressurized and the temperature raised to 37° C (34° C for cremaster arterioles). This constriction of arterioles can be maintained for several hours. Fig 1-1c illustrates the pressure-diameter relationship of an arteriole in the presence (active) or absence (passive) of Ca\(^{2+}\) in the bathing media.

Graphs redrawn from Davis & Hill (Davis and Hill 1999).
that the cerebral circulation is not under nervous control and the blood in the pial arterioles did not show any change in its color, it was concluded that the vascular responses were not neural or chemically mediated, thus the Bayliss response might be the cause of this constriction. Burgi (Burgi 1944), also made similar observations, recording transient and sustained contractions of the bovine mesenteric arteries during step elevations in intra luminal pressure. Apart from these reports there was little work on myogenic responses for five decades subsequent to the Bayliss study in 1902. This was due partly to the persuasive arguments of Anrep and also the majority of work done in the following years was in support of the argument that chemical and neural factors may be responsible for the observed changes in arteriolar diameter following an alteration in intra luminal pressure.

The question of vascular tone was revisited by Folkow (Folkow 1949), who examined the vascular responses to changes (increases and decreases) in intra luminal pressure. He showed that extirpation of adrenals and a complete denervation of hindlimbs (muscular), splanchnic and cutaneous vessels of cats, dogs and rabbits did not abolish vascular tone. Although the flow was double in the denervated preparations he confirmed the findings of Bayliss that an increase in the intra luminal pressure caused stretching of the VSM, which acts as a stimulus for increased tone in the small blood vessels, while a reduction in intra luminal pressure abolished the stimulus thereby leading to vasodilatation. Folkow (Folkow 1952) also demonstrated that autoregulation of the blood flow is a non-neural and pressure-dependent mechanism. His observations indicated that vascular tone is myogenic in origin, albeit strongly influenced by external factors.
Emil Bozler considered as the “Father of the Electrophysiology” of visceral smooth muscle recognized that such muscle could be classified as single or multiunit smooth muscle by using extracellular recording methods in studies performed between 1938 and 1948. Bulbring (Bulbring 1955) was the first to apply microelectrode techniques to study the relationship between smooth muscle contraction, spike activity and $E_m$ of the smooth muscle cells in guinea pig taenia coli. There was an increase in the spike activity and the $E_m$ depolarized with stretch. Bulbring and Kuriyama (Bulbring and Kuriyama 1963), suggested that application of stretch changes the electrical properties of membranes and thus a change in the $Na^+$ permeability which should lead to depolarization and the increased spike activity observed in taenia coli. Refined patch clamp techniques were introduced by Neher and Salkman (Neher and Sakmann 1976; Hamill, Marty et al. 1981) and Hamill (Hamill, Marty et al. 1981) which were important for the study of specific ion channels. VSM work derived using this method is described later.

Pappenheimer and Soto-Rivera (Pappenheimer and Soto-Rivera 1948) combined a continuous measurement of the weight of the organ during perfusion with the flow and arterial and venous pressure which can be used to indirectly determine capillary pressure. Pappenheimer’s studies laid the foundation for various microcirculatory studies of intrinsic tone. In 1959 Johnson (Johnson 1959), studied the myogenic response and autoregulation of the blood flow using sophisticated whole organ methods and concluded that this mechanism can be responsible for changes in vascular resistance in vivo.

To understand better the relation between contractility of a vessel and excitation
coupling it was essential that the vessel be investigated under controlled load. To facilitate this Bevan and Osher (Bevan and Osher 1972), developed the first myograph with vessel segments mounted on two wires, which were clamped at either ends for an isometric response. This was followed by the presumed more physiological arrangement of the pressure myograph, where small blood vessels were cannulated, on glass pipettes to allow control of intra luminal pressure. Studies by Duling in 1981 (Duling, Gore et al. 1981), pioneered the techniques for quantification of myogenic responses and enabled finer analysis of its properties. They used isolated microvessels from hamster cheek pouch, testis and mesentery for the study of their physiology and pharmacology studies. This technique allowed more careful separation of the effects of intra luminal pressure on vascular tone from the effects of neural, chemical and flow-mediated factors. Since then techniques of imaging, fluorescent molecules and tools of molecular biology have enhanced our understanding of the myogenic response.

**Blood flow dynamics**

Blood flowing through a vessel or a tube is determined by several factors including impediment to the blood flow or vascular resistance, and the pressure difference between the proximal and the distal part of the vessel, depicted mathematically as follows:

\[ Q = \frac{\Delta P}{R} \]

Where as \( Q \) is the blood flow, \( \Delta P \) = pressure difference between the two ends of the vessel and \( R \) is the resistance to flow.
This is the simplest expression of resistance to flow and is analogous to Ohm's law. It allows clarification of inter-relationships among pressure, flow and resistance, although it does not expand on the factors responsible for changes in resistance. Poiseuille’s equation developed from the study of steady laminar flow by a Newtonian liquid in narrow rigid glass tubes provides the factors influencing resistance (Poiseuille 1846).

$$Q = \pi \frac{(P_1 - P_2)r^4}{8\eta}$$

Where $Q$ = flow rate, $P_1 - P_2$ = pressure difference across the circuit, $r$ = radius of the tube, $\eta$ = viscosity of liquid and $L$ = length of the tube.

This equation shows the relation between vascular resistance and viscosity and length of the tube. If either of the two, i.e. viscosity or length of the tube halve, resistance will be doubled. This also emphasizes the importance of the radius, because if the radius doubles the blood flow increases sixteen times. Thus this relationship stresses the impact of a change in vascular tone that may follow an alteration in intra luminal pressure.

**Physiological significance of myogenic response**

The myogenic response participates in a number of physiologically important functions, namely, it establishes basal vascular tone, blood flow autoregulation and regulation of capillary hydrostatic pressure (Davis and Hill 1999). Vascular tone results from myogenic mechanisms as suggested by Bayliss and Folkow and is indicative of the average contractile state of musculature of small arteries within a regional circulation (Mellander 1968). Vascular tone is the end result of intrinsic activity of smooth muscle cells of the vascular wall over the extrinsic effect of metabolic, nervous system and
humoral factors. In isolated arteriolar preparations tone is usually achieved when the vessel is pressurized to the in vivo physiological level (Duling, Gore et al. 1981; Davis, Kuo et al. 1995).

Autoregulation, the inherent ability of a vascular bed to preserve blood flow despite changes in arterial pressure, has been observed in a variety of vascular beds (Johnson 1964; Edwards 1983; Sun, Messina et al. 1992; Liao and Kuo 1997; Thorin-Trescases, Bartolotta et al. 1997). It is believed that the mechanism behind autoregulation can be best explained by considering the balance of the two principal local control mechanisms, the myogenic response and metabolite regulation (Johnson 1986). Although the exact contribution from the myogenic mechanism is unknown, it is believed that its contribution is significant (Schubert and Mulvany 1999).

The myogenic response may also regulate capillary hydrostatic pressure if there is any change in the systemic arterial pressure, supporting the possibility that capillary pressure is, indeed, controlled through a range of 30-170 mmHg (Jarhult and Mellander 1974; Davis and Hill 1999). This occurs as capillary pressure depends on the pre-to post-capillary ratio which increases by autoregulatory constriction of the arterioles following an increase in intra luminal pressure.

**Mechanisms underlying the presence of myogenic tone**

The importance of Ca\(^{2+}\) was first demonstrated by Uchida and Bohr (Uchida and Bohr 1969), when they showed that myogenic tone could be abolished by removal of Ca\(^{2+}\) from the extracellular fluid. This is in contrast to skeletal muscles which have no dependence, and cardiac muscles which have partial dependence on the availability of
extracellular Ca\(^{2+}\). Their observations were confirmed by the isolated arteriole studies of Duling and colleagues (Duling, Gore et al. 1981). More recent studies utilizing newer and finer techniques of isolated vascular perfusion, fluorescent imaging, and molecular biology have demonstrated the central role of [Ca\(^{2+}\)]\(_{cyt}\) in the control of myogenic tone. **Fig 1-2** provides a schematic of the possible operative principles. It is generally believed that myogenic responses are independent of the endothelium, and require Ca\(^{2+}\) entry and Ca\(^{2+}\) sensitization processes in the VSM cells. Using a variety of techniques it has been established that stretch activates Ca\(^{2+}\) influx and that this effect can be abolished by inhibitors of VGCC in most vascular beds (Nakayama 1982; Laher and Bevan 1989; Wesselman, VanBavel et al. 1996; Setoguchi, Ohya et al. 1997).

**Membrane events, depolarization and ion channels**

The dominant notion in myogenic signalling is that smooth muscle cell stretch causes depolarization which leads to opening of voltage-gated Ca\(^{2+}\) channels resulting in Ca\(^{2+}\) entry, raising the [Ca\(^{2+}\)]\(_{cyt}\) (**Fig 1-3**). The idea of a role for \(E_m\) in myogenic responses was put forth by Bulbring (Bulbring 1955). The relationship between the intra luminal pressure and \(E_m\) was subsequently demonstrated in cannulated arteries by Harder. Using glass micro-electrodes Harder showed that \(E_m\) becomes less negative when the intra luminal pressure is increased (Harder 1984).

Arteriolar smooth muscle cells *in vitro* exhibit a stable \(E_m\) of between -40mV to -60mV at normal levels of intra luminal pressure (Harder 1984; Harder, Gilbert et al. 1987; Nelson, Patlak et al. 1990; Brayden and Nelson 1992). The reported *in vivo* \(E_m\)s are -40 mV to -55 mV (Neild and Keef 1985; Hirst and Edwards 1989).
Fig 1-8. Schematic of the possible operative principles for myogenic responses in the VSM cells.

Mechanical stimulation by intraluminal pressure → ↑ Intracellular Ca²⁺ → Myogenic constriction

Fig 1-9. Stretch of smooth muscle cell leads to increase in [Ca²⁺]_{cyt}
$E_m$ is a very important regulator of vascular tone with this relationship being very steep such that a few millivolts change in $E_m$ causes a significant change in blood vessel diameter (Nelson, Standen et al. 1988; Kotecha and Hill 2005). $E_m$ regulates the vascular tone primarily by opening of VGCCs (Nelson, Patlak et al. 1990), and Ca$^{2+}$ release through inositol triphosphate (IP$_3$) production (Itoh, Seki et al. 1992).

A major remaining question is how does depolarization occur to initiate myogenic constriction? Suggested mechanisms include the activation of an outward Cl$^-$ current; Na$^+$ entry via NSCC (Byrne and Large 1987; Garland 1987; Benham, Bolton et al. 1987b; Hirst and Edwards 1989); or inhibition of various K$^+$ channels. There is evidence that stretch-activated channels (SACC) (Davis, Donovitz et al. 1992; Wu and Davis 2001) and transient receptor potential channels (TRPC) (Welsh, Morielli et al. 2002; Earley, Waldron et al. 2004) cause pressure-induced depolarization and hence vasoconstriction. McCarron et al. reported that vasoconstriction observed in response to increased intra luminal pressure is dependent on Ca$^{2+}$ entry through VGCCs, since it was abolished by Ca$^{2+}$ removal and by dihydropyridine antagonists of VGCC. Langton et al. concluded that increases in [Ca$^{2+}$]$_{cyt}$ within the range 100-200 nM can account for myogenic contraction, and that stretch-evoked modulation of Ca$^{2+}$ currents may contribute to the myogenic response in cerebral resistance arteries (McCarron, Crichton et al. 1997).

Depolarization thus increases the open probability of the L-type VGCCs and raises [Ca$^{2+}$]$_{cyt}$ which causes contraction via Ca$^{2+}$/calmodulin (Ca$^{2+}$/CAM) dependent activation of myosin light chain kinase. It is reported that an increase in the intra luminal pressure causes a graded membrane depolarization of the smooth muscle cell membrane of the
resistance arteries from -65 mV to -35 mV which causes a corresponding graded constriction (Harder 1984; Brayden and Nelson 1992; Meininger and Davis 1992; Knot and Nelson 1995). This depolarization is sufficient to increase the opening probability of dihydropyridine sensitive Ca\(^{2+}\) channels and increase \([\text{Ca}^{2+}]_{\text{cyt}}\) causing thereby a Ca\(^{2+}\) dependent contraction (Knot and Nelson 1998).

The following section lists the main kinds of ion channels, which may contribute in maintenance of \(E_m\), and thus myogenic tone:

1. **Potassium channels:**

Membrane depolarization of the VSM relies on the activation and the influx of Na\(^{+}\), on the other hand, membrane repolarization and vaso relaxation is dependent on opening of cell membrane K\(^{+}\) channels. Thus K\(^{+}\) channels not only diminish membrane depolarization but also may play a role in maintenance of resting \(E_m\) and the resulting myogenic tone. The following section will discuss the different types of K\(^{+}\) channels whose characteristics have largely been determined using patch clamp techniques.

   *i. Inward rectifier K\(^{+}\) channels (K\(_{\text{IR}}\)):

Inward rectifier current is present in small resistance vessels (i.e. < 200 µm) of various vascular beds including cerebral (Quayle, McCarron et al. 1993), coronary (Robertson, Bonev et al. 1996) and mesenteric, (Edwards and Hirst 1988) vasculature to name a few. The channel shows a time-dependent rectification opposite to the driving force of the K\(^{+}\) gradient, opening when the membrane is hyperpolarized; however a small sustained outward current is present at a positive Nernst equilibrium potential). The K\(_{\text{IR}}\) channel is the only channel which is open when extracellular \([\text{K}^{+}]\) is elevated in the
range of 5-25 mM. This shifts the $E_m$ towards the $E_K$. This means that the membrane moves away from the basal potential of -50 causing vascular hyperpolarization. This leads to the inhibition of voltage dependent $Ca^{\text{+}}$ entry thereby causing vasodilatation. Although in arterial smooth muscle the roles of $K_{iR}$ channel are incompletely understood, one of the physiological roles of the $K_{iR}$ channel is suggested to be regulating the resting $E_m$. Thus $K_{iR}$ can be an important regulator of vascular tone (Nelson and Quayle 1995b). Further, in the cerebral circulation, increased neuronal activity is associated with release of metabolites including $K^+$. One of the mechanisms by which increase in the extracellular $K^+$ arterioles is via activation of $K_{iR}$ channels (Edwards and Hirst 1988). Thus $K_{iR}$ may be one of the mechanisms of vasodilatation by which smooth muscle cells might respond to patho-physiological situations like hypoxia and ischemia where extracellular $[K^+]$ is elevated (Edwards, Hirst et al. 1988a; McCarron and Halpern 1990).

**ii. Voltage-dependent $K^+$ channels ($K_v$):**

$K_v$ channels are $K^+$ channels found in many vascular beds including coronary (Volk and Shibata 1993), cerebral (Bonnet, Rusch et al. 1991), and the pulmonary (Okabe, Kitamura et al. 1987) vasculature. These channels carry outward rectifying current with a brief delay following depolarization beyond -30mV which is called the delayed rectifier $K^+$ current (Jan and Jan 1992). This channel is important in decreasing the excitation by providing a mechanism of membrane hyperpolarization and secondly contributes toward the VSM resting $E_m$. In pressurized small cerebral arteries from rabbit, inhibition of $K_v$ with agents such as 4-aminopyridine and 3,4-diaminopyridine caused depolarization and an increased level of myogenic tone to graded elevations in transmural pressure (20-100 mmHg) (Knot and Nelson 1995).
iii. ATP-dependent $K^+$ channels ($K_{\text{ATP}}$):

These are voltage independent and adenosine triphosphate (ATP)-dependent channels, identified in various vascular beds including coronary (Xu and Lee 1994), pulmonary (Clapp and Gurney 1992) and mesenteric (Silberberg and van Breemen 1992) circulations. The open probability of $K_{\text{ATP}}$ increases when the intracellular ATP falls to a very low level or when adenosine, ADP or $H^+$ concentrations are increased as happens during hypoxia. Glibenclamide, a $K_{\text{ATP}}$ blocker, causes partial depolarization and vasoconstriction demonstrates that $K_{\text{ATP}}$ is important in the regulation of vascular tone in some vascular beds including the coronary circulation (Eckman, Frankovich et al. 1992; Imamura, Tomoike et al. 1992), and mesenteric arteries (Garland and McPherson 1992). Inhibition of $K_{\text{ATP}}$ channels also causes disruption of coronary (Komaru, Lamping et al. 1991; Narishige, Egashira et al. 1993) and cerebral (Hong, Pyo et al. 1994) autoregulation of blood flow. This indicates the role of $K_{\text{ATP}}$ channels in regulation of vascular tone and autoregulation of blood flow.

iv. Ca$^{2+}$-dependent $K^+$ channels ($K_{\text{Ca}}$):

Large conductance $K^+$ channels, found in most smooth muscle cells (also called big $K_{\text{Ca}}$ (BK$_{\text{Ca}}$) or maxi $K_{\text{Ca}}$ channels), are activated by $[\text{Ca}^{2+}]_{\text{cyt}}$ and membrane depolarization (Nelson 1993). The open state probability of the BK$_{\text{Ca}}$ channel increases with membrane depolarization (2.7 fold increase per 12-14 mV depolarization) (Benham, Bolton et al. 1986a; Langton, Nelson et al. 1991). The open probability of the channel increases with elevation of $[\text{Ca}^{2+}]_{\text{cyt}}$. This activation is under partial control of small concentrated bursts of Ca$^{2+}$ release (Ca$^{2+}$ sparks) from the sarcoplasmic reticulum (Nelson, Cheng et al. 1995; Jaggar, Wellman et al. 1998), estimated to be around 10 µM (Zhuge, Fogarty et al. 2002). Activation of Ca$^{2+}$ sparks is due to release channels sensitive to ryanodine.
Chapter 1  

and activated by Ca\(^{2+}\) influx (Jaggar, Wellman et al. 1998). BK\(_{\text{Ca}}\) is also activated by cyclic nucleotides (Kume, Takai et al. 1989; Taniguchi, Furukawa et al. 1993) apart from being regulated by fatty acids and stretch as shown by Kirber in pulmonary artery smooth muscle. These are a direct effect and thus independent of intracellular Ca\(^{2+}\) changes or of channel phosphorylation of BK\(_{\text{Ca}}\) (Kirber, Ordway et al. 1992).

BK\(_{\text{Ca}}\) channels have been suggested as a target for the arachidonic acid (AA) metabolite 20 HETE (a Cytochrome P450 monooxygenase pathway product). 20 HETE is produced after elevation in intra luminal pressure and causes both depolarization and vasoconstriction that acts in part by inhibition of the opening of the BK\(_{\text{Ca}}\) (Harder, Lange et al. 1997; Wesselman, Schubert et al. 1997; Gebremedhin, Lange et al. 1998; Roman 2002).

Other types of K\(_{\text{Ca}}\) channels, namely small- and intermediate-conductance Ca\(^{2+}\) activated potassium channels (SK\(_{\text{Ca}}\) and IK\(_{\text{Ca}}\)) are present in arterial endothelial cells but not on the smooth muscle (Edwards, Dora et al. 1998; Doughty, Plane et al. 1999). This is pivotal as activation of these channels causes endothelial cell hyperpolarization which leads to hyperpolarization of smooth muscle and relaxation.

The activation of K\(_{\text{Ca}}\) leads to K\(^{+}\) efflux and hyperpolarisation and therefore closure of voltage-gated Ca\(^{2+}\) channels and VSM relaxation (Brayden and Nelson 1992). It is further suggested that these channels have a physiological role in the regulation of vascular tone (Toro, Amador et al. 1990; Kume and Kotlikoff 1991; Scornik and Toro 1992) and specifically to have a role in limiting the potential feed-forward nature of the myogenic response (Nelson, Cheng et al. 1995).
2. Ca\(^{2+}\) activated chloride channels (Cl\(_{\text{Ca}}\)):

The chloride conductance reported in smooth muscle is that of Ca\(^{2+}\) dependant (I\(_{\text{Cl(Ca)}}\)) and volume sensitive Cl\(^-\) currents (Cl\(^-\)_VR) (Pacaud, Loirand et al. 1991; Large and Wang 1996; Yamazaki, Duan et al. 1998). I\(_{\text{Cl(Ca)}}\) has been identified in number of vascular beds namely, mesenteric (Klockner and Isenberg 1991) and pulmonary arteries (Leblanc and Hume 1990). Although I\(_{\text{Cl(Ca)}}\) is strongly dependent on [Ca\(^{2+}\)] it is not as sensitive as K\(_{\text{Ca}}\). Due to the presence of other transport mechanisms (Cl\(^-\)/HCO\(_3^-\) exchanger and Na\(^+\)/K\(^+\)/2Cl\(^-\)) in the smooth muscle membrane there is a build up of Cl\(^-\) over and above the expected level due to the passive diffusion of the ion (Casteels 1971; Koncz and Daugirdas 1994). As the E\(_m\) of the smooth muscle cell is approximately -50mV, which is lower than the equilibrium potential for Cl\(^-\) (-20mV), there is an efflux of Cl\(^-\) from the cell (once the Cl\(^-\) channels are activated) and this results in depolarization. Release of Ca\(^{2+}\) from intracellular stores is one mechanism which may mediate the activation of Cl\(^-\) channels (Large and Wang 1996). The resulting membrane depolarization may result in the activation and opening of VGCC and cause contraction. It is suggested that Cl\(_{\text{Ca}}\) may play a role in regulation of resting E\(_m\). However due to the non-specific effects of available Cl\(^-\) channel inhibitors (Doughty, Miller et al. 1998), convincing functional evidence has been hard to obtain.

Welsh et al. reported that swelling-induced Cl\(^-\) currents might be involved in myogenic depolarization. A hyposmotic challenge was able to elicit a response that was similar to myogenic response which was abolished by Cl\(^-\) channel antagonists (Welsh, Nelson et al. 2000). However, it is uncertain whether the events activated by osmotic cell swelling exactly mimic that occurring in VSM during an increase in intra luminal pressure.
3. **Stretch-activated Ca\textsuperscript{2+} channels (SACC):**

Stretch-activated or mechano-sensitive channels have been shown to be present on variety of vascular and visceral smooth muscle using patch clamp techniques. Mechano-sensitive channels with Na\textsuperscript{+}, K\textsuperscript{+} or Cl\textsuperscript{−} selectivity have been identified (Morris 1990). As per Goldman-Hodgkin-Katz equation, the $E_m$ is predominantly determined by most permeable ion which at rest it is the K\textsuperscript{+} ion (Nelson, Patlak et al. 1990). Stretch can lead to depolarization by the opening of cation channels (leading to entry of positive ions Ca\textsuperscript{2+} or Na\textsuperscript{+}), the closure of K\textsuperscript{+} channels or the opening of Cl\textsuperscript{−} channels (Fig 1-4). Under physiological conditions there is a relative permeability of K\textsuperscript{+} ≥ Na\textsuperscript{+} ≥ Ca\textsuperscript{2+} (Davis, Donovitz et al. 1992; Wellner and Isenberg 1994; Setoguchi, Ohya et al. 1997).

There is evidence for mechano-sensitive ion channels in arteriolar VSM (Davis, Meininger et al. 1992a). These are proposed to form the link between stretch and depolarization and bring the cell closer to threshold for VGCC opening. This hypothesis is supported by the facts that stretch causes an increase in intracellular Ca\textsuperscript{2+} of isolated VSM cells (Davis, Meininger et al. 1992a), pressure causes increase in intracellular Ca\textsuperscript{2+} in isolated arteries (Meininger, Zawieja et al. 1991), VGCC antagonists do not block development of depolarization and VGCC antagonists only partially block the stretch-induced increase in Ca\textsuperscript{2+} (Davis, Meininger et al. 1992a; Davis 1993; Zou, Ratz et al. 1995).

4. **Transient receptor potential channels (TRPC):**

TRP channels were first described in Drosophila photoreceptors, when a mutation of the TRP gene led to a more transient voltage response to continuous bright light (Minke 1977; Montell, Jones et al. 1985; Minke and Cook 2002). In recent years a number of
homologs of TRP have been found; at least 20 genes encode for related cation channels. These TRP channels can be classified into six related protein families (Clapham 2003).

TRP channels TRPC3, TRPC6 and TRPC7 have relatively low selectivity of Ca$^{2+}$ over Na$^+$ and are sensitive to [Ca$^{2+}$]$_{cyt}$ changes (Nilius and Droogmans 2001; Minke and Cook 2002; Clapham 2003). It is also suggested that these channels may have roles in the regulation of myogenic tone, airway resistance and cardiac function (Clapham 2003).

Recent studies suggest that TRPC6 and TRPM4 play an essential role in the myogenic response of intact cerebral arteries. TRPC6 and TRPM4 antisense ODNs reduced the vasoconstriction and membrane depolarization induced by increased intra luminal pressure (Welsh, Morielli et al. 2002; Earley, Waldron et al. 2004). Although TRPM4 is not known to be stretch-activated, it is important for the myogenic response. It is hence hypothesized that Ca$^{2+}$ entering through TRPC6 channels stimulates TRPM4 channels so that the response of TRPC6 is amplified (Beech 2005). The mechanism by which stretch activates TRPC6 is unknown, but it is proposed that intra luminal pressure may cause increased TRPC6 activity via the phospholipase C (PLC)/Diacylglycerol (DAG) pathway, causing membrane depolarization and increased myogenic tone (Slish, Welsh et al. 2002; Beech 2005).

**Ca$^{2+}$ homeostasis**

Ca$^{2+}$ is the most abundant cation in the human body (Peng, Brown et al. 2003). Sydney Ringer (Ringer 1883) was the first to describe the physiological effect of Ca$^{2+}$ on cardiac
Fig 1-10. Stretch activation of $\text{Ca}^{2+}$ channels leading to depolarization and VSM contraction.

Stretch can lead to depolarization by opening of cation channels, closure of $K^+$ channels or opening of $\text{Cl}^-$ channels (Fig 1-4). This leads to entry of positive ions ($\text{Ca}^{2+}$ or $\text{Na}^+$), depolarization and hence opening of VGCC and contraction.
contraction. It was in 1928, that Lewis Victor Heilbrunn (Steinbach 1960; Campbell 1986) recognized the wide-ranging importance of Ca\(^{2+}\) in cellular functions including promotion of cellular adhesion and/or intercellular communication; effects on various enzyme systems, including ATPases and lipases; regulation of permeability properties of cell membranes; regulation of cell division; control of metabolic activity of the cell; and apoptosis. The ionic form of Ca\(^{2+}\) is a universal intracellular messenger (Clapham 1995) to modulate many intracellular processes.

Ca\(^{2+}\) homeostasis is vital for VSM cells with various stimuli affecting the state of contraction by modulating intracellular Ca\(^{2+}\) concentrations. It is, therefore, of major importance to maintain and regulate the Ca\(^{2+}\) gradient across the cell membrane. The free (ionic) [Ca\(^{2+}\)]\(_{cyt}\) level is approximately 0.1 \(\mu\)M under basal conditions. This is about 10,000 times lower than that present in the extracellular space which is in the order of 1-2 mM (Benham, Hess et al. 1987a; Marin 1993). Since the \(E_m\) is negative (inside to outside), the electrical gradient also favors Ca\(^{2+}\) entry. Despite this favorable concentration and electrical gradient, Ca\(^{2+}\) influx remains very low in the resting state. This is due to the fact that in the resting state the Ca\(^{2+}\) channels are largely closed and the passive diffusion is very poor due to the Ca\(^{2+}\) being highly water soluble (Godfraind 1994). VSM also has a rich intracellular source of Ca\(^{2+}\), the sarcoplasmic reticulum. Resting sarcoplasmic reticulum Ca\(^{2+}\) is 75-130 \(\mu\)M (Sugiyama and Goldman 1995) which can be released upon stimulation. Thus, there are two principal potential sources for raising intracellular Ca\(^{2+}\); entry of Ca\(^{2+}\) through VSM plasma membrane and/or from sarcoplasmic reticulum. Similarly, Ca\(^{2+}\) removal occurs via the plasma membrane and/or sequestration to the intracellular stores (Karaki, Ozaki et al. 1997).
Ca\(^{2+}\) influx across plasma membrane and sarcoplasmic reticulum

To maintain \([\text{Ca}^{2+}]_{\text{cyt}}\) several pathways for increasing Ca\(^{2+}\) in the cell have been demonstrated and reported in the literature (Fig 1-5):

**ii. Voltage-gated Ca\(^{2+}\) channels (VGCC):**

There are six types of voltage-gated Ca\(^{2+}\) channels identified (Godfraind and Govoni 1995). Out of these six, two are present in VSM, long lasting or L-type representing the major Ca\(^{2+}\) influx pathway (Bean, Sturek et al. 1986; Benham, Hess et al. 1987a; Akaike, Kanaide et al. 1989; Kuga, Sadoshima et al. 1990; Ganitkevich and Isenberg 1991; Vogalis, Publicover et al. 1991; Kuriyama, Kitamura et al. 1995; Matsuda, Hagiwara et al. 1996) and the second channel being the transient or T-type channels (Hurwitz 1986; Pelzer, Pelzer et al. 1990; Tsien, Ellinor et al. 1991). T-type channels or low-voltage-activated channels have a threshold \(E_m\) for activation that is relatively negative, and a small single channel conductance as compared to L-type VGCC (Nowycky, Fox et al. 1985). Ca\(^{2+}\) entry through T-type channels causes an increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) which may increase further the release of Ca\(^{2+}\) from intracellular stores i.e. Ca\(^{2+}\) induced Ca\(^{2+}\) release (CICR) from the IP\(_3\) compartment and also from the ryanodine-sensitive compartments (Wong and Klassen 1993).

L-type VGCCs are activated by strong depolarization (threshold around -40 mV) and inactivated less rapidly as compared to T-type channels (McDonald, Pelzer et al. 1994), thereby leading to a greater increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) as compared to T-type channels. This rise directly activates contractile filaments, although a smaller portion may contribute to Ca\(^{2+}\)-induced-Ca\(^{2+}\) release (CICR) (Karaki and Weiss 1988), discussed later. L-channel inactivation is both voltage and \([\text{Ca}^{2+}]_{\text{cyt}}\) dependent (McDonald, Pelzer et al. 1994). Very
Fig 1-11. Regulation of [Ca]\textsubscript{cyt}.

NCX plays an important role in the homeostasis of intracellular \( \text{Ca}^{2+} \) by causing \( \text{Ca}^{2+} \) influx (reverse) and \( \text{Ca}^{2+} \) efflux (forward mode) across the plasma membrane. PMCA, plasma membrane \( \text{Ca}^{2+} \) ATPase; SERCA, sarcoplasmic reticulum \( \text{Ca}^{2+} \) ATPase; RyR, Ryanodine receptors; IP3R, inositol triphosphate receptor; SOCC, store-operated \( \text{Ca}^{2+} \) channels; VGCC, voltage-gated \( \text{Ca}^{2+} \) channels; ROCC, receptor operated \( \text{Ca}^{2+} \) channels.

Redrawn from Matsuda et al. (Matsuda, Koyama et al. 2005).
high \([\text{Ca}^{2+}]_{\text{cyt}}\) (Stanfield 1986) and positive potentials (McDonald, Pelzer et al. 1994) cause closure of L-type VGCC. L-type VGCC is also regulated by sarcoplasmic reticulum. Depletion of sarcoplasmic reticulum \(\text{Ca}^{2+}\) increased \([\text{Ca}^{2+}]_{\text{cyt}}\) and muscle tone (Kojima, Dohi et al. 1994). Hence, \(\text{Ca}^{2+}\) entry through L-type VGCC is important for the maintenance of basal tone of smooth muscle (Rubart, Patlak et al. 1996). There is strong evidence that during the myogenic response \(\text{Ca}^{2+}\) enters the VSM cells via L-type VGCC (Harder, Gilbert et al. 1987; Karaki and Weiss 1988; Nakayama, Tanaka et al. 1989; Nakayama and Tanaka 1993; Knot and Nelson 1995; Wesselman, VanBavel et al. 1996; McCarron, Crichton et al. 1997).

iii. Receptor operated \(\text{Ca}^{2+}\) channels (ROCC):

ROCCs are \(\text{Ca}^{2+}\) influx channels which are not dependent on \(E_m\) of smooth muscle cells for activation. It was demonstrated that in the presence of \(\text{Ca}^{2+}\), noradrenaline can still evoke contractions in an already depolarized muscle where no change in \(E_m\) was possible (Bolton 1979). It was inferred that there existed channels permeable to \(\text{Ca}^{2+}\), insensitive to \(E_m\) and activated by an agonist receptor. Thus Bolton (Bolton 1979) and Van Breemen (Van Breemen, Aaronson et al. 1978) introduced the term ROCC.

It was reported that ROCCs may be coupled to \(\alpha_1\) adrenergic receptors by G proteins (Ruffolo, Nichols et al. 1991). The main evidence in favor of ROCCs came when it was reported that noradrenaline increased \(^{45}\text{Ca}^{2+}\) influx and force without a substantial change in membrane depolarization in aortic smooth muscle, which was less sensitive to organic \(\text{Ca}^{2+}\) channel blockers. Further, noradrenaline was able to elicit extra tension in smooth muscle already depolarized by high \(K^+\) (Nelson, Patlak et al. 1990). Thus \(\text{Ca}^{2+}\) entry activated via vasoconstrictors can have profound implications on vascular tone.
with changing $E_m$. Hyperpolarization increases the driving force for $Ca^{2+}$ exit across the plasma membrane, and at the same time decreases $Ca^{2+}$ influx via VGCCs by reducing their open probability (Nelson, Patlak et al. 1990; Large 2002).

ROCCs are activated by norepinephrine and acetylcholine via G-protein coupled receptors linked to phospholipase C and by DAG. The action of DAG occurs by a mechanism independent of protein kinase C, but other kinases may mediate the responses to norepinephrine and DAG. In addition, activation of tyrosine kinases leads to opening of this channel (Large 2002).

**iv. Store-operated (SOCC) and stretch-activated (SACC) $Ca^{2+}$ channels:**

$Ca^{2+}$ can also enter VSM cells via store-operated (Gibson, McFadzean et al. 1998) and stretch-activated channels (Davis, Donovitz et al. 1992; Davis, Meininger et al. 1992a). First described by Putney (Putney 1986), store-operated $Ca^{2+}$ channels are activated when the intracellular stores for $Ca^{2+}$ empty, thus providing a means to refill the stores. This process is important in the regulation of vascular tone (Berridge 1997; Gibson, McFadzean et al. 1998). Such $Ca^{2+}$ influx, also called “capacitative $Ca^{2+}$ entry”, is inhibited by increased $[Ca^{2+}]_{cyt}$ (Clapham 1995a). It is suggested that there is a capacitative $Ca^{2+}$ entry channel which is a type III IP$_3$ receptor present in the plasma membrane associated with a $Ca^{2+}$ channel. This channel is regulated by depletion of intracellular $Ca^{2+}$ stores rather than IP$_3$ (Putney 1986; Parekh and Putney 2005). Recently a $Ca^{2+}$ current called $Ca^{2+}$ release-activated $Ca^{2+}$ current has been described that is activated by store depletion (Parekh and Penner 1997).
As already described earlier in the chapter, Ca^{2+} can also enter the VSM cells via SACCs. Thus it is possible that stretch can be transduced into membrane depolarization and development of force and leading to contraction of VSM. Although at physiological Ca^{2+} concentrations, entry of Ca^{2+} via SACC is low (Kirber, Walsh et al. 1988) and may not produce a contraction per se. However, non selective cation entry of Na^{+} and Ca^{2+} (Kirber, Walsh et al. 1988; Hisada, Ordway et al. 1991) through SACC is sufficient to depolarize the smooth muscle cell and open the VGCC.

**v. Ca^{2+} release from sarcoplasmic reticulum:**

The intracellular stores (sarcoplasmic reticulum) also release Ca^{2+} upon stimulation through sarcoplasmic reticulum channels (Stout and Diecke 1983; Yamamoto and van Breemen 1986). There are two mechanisms for Ca^{2+} release from sarcoplasmic reticulum in smooth muscle cells; Ca^{2+} induced Ca^{2+} release (CICR) and IP_{3} induced Ca^{2+} release (IIICR). The first direct evidence of CICR in smooth muscle was reported by Iino et al. in muscle segments from the taenia caeci (Iino 1989). CICR is a term given to the phenomenon whereby Ca^{2+} influx by the voltage-dependent Ca^{2+} current regulates the ryanodine receptors (RYRs) (Fabiato 1983) or IP_{3} receptors (IP_{3}R), to cause rapid release of Ca^{2+} from intracellular stores. RYR is a Ca^{2+} channel present in the sarcoplasmic reticulum, and with the increase in [Ca^{2+}]_{cyt} open probability of RYR also increases (Meissner 1994; Striggow and Ehrlich 1996). Three potential physiological roles have been proposed for CICR; first is that CICR causes amplification of the agonist induced Ca^{2+} signal. In smooth muscle cells, RYR cause CICR in the physiological range of [Ca^{2+}]_{cyt} thus, CICR increases [Ca^{2+}]_{cyt} levels with Ca^{2+} entry through the plasma membrane channels (Saida and van Breemen 1983; Ito, Ikemoto et al. 1991). Secondly, CICR sets up Ca^{2+} oscillations; by intermittently releasing Ca^{2+} from the intracellular
stores (Berridge and Galione 1988). This saves the cell from persistently high levels of free \([\text{Ca}^{2+}]_{\text{cyt}}\) (Miyazaki 1993). Thirdly, CICR also induces \(\text{Ca}^{2+}\) waves in smooth muscle cells, by which the initial increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) in a specific region of the cell spreads over the rest of the cell (Johnson, Theler et al. 1991; Mayer, Kodner et al. 1992; Neylon, Nickashin et al. 1992).

IICR involves regulation of IP_3R by IP_3 and \(\text{Ca}^{2+}\) working together as co-agonists and co-inhibitors (Bezprozvanny, Watras et al. 1991; Finch, Turner et al. 1991; Missiaen, De Smedt et al. 1992). IICR is increased with \([\text{Ca}^{2+}]_{\text{cyt}}\) below 300 nM and inhibited above this concentration (Iino 1990; Iino and Endo 1992; Iino and Tsukioka 1994). There is evidence in support of IICR, studies demonstrating accumulation of inositol phosphates following intra luminal pressure changes (Hilgemann 1994; Narayanan, Imig et al. 1994). IICR has two components, a fast release component followed by a slow release. This biphasic release of \(\text{Ca}^{2+}\) has been termed as a “Quantal \(\text{Ca}^{2+}\) release” (Parys, Missiaen et al. 1996), which is proposed to be due to inactivation of the IP_3R (Finch, Turner et al. 1991).

Total binding sites for IP_3 were 9-10 times more than for ryanodine in intestinal longitudinal smooth muscle (Wibo and Godfraind 1994). There is evidence to support that IP_3 receptors were concentrated in the ribosome-coated portion of the sarcoplasmic reticulum as the stoichiometric ratio of IP_3 to ryanodine receptors was higher in ribosome-coated regions. This can explain the presence of a \(\text{Ca}^{2+}\)-storage compartment without CICR (Wibo and Godfraind 1994). IICR appears to play an important physiological role as it is present in all VSM cells and increases the \([\text{Ca}^{2+}]_{\text{cyt}}\) upon

**Ca\textsuperscript{2+} efflux across plasma membrane and sarcoplasmic reticulum**

To maintain optimum intracellular Ca\textsuperscript{2+} levels and thus to maintain Ca\textsuperscript{2+} homeostasis it is important to have mechanisms that can reduce Ca\textsuperscript{2+} levels such as plasmalemmal Ca\textsuperscript{2+} ATPase (PMCA), sarcoplasmic reticulum Ca\textsuperscript{2+} ATPase (SERCA) and Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX). The following section will deal with PMCA and SERCA; NCX will be reviewed in later parts of the chapter.

The PMCA pumps produce Ca\textsuperscript{2+} efflux in exchange for protons using the energy from ATP (Van Breemen, Aaronson et al. 1978; Horowitz, Menice et al. 1996). The coupling ratio has been estimated as both electroneutral (Carafoli 1991) and electrogenic (Gimble, Waisman et al. 1982) although this is not yet established for VSM. PMCA pumps have high affinity but low capacity to pump Ca\textsuperscript{2+} out of the cell (Khalil, Lodge et al. 1990). They represent only < 0.1% (Wuytack and Raeymaekers 1992) of the intrinsic membrane proteins as compared to SERCA pumps which are around 90% of the total sarcoplasmic reticulum protein content (Orallo 1996). This ATPase is CAM-dependent and 4Ca\textsuperscript{2+}/CAM stimulates its activity (Horowitz, Menice et al. 1996). Apart from CAM, the PMCA pump is also regulated by protein kinases (Wuytack and Raeymaekers 1992) and phospholipids (Monteith and Roufogalis 1995).

The SERCA pump has been more extensively studied as compared to the PMCA pumps. Present in sarcoplasmic reticulum, it has high Ca\textsuperscript{2+} affinity, and is also an electroneutral pump, allowing one K\textsuperscript{+} and H\textsuperscript{+} influx for one Ca\textsuperscript{2+} efflux (Grover and Khan
1992). However, some authors have suggested this pump to be electrogenic (Sumida, Okuda et al. 1984) There are three isoforms of SERCA pumps with SERCA 2a being found in smooth muscle (Amrani, Magnier et al. 1995). This pump is regulated by the sarcoplasmic reticulum-associated regulatory protein, phospholamban which plays an important role in regulation of Ca\(^{2+}\) uptake in the VSM cells (Lalli, Harrer et al. 1997). Pump activity can be reversed by phosphorylation of phospholamban with cyclic AMP dependent protein kinase A (Toyofuku, Kurzydlowski et al. 1994).

**Excitation-contraction (EC) coupling**

Under normal *in vivo* conditions VSM cells are in a state of partial contraction called vascular tone. This may be due to the fact that the level of \([\text{Ca}^{2+}]_{\text{cyt}}\) has exceeded the 0.1 µM threshold (basal Ca\(^{2+}\)) and the \(E_m\) is typically 10-20 mV more positive than in the resting state. Tone is thought to reflect this and influx of Ca\(^{2+}\) is through VGCC because this is the voltage range favorable to the opening of VGCC and this Ca\(^{2+}\) influx and tone can usually be blocked by VCCC inhibitors (Nelson, Patlak et al. 1990). An increase in the \([\text{Ca}^{2+}]_{\text{cyt}}\) can be caused by stimuli leading to membrane depolarization and therefore influx of Ca\(^{2+}\) through L-type VGCC (Harder 1984) (Electro-mechanical coupling) and/or binding of a contractile agonist to a particular receptor (Pharmacomechanical coupling), both of which may also cause redundant Ca\(^{2+}\) efflux from sarcoplasmic reticulum (Ashida and Blaustein 1987; Meiningher, Zawieja et al. 1991; Somlyo and Somlyo 1994).
**Electro-mechanical coupling**

In the resting state the $E_m$ of the VSM cell is near the opening range of the VGCCs. This means that any small depolarization has a potential of increasing the open probability of these channels leading to $\text{Ca}^{2+}$ entry. The action potential being an electric phenomenon does not cause contraction directly but requires a chemical mediator, which is $\text{Ca}^{2+}$.

As discussed in the previous sections $\text{Ca}^{2+}$ enters the cell mainly via VGCC. $\text{Ca}^{2+}$ also enters the VSM cell through SACC, thus being partly responsible for stretch-induced depolarization. Similar results have been found in mesenteric arteries in guinea pigs (Setoguchi, Ohya et al. 1997). Depolarization plays an important role in the response of a VSM to stretch or an increase in the intraluminal pressure (Harder, Gilbert et al. 1987; VanBavel and Mulvany 1994; Knot and Nelson 1995). The exact mechanism of sensing of an increase in intraluminal pressure or stretch is not yet clear, but the basic mechanism involves alterations in the sarcolemmal properties. Stretch causes depolarization and activation of VGCC as described in the earlier section. These channels are non-selective and are sensitive to diltiazem and amiloride (Hamill, Marty et al. 1981) but not sensitive to dihydroxypyridine receptor (DHPR) antagonists (Bevan, Bevan et al. 1986). The depolarization if sufficient causes opening of the VGCC. The $\text{Ca}^{2+}$ influx overcomes the buffering ability of the sarcoplasmic reticulum and directly activates the contractile machinery of the smooth muscle.

Action potentials are not the only means by which contractions are generated as contractions have been seen in depolarizing solutions (high $K^+$) where no spikes occur (Farley and Miles 1977). Vasoactive agents can also generate tonic contractions after spikes have been blocked, thus contraction of a fully depolarized VSM can be enhanced
by vasoconstrictor agents and inhibited by vasodilator agents (Waugh 1962; Somlyo and Somlyo 1968). The observations that pharmacological substances can influence the contractility of a smooth muscle through mechanisms in addition to changes in $E_m$, drew attention to the importance of pharmaco-mechanical coupling in VSM.

**Pharmaco-mechanical coupling**

Pharmaco-mechanical coupling is defined as a process through which a drug or neurohumoral factors can cause a relaxation or contraction without a necessary change in the resting $E_m$ or in action potential frequency (Somlyo and Somlyo 1968). Agonists can cause increases in $[\text{Ca}^{2+}]_{\text{cyt}}$ by binding to their receptors and activate the phosphatidyl-inositol cascade, thereby causing release of secondary messengers including IP$_3$, which causes a release of Ca$^{2+}$ from intracellular stores (Orallo 1996).

The amount of Ca$^{2+}$ and the amplitude of contraction are not always in proportion to each other. This process, named Ca$^{2+}$ sensitization, can be modulated by either myosin-linked or actin-linked regulatory mechanisms (Kuriyama, Kitamura et al. 1998). The process of Ca$^{2+}$ sensitization involve intracellular signalling mechanisms, which include small molecular weight G proteins such as Rho and Ras proteins, PKC activated by DAG, or arachidonic acid produced by the activation of phospholipase A$_2$ (Somlyo and Somlyo 1998).

Vasoconstrictors (noradrenaline, endothelin1) can cause increases in the $[\text{Ca}^{2+}]_{\text{cyt}}$ by binding to phospholipase (PLC)-coupled receptors, such as $\alpha_1$ adrenoreceptors and endothelin1 subtypes (Bylund, Eikenberg et al. 1994; Dalziel and Westfall 1994; Masaki, Vane et al. 1994). PLC catalyses the hydrolysis of phosphatidyl inositol biphosphate
present in the cell membrane, to produce signalling proteins, IP₃ and diacylglycerol (DAG) (Hirasawa and Nishizuka 1985). IP₃ mobilises Ca²⁺ from the sarcoplasmic reticulum via IP₃R (Tawada, Furukawa et al. 1987; Little, Neylon et al. 1992) and DAG activates protein kinase C (PKC). Once activated, PKC causes contraction of VSM via voltage-independent opening of Ca²⁺ channels and thus increasing the Ca²⁺ influx (van Breemen and Saida 1989; Lepretre, Mironneau et al. 1994). It also increases the Ca²⁺ sensitivity of the myofilaments (Ikebe, Inagaki et al. 1985) and increases the interaction between IP₃ and its receptor on the sarcoplasmic reticulum membrane (Farago and Nishizuka 1990), thereby increasing vascular tone. This increase in the [Ca²⁺]ₘₜ can also cause an increase in the Ca²⁺ influx from the extracellular compartment. Depolarization-independent contraction also occurs because of non-PLC coupled membrane receptors (α₂ adrenergic receptors) (Hieble and Bond 1994) which cause an increase in the [Ca²⁺]ₘₜ by Ca²⁺ influx via receptor-operated Ca²⁺ channels (Lepretre, Mironneau et al. 1994).

Exogenously applied AA was able to increase the tension level and 20 kDa myosin light chain (MLC₂₀) phosphorylation (Gong, Fuglsang et al. 1992). AA may act as a messenger promoting protein phosphorylation by inhibiting protein phosphatases that dephosphorylate MLC₂₀, contributing to Ca²⁺ sensitivity (Kuriyama, Kitamura et al. 1998). It was reported that Ca²⁺-mobilising agonists increase the AA and DAG which precede the development of force in intact smooth muscle (Gong, Kinter et al. 1995). 20 HETE, a metabolite of AA, is a potent vasoconstrictor, activates PKC (Lange, Gebremedhin et al. 1997) and depolarizes VSM by inhibiting the large conductance BKCa channel (Harder, Lange et al. 1997; Lange, Gebremedhin et al. 1997; Gebremedhin, Lange et al. 1998) and increases Ca²⁺ influx via L-type VGCCs (Gebremedhin, Lange et
al. 1998). 20 HETE increases Ca\(^{2+}\) sensitivity by a Rho kinase-dependent mechanism leading to increased vascular responsiveness. Gebremedhin et al. have shown a pressure-dependent production of 20 HETE for intra luminal pressures ranging from 20 to 140 mmHg. These investigators reported that an inhibition of its production attenuated myogenic reactivity, therefore suggesting an important role for 20 HETE in the autoregulation of blood flow (Gebremedhin, Lange et al. 2000).

Studies have shown that Ca\(^{2+}\) sensitivity in smooth muscle contraction also involves Rho A and Rho kinase (Somlyo and Somlyo 2003). The RhoA-associated Rho kinase (RhoA/ROK) pathway plays an important role in regulating Ca\(^{2+}\) sensitivity. It was recently identified that G protein-coupled regulation of 130 kDa myosin light chain phosphatase was responsible for dephosphorylation of the regulatory light chain of myosin (Somlyo and Somlyo 2000). Recent studies have proposed that RhoA/ROK-dependent tone is present under basal conditions in arterioles and contributes importantly to the steady-state myogenic tone (Schubert and Mulvany 1999; VanBavel, van der Meulen et al. 2001).

Thus the Ca\(^{2+}\) available by either electro-mechanical or pharmaco-mechanical coupling plays a significant role in the regulation of vascular tone and smooth muscle contraction. Ca\(^{2+}\) binds to CAM, forming a complex containing 4Ca\(^{2+}\) ions for every CAM molecule (Watterson, Sharief et al. 1980; Means, VanBerkum et al. 1991). This complex binds to myosin light chain kinase which leads to contraction (Moreland, Antes et al. 1990; Somlyo and Somlyo 1994; Zou, Ratz et al. 1995).

**Na\(^{+}\)/Ca\(^{2+}\) exchanger (NCX)**
The Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) is a counter-transport mechanism located in the plasma membrane of almost every type of mammalian cell. As the name suggests it transports Ca\(^{2+}\) and Na\(^+\) in opposite directions across the cell membrane. The NCX exchanges Na\(^+\) and Ca\(^{2+}\) in Ca\(^{2+}\) efflux or Ca\(^{2+}\) influx mode, depending on the ion gradients across the plasma membrane and the E\(_m\) (Blaustein and Lederer 1999; Egger and Niggli 1999; Philipson, Nicoll et al. 2002). In heart, smooth muscle cells, neurons, and basolateral membrane of membrane of cells in the proximal, distal and connecting tubule of nephron cells the NCX plays an important role in the regulation of intracellular Ca\(^{2+}\) concentration (Iwamoto and Kita 2004b). The NCX is also thought to play an important role in excitation-contraction coupling in heart and smooth muscle (Blaustein and Lederer 1999).

**Historical perspectives**

It was known for a very long time that the contractility of the myocardium depended on the ratio of Na\(^+\) and Ca\(^{2+}\) concentration in the bathing solution rather than on the concentration of Ca\(^{2+}\) alone. In 1921, Daly and Clark (Daly and Clark 1921) showed that removal of extracellular Na\(^+\) led to enhanced cardiac muscle contraction. In 1948, Wilbrandt and Koller (Wilbrandt and Koller 1948) demonstrated an interaction between Na\(^+\) and Ca\(^{2+}\) at the plasma membrane. They found that the strength of contraction of the frog's heart was directly related to the concentration ratio of Ca\(^{2+}\) and Na\(^+\) in the extracellular space i.e. \([\text{Ca}^{2+}]_o/([\text{Na}^+]_o)^2\). Later in 1963, Niedergerke (Niedergerke 1963) demonstrated that amount of Ca\(^{2+}\) in the cells was dependent on the external Na\(^+\) concentration [Na\(^+\)]\(_o\). The ventricles gained Ca\(^{2+}\) when exposed to Na\(^+\)-depleted solutions. These ventricles promptly lost Ca\(^{2+}\) and relaxed when they were returned to control (Na\(^+\)-repleted) Ringer solution. On the basis of these observations, Niedergerke
Chapter 1

postulated that Ca^{2+} enters the muscle fibers via a carrier mechanism for which external Na^{+} and Ca^{2+} compete, presumably in the ratio 2 Na^{+}: 1 Ca^{2+}.

During 1960’s research was directed towards the possible role of increasing intracellular [Na^{+}] in enhancing the positive inotropic effects of various cardiotonic compounds (Blaustein and Lederer 1999). At that time, it was interpreted as some form of competition between Na^{+} and Ca^{2+} at intracellular sites. These findings were reinterpreted and extended in the late 1960s by investigators in three different laboratories: Reuter and co-workers in Germany and Switzerland (Reuter and Seitz 1968); Baker, Blaustein, and Hodgkin and their co-workers in England (Baker and Blaustein 1968); and Martin and DeLuca in the United States (Martin and DeLuca 1969). While studying different tissues, they independently reached the conclusion that a coupled counter-transport mechanism, involving the exchange of Na^{+} for Ca^{2+}, may be involved in Ca^{2+} transport across the plasma membrane in mammalian cardiac muscle, in invertebrate neurons and in mammalian small intestinal epithelium.

Working on ⁴⁵Ca^{2+} loaded auricular and ventricular muscle of guinea pig heart, Reuter and Seitz showed that removal of extracellular Na^{+} and Ca^{2+} was associated with a decrease in the Ca^{2+} flux. They interpreted these observations as evidence for a Na^{+}/Ca^{2+} exchange mechanism (Reuter and Seitz 1968). Martin and Deluca studied the transepithelial transport in rat small intestine. They found that there was a decrease in the transport of Ca^{2+} by gut sacs in the absence of Na^{+}. Hence, they suggested that the Ca^{2+} extrusion was a result of altered Na^{+} gradient across basolateral membrane of epithelial cells (Martin and DeLuca 1969).
Chapter 1

After the identification of exchange phenomenon, research was directed towards elucidating the stoichiometry, steady state kinetics and electrogenericity of the exchanger (Baker, Blaustein et al. 1969; Blaustein and Santiago 1977; Mullins 1984). Reeves and Sutko applied the biochemical approaches to the exchanger with their membrane vesicle studies of \( \text{Na}^+ / \text{Ca}^{2+} \) exchange in rabbit ventricular tissue. The sarcolemmal vesicles accumulated \( \text{Ca}^{2+} \) when an outwardly-directed \( \text{Na}^+ \) gradient was formed across the vesicle membrane. However, this \( \text{Ca}^{2+} \) activity was not associated with \( \text{K}^+ \) loaded vesicles. (Reeves and Sutko 1979). This was followed by the partial purification of the cardiac exchanger by Philipson et al. (Philipson, Longoni et al. 1988) and the cloning of this exchanger by Nicoll et al. (Nicoll, Longoni et al. 1990). Since then different isoforms have been identified. Studies were initiated into understanding the physiological roles played by exchanger in contraction and relaxation (O'Neill, Valdeolmillos et al. 1988; Bers and Bridge 1989). Yau and Nakatani used patch clamp techniques to measure the current carried by NCX (\( I_{\text{Ca}/\text{Na}} \)) in isolated cells (retinal rods) in 1984 and this was followed by description of various features of this current (Yau and Nakatani 1984). Additional properties of the exchanger were elucidated as a result of the application of other novel methods: ion-selective \( \text{Ca}^{2+} \) and \( \text{Na}^+ \) electrodes (Diederichs 1994), release of caged \( \text{Ca}^{2+} \) (Niggli and Lederer 1993), and electrical measurements of transport in reconstituted proteoliposomes fused to a planar lipid bilayer (Eisenrauch, Juhaszova et al. 1995).

**Molecular biology and structure**

The mammalian NCX forms a multigene family of highly homologous proteins (~70% identical to one another) comprising three isoforms, NCX1, NCX2, and NCX3. NCX1 is highly expressed in the heart and brain (Reuter and Porzig 1995) and at much lower
levels in other tissues (Nicoll, Longoni et al. 1990; Kofuji, Hadley et al. 1992; Komuro, Wenninger et al. 1992; Low, Kasir et al. 1993; Kofuji, Lederer et al. 1994; Li, Matsuoka et al. 1994). The expression of NCX2 and NCX3 is thought to be limited mainly to the brain and skeletal muscle (Nicoll, Quednau et al. 1996a; Quednau, Nicoll et al. 1997).

**Structure of NCX**

NCX is formed by 938 amino acids and the initial hydrophobic analysis suggested up to 11 transmembrane segments (TMS) and a large cytoplasmic domain (loop f) between 5 and 6 TMS (Philipson and Nicoll 1993). The model was later revised to 9 alpha helical TMS and a few non-helical receptor-entrant loops associated with α repeat regions. (Iwamoto, Nakamura et al. 1999; Nicoll, Ottolia et al. 1999; Qiu, Nicoll et al. 2001). Five of these TMSs are present in the amino part of the protein and separated from the other four TMSs by a hydrophilic cytoplasmic loop f (Fig 1-6). There is the presence of a leader peptide, which corresponds to the first amino acids of the protein (Durkin, Ahrens et al. 1991). Removal of this leader peptide during processing of the protein facilitates its extracellular location and glycosalation (Hryshko, Nicoll et al. 1993).

The transmembrane sequence, particularly the α repeat regions, may participate in ion transport and the interaction with NCX inhibitors and ionic modulators (Nicoll, Hryshko et al. 1996b; Doering, Nicoll et al. 1998; Iwamoto, Uehara et al. 2000). The intracellular cytoplasmic loop, possessing the exchanger inhibitory peptide (XIP) region (Li, Nicoll et al. 1991; Matsuoka, Nicoll et al. 1997), regulatory Ca$^{2+}$ binding site (Levitsky, Nicoll et al. 1994; Matsuoka, Nicoll et al. 1995), and phosphorylation site, is primarily involved in various regulatory properties.
**Modes of operation**

Movement of net Ca\(^{2+}\) either out of or into cells, via NCX, depends on the prevailing electrochemical driving forces (i.e., the Na\(^+\) and Ca\(^{2+}\) concentration gradients and the \(E_m\)).

The two modes are referred to as Ca\(^{2+}\) efflux (also called exit or the forward mode) and Ca\(^{2+}\) influx (also called entry or the reverse mode) (Blaustein and Lederer 1999). Ca\(^{2+}\) entry mode results in Ca\(^{2+}\) influx into the cell dependent on cytosolic Na\(^+\) concentration ([Na\(^+\)]\(_{cyt}\)) and ouabain-insensitive Na\(^+\) efflux which is dependent on extracellular Ca\(^{2+}\) concentration [Ca]\(_o\) (Blaustein and Lederer 1999). The [Ca\(^{2+}\)]\(_{cyt}\) required for half-maximal activation of Ca\(^{2+}\) influx mode is on the order of 1 µM under physiological conditions (DiPolo and Beaugé 1988; Hilgemann 1990; Blaustein and Lederer 1999). Therefore, only a small fraction of the exchangers are predicted to be active at the normal resting [Ca\(^{2+}\)]\(_{cyt}\) (~ 0.1 µM) in most cells. However, as the [Ca\(^{2+}\)]\(_{cyt}\) is in the low micro molar range this exchanger is fully activated during peak activity in many types of excitable and secretory cells (Blaustein and Lederer 1999).

The Ca\(^{2+}\) exit mode results in Ca\(^{2+}\) efflux dependent on extracellular Na\(^+\) ([Na\(^+\)]\(_o\)) and ouabain-and tetrodotoxin (TTX)-insensitive [Ca]\(_{cyt}\) dependent influx of Na\(^+\) (Blaustein and Lederer 1999). In contrast to Ca\(^{2+}\) entry mode, Ca\(^{2+}\) efflux does not require extracellular Ca\(^{2+}\) for activation, demonstrating that the exchanger is asymmetric. Hydrolysis of ATP is known to modulate the kinetics of the exchanger, however it is not required to power net Ca\(^{2+}\) extrusion mediated by the NCX (Blaustein and Lederer 1999).
The exchanger can also work where external Ca\textsuperscript{2+} or Na\textsuperscript{+} can be exchanged for internal Ca\textsuperscript{2+} and Na\textsuperscript{+} respectively, i.e. Ca\textsuperscript{2+}/Ca\textsuperscript{2+} exchange or Na\textsuperscript{+}/Na\textsuperscript{+} exchange or the homo exchange form (Blaustein and Lederer 1999). Both the Ca\textsuperscript{2+} influx mode and the Ca\textsuperscript{2+} efflux mode of NCX are dependent on the intracellular Ca\textsuperscript{2+} level (Baker and McNaughton 1976; DiPolo 1979; Hilgemann 1990), however, the Ca\textsuperscript{2+} exit mode is not dependent on extracellular Ca\textsuperscript{2+} for activation. The Ca\textsuperscript{2+} influx mode is activated by external alkali metal ions including Na\textsuperscript{+} (Blaustein and Santiago 1977). In contrast, Ca\textsuperscript{2+} efflux is activated by internal alkali metal ions yet inhibited by high [Na\textsuperscript{+}]\textsubscript{cyt} (Fontana, Rogowski et al. 1995; Blaustein and Lederer 1999).

**Kinetics**

Obtaining the NCX exchange turnover rate (i.e., the number of ions transported per exchanger per unit time of a protein) is more difficult than for other ion channels. This is due to the fact that the electrical signal generated by the NCX is much smaller and also because there is no methodology for determining how many exchangers are contributing to the current.

By measuring the Na\textsuperscript{+} dependent flux of Ca\textsuperscript{2+} and the density of NCX protein maximum turnover rate of the NCX have been established. Estimates between 1,000-5,000 per second (Cheon and Reeves 1988; Hilgemann, Nicoll et al. 1991; Niggli and Lederer 1991) have been provided for turnover rate for NCX, which seem to suggest that NCX is a transport protein with a high turnover rate as compared to other transport proteins for e.g. Na\textsuperscript{+}/K\textsuperscript{+} ATPase (60-200 per second) (Egger and Niggli 1999). On the other hand Powell et al. have suggested a turn over rate of < 300 per second (Powell, Noma et al. 1993).
Fig 1-12. Topological model of the NCX.

Shown are the 9 TMSs represented by numbered cylinders. The start of the first intracellular segment is glycosylated (CH$_2$O). Also shown are the 2α repeat regions of intramolecular homology, responsible for ion translocation. The intracellular loop (f) contains the regulatory Ca$^{2+}$ binding site and the region for Na$^+$ inactivation (endogenous XIP region). Between the exchanger inhibitor peptide (XIP) and Ca$^{2+}$ binding sites there is a potential protein kinase A phosphorylation site.
Chapter 1  

The coupling ratio and stoichiometry

Coupling refers to the number of ions transported while the stoichiometry refers to the number of bound ions. Initially the Hill coefficient corresponding to the cooperation between external Na\(^+\) in activating Ca\(^{2+}\) efflux was used (Baker, Blaustein et al. 1969) to estimate the coupling between Na\(^+\) and Ca\(^{2+}\). Over years it has been an issue of controversy whether the stoichiometry of NCX is 2:1, 3:1 or 4:1 between Na\(^+\) and Ca\(^{2+}\).

Earlier work assumed an electroneutral coupling ratio of 2:1 (Luttgau and Niedergerke 1958) followed by a ratio of 4:1 between Na\(^+\) and Ca\(^{2+}\) (Brinley and Mullins 1974). Blaustein and Hodgkin have shown that to maintain the \([\text{Ca}^{2+}]_{\text{cyt}}\) to 100 nM it is imperative that the coupling ratio between Na\(^+\) and Ca\(^{2+}\) be maintained at 3:1. As there is unequal movement of charge across the membrane this might contribute to \(E_m\). It also means that \(E_m\) might have a role to play in the Na\(^+\)/Ca\(^{2+}\) exchange (Blaustein and Hodgkin 1969).

Blaustein made the first direct measurement of the coupling ratio (i.e., measurement of the coupled fluxes of Na\(^+\) and Ca\(^{2+}\)) in internally dialyzed squid axons. Their Na\(^+\) influx and Ca\(^{2+}\) efflux data suggested that the fluxes were coupled with a stoichiometry of approximately 3 Na\(^+\) to 1 Ca\(^{2+}\) (Blaustein and Russell 1975). Since then, the coupling ratio of 3:1 has been confirmed by many worker using different methodologies like radioactive tracer flux experiments, intracellular microelectrodes (Glitsch, Reuter et al. 1970; DiPolo and Beauge 1984; Reeves and Hale 1984; Rasgado-Flores, Santiago et al. 1989), patch clamp methods (Kimura, Miyamae et al. 1987; Hilgemann 1990), and the photo chemical release of caged Ca\(^{2+}\) (Niggli and Lederer 1993).
Fujioka et al. used inside-out "macro patches" in guinea-pig ventricular myocytes and measured the reversal potential of Na\(^+\)/Ca\(^{2+}\) exchange current. Their study suggested that the coupling ratio between Na\(^+\) and Ca\(^{2+}\) might be 4:1 (Fujioka, Komeda et al. 2000). Recently, Kang and Hilgemann, utilized an ion-selective electrode technique to quantify ion fluxes in giant patches. They confirmed that NCX stoichiometry is 3.2:1 and cannot be greater than 3.4:1. Kang and Hilgemann proposed a refined Na\(^+\)/Ca\(^{2+}\) exchange model. They proposed that NCX1 can transport not only 1 Ca\(^{2+}\) or 3 Na\(^+\) ions, but also 1 Ca\(^{2+}\) with 1 Na\(^+\) ion at a low rate. Therefore, in addition to the major 3:1 transport mode, import of 1 Na\(^+\) with 1 Ca\(^{2+}\) defines a Na\(^+\) conducting mode that exports 1 Ca\(^{2+}\), and an electroneutral Ca\(^{2+}\) influx mode that exports 3 Na\(^+\). The two minor transport modes can potentially determine resting free Ca\(^{2+}\) and background inward current (Kang and Hilgemann 2004).

**Role of NCX in VSM**

NCX1 is believed to extrude [Ca\(^{2+}\)]\(_{cyt}\) during EC coupling to maintain Ca\(^{2+}\) homeostasis in VSM (Iwamoto, Kita et al. 2005). Direct functional evidence for NCX in VSM was first described 25 years ago (Reuter, Blaustein et al. 1973). Numerous studies on VSM have shown that a reduction in Na\(^+\) gradient across the plasma membrane, increases VSM tone (Pritchard and Ashley 1986; Smith and Smith 1987; Woolfson, Hilton et al. 1990; Sato and Aoki 1991; Slodzinski, Juhaszova et al. 1995). It was concluded that Na\(^+\)/Ca\(^{2+}\) exchange may play a pivotal role in this mechanism.

Recent studies have suggested that NCX knockdown utilizing antisense ODNs prolongs agonist responses by delaying the return of [Ca\(^{2+}\)]\(_{cyt}\) to the resting level. Further, ouabain augmented the agonist-induced rise in [Ca\(^{2+}\)]\(_{cyt}\) in controls but not in antisense-treated
cultured VSM cells (Slodzinski, Juhaszova et al. 1995; Slodzinski and Blaustein 1998). The functional studies were supported by the direct demonstration of NCX mRNA and protein in primary cultured VSM cells (Juhaszova, Ambesi et al. 1994). Further, immunocytochemical studies revealed that the exchanger, in smooth muscle, appears to be restricted primarily to plasma membrane regions that are adjacent to junctional sarcoplasmic reticulum (Moore, Etter et al. 1993; Juhaszova, Ambesi et al. 1994). In contrast, PMCA pump is distributed much more uniformly over the surface of the VSM cells. This localization of the NCX may imply that a major role of the exchanger in smooth muscles is to indirectly modulate the Ca\textsuperscript{2+} content of the sarcoplasmic reticulum stores and thereby influence Ca\textsuperscript{2+} signalling and tension development (Blaustein and Lederer 1999). Although the precise role(s) of the NCX in VSM is (are) unresolved, extrusion of Ca\textsuperscript{2+} via the exchanger dominates over extrusion via the PMCA pump, at least under some circumstances (Blaustein and Lederer 1999).

**Regulation of NCX**

NCX is regulated by a variety of factors; some of the widely investigated ones will be discussed in this section. Although Ca\textsuperscript{2+} and Na\textsuperscript{+} are the substrates for the exchanger they are also involved in the regulation of NCX.

When Na\textsuperscript{+} is applied to the intracellular surface of the excised sarcolemmal patch, the outward exchange current is increased (Hilgemann, Collins et al. 1992). However, when intracellular Na\textsuperscript{+} was increased in a step-wise fashion the current increased instantaneously but transiently. Following the peak, there was time-dependent decline in the current, due to the entry of exchanger into an inactive form, called Na\textsuperscript{+} dependent inactivation (I\textsubscript{1}). When the Na\textsuperscript{+} binds to the exchanger, it can either be translocated
across the membrane or the exchanger can enter the inactive state $I_1$ (Hilgemann, Collins et al. 1992).

In the absence of $[\text{Ca}^{2+}]_{\text{cyt}}$ NCX enters another inactive state called $I_2$. Levitsky identified a high-affinity $\text{Ca}^{2+}$ binding regulatory domain on the intracellular, hydrophilic loop of the exchanger by using fusion proteins and the overlay $^{45}\text{Ca}^{2+}$ technique (Levitsky, Nicoll et al. 1994). $I_2$ is removed when $\text{Ca}^{2+}$ binds to a high affinity regulatory site on the intracellular loop (Levitsky, Nicoll et al. 1994; Matsuoka, Nicoll et al. 1995), which is different from the transport site for $\text{Ca}^{2+}$. Two highly acidic regions of 138 amino acids, each characterized by 3 consecutive aspartic acid residues present in the intracellular loop of the exchanger are thought to be important in regulation of NCX by $\text{Ca}^{2+}$ (Doering and Lederer 1993).

Although $\text{Na}^+$/Ca$^{2+}$ current was stimulated by ATP application to the intracellular surface of the excised patches from cardiac myocytes, no ATP is required to drive the exchange (Hilgemann 1990). This may be due to variety of mechanisms, which include; production of PIP$_2$ from phosphoinositol in the plasma membrane which may modulate $\text{Na}^+$/Ca$^{2+}$ exchange current, phosphorylation of the NCX by ATP-dependent protein kinases, or a direct effect of ATP on the NCX (Egger and Niggli 1999). Acidification can inhibit the activity of NCX (Philipson, Bersohn et al. 1982; Doering and Lederer 1993) by reducing the affinity of Ca$^{2+}$ for the cytoplasmic regulatory site (Hilgemann, Collins et al. 1992). Any variation of intracellular pH changes the $\text{Na}^+$/Ca$^{2+}$ exchanger transport with inhibition occurring at more acidic pH and augmentation occurring at more basic pH (Fontana, Rogowski et al. 1995; Blaustein and Lederer 1999).
Stimulation of Na\(^+\)/Ca\(^{2+}\) exchange activity by phosphorylation has been recorded (Blaustein, Fontana et al. 1996; Shigekawa, Iwamoto et al. 1996; Iwamoto, Pan et al. 1996b). The mechanism is due to an increase in the affinity for Ca\(^{2+}\) on the inside and Na\(^+\) from the outside involving a Ca\(^{2+}\) dependent kinase reaction, thereby causing activation of the exchanger.

**NCX inhibition**

An important approach for examining the role of a transport system is with the use of highly selective inhibitors. Regrettably there are only limited numbers of selective NCX inhibitors. Further, as NCX mediates both Ca\(^{2+}\) influx and efflux it is possible that different inhibitors have disparate effects on the kinetics of these two modes.

A variety of divalent and trivalent cations have been used as the inhibitors of NCX because their high density of positive charge enables interaction with Ca\(^{2+}\) binding sites. There are reports suggesting that La\(^{3+}\) inhibits NCX in many different cell types (Brommundt and Kavaler 1987; Kimura, Miyamae et al. 1987; Caputo, Bezanilla et al. 1989). This has also been shown in VSM where the IC\(_{50}\) was approximately 0.5 mM (Furukawa, Tawada et al. 1988; Shimizu, Borin et al. 1997). Ni\(^{2+}\) and other divalent cations have also been used as NCX inhibitors at the concentration of 2-5 mM (Kimura, Miyamae et al. 1987; Niggli and Lederer 1991; Niggli and Lederer 1993; Main, Grantham et al. 1997). Other cations inhibit the NCX, including Mg\(^{2+}\) (DiPolo and Beauge 1984; Kimura 1996), Cd\(^{2+}\) (Trosper and Philipson 1983; Hobai, Bates et al. 1997), Co\(^{2+}\) (Hilgemann 1989), Mn\(^{2+}\) (Russell and Blaustein 1974; Trosper and Philipson 1983) and Zn\(^{2+}\) (Colvin 1998), but none of these are specific inhibitors.
Amiloride, an acylguanididne, has an IC$_{50}$ of 1 mM, and is a weak inhibitor of NCX, but is a more potent blocker of Na$^+$ channels (Blaustein and Lederer 1999). Amiloride analogs have been used as more potent NCX inhibitors, for example Benzamil, DBM (N$^5$- 2,4-dimethylbenzyl-amiloride) and DCM (3,4-dichlorobenzamil) have IC$_{50}$ values of 100 µM, 10 µM and 17 µM, respectively (Siegl, Cragoe et al. 1984; Karwatowska-Prokopczuk, Nordberg et al. 1998) but they have limited (Trosper and Philipson 1983) usefulness because they have a relatively poor selectivity (they can also block the Na$^+$/H$^+$ exchanger and VGCC). Also the mechanism of block by amiloride analogs appears complex; at higher concentrations they bind to Na$^+$, K$^+$ and Ca$^{2+}$ binding sites, whereas at lower concentrations they bind only to a Na$^+$ binding site (Slaughter, Garcia et al. 1988).

A peptide of 20 amino acids, having a sequence similar to a part of the loop f of the NCX protein, was synthesized to examine the possibility that it might be auto-inhibitory (Li, Nicoll et al. 1991). This sequence did inhibit NCX and was named XIP. As XIP binds indiscriminately to other CAM binding proteins the actions of XIP are difficult to interpret (Blaustein and Lederer 1999). This would be particularly troublesome in studies of VSM contraction.

In 1996, KB-R7943 was introduced for the first time as a selective NCX inhibitor (Watano, Kimura et al. 1996; Iwamoto, Watano et al. 1996a). This agent demonstrates interesting pharmacology. KB-R7943 inhibits the reverse mode (i.e. Ca$^{2+}$ influx) by NCX much more effectively than the forward mode (Watano, Kimura et al. 1996; Iwamoto, Pan et al. 1996b; Satoh, Ginsburg et al. 2000; Elias, Lukas et al. 2001). In intact cells, the IC$_{50}$ for Ca$^{2+}$ entry mode is 1.2-2.4 µM, whereas for the Ca$^{2+}$ exit mode it is 30 µM.
In single cardiac ventricular cells of guinea pig utilizing whole cell patch clamp, Watano et al. reported that, at 0.1-10 µM KB-R7943 suppressed the outward Na\(^+\)/Ca\(^{2+}\) exchange current with an IC\(_{50}\) value of approximately 0.32 µM. Further, at 5-50 µM, KB-R7943 suppressed the inward Na\(^+\)/Ca\(^{2+}\) exchange current with a higher IC\(_{50}\) value of approximately 17 µM (Watano, Kimura et al. 1996). In addition, KB-R7943 is 3-fold more effective on NCX3 than NCX1 and NCX2 (Iwamoto, Kita et al. 2001). The highly conserved \(\alpha2\) repeat of the exchanger is almost exclusively responsible for the difference in drug response of the isoforms (Iwamoto, Kita et al. 2001), with amino acids Val-820, Gln-826, and Gly-833 in the \(\alpha2\) repeat being essential for the interaction of the exchanger with KB-R7943 (Iwamoto, Kita et al. 2004).

KB-R7943 has been widely used as a pharmacological tool to study the roles of the exchanger at the cellular and organ levels. For example, KB-R7943 has been shown to efficiently guard against toxicity by cardiac glycosides and ischemia/reperfusion injury of the heart, kidney and brain (Iwamoto, Kita et al. 2004). However, non-specific effects of KB-R7943 have been suggested. KB-R7943 at relatively low doses is reported to block ion channels, the neuronal nicotinic acetylcholine receptor, the N-methyl-D-aspartate receptor, and the norepinephrine transporter. This agent has a lower potency for ion transporters, such as the Na\(^+/H^+\) exchanger, Na\(^+\)/K\(^+\) ATPase, Ca\(^{2+}\) ATPase up to 10 µM concentration, hence, is fairly specific to NCX (Iwamoto, Kita et al. 2004). KB-R7943 also inhibits L-type VGCC, which in turn decreases the intracellular Ca\(^{2+}\) load. This changes the magnitude of NCX, hence making the interpretation of its anti-arrhythmic effect quite uncertain (Nagy, Virag et al. 2004).
In 2001, Matsuda et al. (Matsuda, Arakawa et al. 2001) reported on SEA0400, a newly developed selective inhibitor of NCX is approximately 10 times more potent than KB-R7943. SEA0400 inhibits \([\text{Na}^+]_{\text{cyt}}\) dependent \(\text{Ca}^{2+}\) uptake via the reverse mode of the exchanger in three kinds of cultured neuronal cells and in cultured cardiomyocytes (Takahashi, Takahashi et al. 2003); their IC\(_{50}\) values were 5-33 nM and 92 nM (Matsuda, Arakawa et al. 2001) respectively. The inhibitory potency of SEA0400 was 80-100 times more powerful than that of KB-R7943.

However, Reuter et al. have shown that application of 5 µM KB-R7943 or 100 nM SEA0400 to heart tubes from NCX1 knockout mice can significantly depress \(\text{Ca}^{2+}\) transients. Both drugs deprived the \(\text{Ca}^{2+}\) transients at low concentrations even in the absence of any \(\text{Na}^+\)/\(\text{Ca}^{2+}\) exchanger. This would be due to the fact that the drugs are affecting other significant \(\text{Ca}^{2+}\) transport pathways besides NCX (Reuter, Henderson et al. 2002).

Recently, a newly synthesized and selective inhibitor of NCX was developed 2-(4- \([4\text{-nitrobenzyloxy}]\) benzyl) thiazolidine-4-carboxylic acid ethyl ester (SN-6). This compound attenuated hypoxia/reoxygenation-induced renal tubular cell damage and ischemia/reperfusion-induced renal failure (Inoue, Ito et al. 2004). Hence SN-6 is expected to be an important pharmacological tool to study the roles of the exchanger as well as a new anti-ischemic injury drug (Inoue, Ito et al. 2004).

**Antisense oligonucleotides**

Antisense ODNs are short DNA molecules, which are targeted against specific mRNAs through complementary Watson-Crick base pairing. Inhibition of gene expression by
antisense ODNs is dependent on their ability to bind a complimentary mRNA sequence and thus inhibit translation (Milligan, Matteucci et al. 1993) and inhibit gene expression of a specific protein in a cell. Importantly, in the absence of specific inhibitors against NCX, antisense ODNs against NCX can be used to inhibit the expression of the exchanger protein. Antisense ODNs have repeatedly demonstrated efficacy in changing the expression of various genes, thus providing important insights into their roles in tumoro-genesis or normal growth and development (Wagner 1994; Branch 1998).

There are a number of issues related to antisense treatment, which will be mentioned briefly in the coming section and discussed further in the body of the thesis. There are concerns regarding the rapid degradation of the ODNs with phosphodiester linkages, which have a half-life of approximately 20 minutes. This can be overcome by use of phosphothioate linkages which are more stable and potent (Fisher, Terhorst et al. 1993; Milligan, Matteucci et al. 1993). There should be inclusion of adequate controls including mismatch ODNs to determine non-specific, or non-antisense, effects of the antisense ODNs (Wagner 1994). The delivery of antisense ODNs has been a major concern due to their inability to cross, cellular membranes, warranting the use of cell permeabilization techniques to introduce ODNs directly into the cytoplasm.

Antisense and sense ODNs sequences can be highly specific owing to the fact that the haploid human genome comprises $3\times10^9$ bases thus it is very rare that a nucleotide sequence of more than 17 bases will occur more than once. This makes antisense ODNs a specific and selective inhibitor and thus in the absence of any specific and sensitive NCX inhibitors, antisense ODNs can be utilized to determine more about the physiological roles of NCX in the myogenic response of a skeletal muscle arteriole.
AIMS

The arteriolar myogenic response, typified by the ability of arterioles to adjust their diameter to a change in intra luminal pressure, is fundamental to the control of vascular resistance. Despite this vital physiological role, the exact signalling pathways underlying this mechanically-induced vasomotor response remain uncertain.

The overall hypothesis underpinning this work is that the mechanical stimulus provided by an increase in intra luminal pressure activates a series of membrane ion gating mechanisms in arteriolar smooth muscle that regulate $E_m$, Ca$^{2+}$ entry and intracellular release, and ultimately the contractile response. Key to the initiation of this sequence of events is a depolarization of the membrane as a result of non-selective cation entry (predominately Na$^+$ under physiological conditions) with subsequent opening of L-type VGCC. With respect to the studies outlined in this proposal it is specifically hypothesized that the NCX, in myogenically active arteriolar smooth muscle, functions in the reverse mode to remove Na$^+$ from the restricted space (between the plasma membrane and sarcoplasmic reticulum) and supply Ca$^{2+}$ from the extracellular space.

Using isolated, cannulated, arteriolar preparations in conjunction with fluorescence imaging and cell biology approaches (gel electrophoresis/Western transfer, PCR, and ODN treatment) the studies aim to:

1. demonstrate the functional significance of the NCX in the setting of arteriolar myogenic tone by manipulation of extracellular Na$^+$ and the use of pharmacological inhibitors showing specificity for the NCX.
2. demonstrate the existence of the NCX in arteriolar smooth muscle using Western blotting.

3. identify at the mRNA level, using real-time multiplex polymerase chain reaction, which exchanger isoforms (NCX 1, 2 and 3) are expressed in arteriolar smooth muscle.

4. further examine the significance of the NCX in arteriolar myogenic reactivity by utilizing a knockdown antisense approach to decrease levels of functional exchanger.
CHAPTER 2

GENERAL METHODS
Animals

In the present study healthy male Sprague-Dawley rats between ages 6-9 weeks were used. Rats were housed in a controlled environment animal holding facility with temperature (15-20° C) and lighting (12 hour light, 12 hour dark cycle). Free access to water and food was allowed during the stay. The Animal Experimental Ethics Committee at RMIT University approved all protocols involving rats.

Isolated arteriole dissection and preparation

In vitro smooth muscle preparations are an important tool in the study of myogenic responses as it is without the complicating interference from parenchymal cells, nerves and circulating vaso-active substances (Jackson and Duling 1989).

Rats were anesthetized with an intra-peritoneal injection of sodium thiopentone (100 mg/kg). After ensuring signs of a surgical plane of anaesthesia (for example loss of reflexes to food pad pinch and corneal touch), the animal was placed on a dissecting board with the ventral side up to allow a clear access to the scrotum. After making a longitudinal incision in the scrotum, the cremaster muscle, which surrounds the testis, was reached by clearing the overlying connective tissue with the help of curved forceps. The preparation was periodically irrigated with cold Krebs-bicarbonate buffer solution (4° C) solution to remove any blood, prevent drying of the muscle tissue and to keep the inflammation to a minimum. The cremaster was then, excised from the animal, and placed in a cooled (4° C) chamber containing dissection buffer (3 mM MOPS, 145 mM NaCl, 5 mM KCl, 2.5 mM CaCl2, 1 mM MgSO4, 1 mM NaH2PO4, 0.02 mM EDTA, 2 mM pyruvate, 5 mM glucose, and 1% endotoxin and fatty acid free albumin) (Duling, Gore et
In preparation for dissecting of the arterioles, the cremaster muscle was pinned onto a silastic layer (Dow Corning USA) at the base of the refrigerated dissection chamber.

Segments of the main intramuscular arteriole (1A) were dissected (Fig 2-1a) (approximately 2-3 mm long) from the muscle using fine dissection instruments (fine Vannas Scissors and Forceps FST (USA) Inc.) (Meininger, Zawieja et al. 1991; Zou, Ratz et al. 1995). Individual vessel segments were then transferred to a custom-designed tissue chamber (volume: 5 ml) with a glass cover slip bottom (50 µm thickness). The chamber was filled with Krebs-bicarbonate buffer solution containing 111 mM NaCl, 25.7 mM NaHCO₃, 4.9 mM KCl, 2 mM 5 CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 11.5 mM glucose, and 10 mM HEPES. The pH of the Krebs-bicarbonate buffer solution was 7.27 at room temperature, which reached 7.4 once at 34º C. The pH was maintained by bubbling the Krebs-bicarbonate buffer solution with 5% CO₂ and 95% N₂ during the course of experiment.

The arterioles were cannulated at both ends on glass micropipettes (60-80 µm tip diameter) that had been filled with Krebs-bicarbonate buffer solution (Fig 2-1b). The vessel lumen was cleared of cellular material and subsequently filled with Krebs-bicarbonate buffer solution by applying a slight positive pressure by raising one end of the tubing connected to the cannulation pipette. The vessels were secured to the pipettes using 10-0 monofilament silk (Dynex) sutures at both the cannulated ends. One pipette was connected to an adjustable pressure system or servo-controlled pump and the other pipette connected to a closed three-way stopcock.
Fig 2-1a.

Fig 2-1b.

**Fig 2-4. Video microscopy system for study of cannulated microvessels.**

Arrow showing the IA intramuscular arteriole (a) (Hill, Trippe et al. 1992) and arterioles dissected from skeletal muscle and cannulated on glass micropipettes (b).
Glass micropipettes were prepared using glass micro-tubes of outer diameter 0.047 inches and an internal diameter of 0.04 inches (Drummond Scientific Co., USA). By using a micro-pipette puller (Sachs Flaming micro-pipette puller, Model PC 84, Sutter Instruments USA) the glass tubes were tapered. The tips were broken at around diameter of 80-100 µm using fine forceps. The tips were heat polished to reduce damage to the vessel lumen and to obtain outer diameters of approximately 60-80 µm after which they were bent, to an angle of approximately 30-45 degrees to the longitudinal axis of the pipette, using a microforge (Stoelting Scientific Instruments).

This arteriole preparation was placed on the stage of an inverted microscope (Olympus IX-70 or Nikon Diaphot). The microscope was equipped with a computer-based video recording system with appropriate software (Apple Macintosh and MacLab 8e). Vessel images were collected using a charged coupled device (CCD) video camera (Panasonic WV-BP312) (Fig 2-2). Inner vessel diameters (in µm) were measured using electronic video calipers (Microcirculation Research Institute, Texas A&M, College Station, Texas) and were displayed on a video screen (Panasonic WV-5420). The video calipers were calibrated using a graticule (Reichert Jung, Germany) with major divisions of 2 mm and minor divisions of 10 µm with a calibration error of ± 2 µm. The diameters were recorded for later analysis.

The abluminal surface of the cannulated arterioles was continually superfused (2–4 ml/minute) with Krebs-bicarbonate buffer solution. The intra luminal pressure was manipulated either by a servo-controlled pump (Living Systems; VA, USA) or by alteration in the height of a fluid reservoir. These pressure systems were connected via
Fig 2-5. Ca$^{2+}$ measurement and imaging system.

Inverted microscope coupled to Ca$^{2+}$ imaging or photometry systems; electrophysiology instrumentation; high resolution video monitor; video calipers; and data storage systems. Adapted from Meininger et al (Meininger, Zawieja et al. 1991).
a small diameter polyethylene tube (Biocorp Australia) filled with distilled water to one of the cannulating micro-pipette holders. Pressure was monitored using a pressure transducer attached to the pressure system which gives a feedback to maintain a constant pressure in the case of the servo-controlled pump. The pressure monitoring systems were calibrated using a mercury sphygmomanometer, with major divisions of 10 mmHg and minor divisions of 2 mmHg with a calibration error of ± 1 mmHg vessel.

Vessel segments were then gradually pressurized from 40 to 70 mmHg (10 mmHg increased every 15 minutes), as 70 mmHg is the \textit{in vivo} intravascular pressure for the 1A arteriole (Meininger, Mack et al. 1987). Arterioles were gradually warmed to 34 ± 0.5°C (\textit{in vivo} temperature of rat cremaster muscle) during the 60 minute equilibration period. This allowed the vessels to develop spontaneous basal tone; vessels failing to develop tone or having leaks were discarded. Passive diameters were recorded by superfusing the blood vessel with 0 mM Ca\textsuperscript{2+}, Krebs-bicarbonate buffer solution containing 2 mM EGTA, for 20 minutes. For functional studies, vessel diameter data were typically normalized to each vessel’s passive diameter at 70 mmHg (Fig 2-3). This normalization procedure was applied due to small differences in the initial diameters of the arterioles.

To denude the endothelium, an air bubble was introduced into the pipette connected to the pressure reservoir. To pass the bubble into the cannulated vessel segment, the stop cock on the distal cannulation pipette was opened. Passage of the air bubble through the vessel caused physical disruption of the endothelium (Potocnik and Hill 2001). To confirm that the vessels were denuded of endothelium the effects of 10^{-8} M and 10^{-4} M acetylcholine and adenosine respectively, were tested on the vessel. Endothelium was
Spontaneous tone at 70 mmHg       Passive diameter at 70 mmHg

Fig 2-6. Isolated and cannulated cremaster muscle arterioles with and without tone at 70 mmHg.
considered to be removed in arterioles, which dilated in response to adenosine but not to acetylcholine. A maintained response to adenosine was taken as evidence that the air bubble had not damaged the underlying smooth muscle layer.

**Measurement of membrane potential**

Intracellular recordings of $E_m$ were performed using glass microelectrodes filled with 1 mM KCl (tip resistances of 100-200 MΩ) and an Axoclamp 2B amplifier (Axon Instruments, USA). Recordings were made in pressurized arteriole preparations as described above. Impalements were made using a Leitz precision micromanipulator (Leica Microsystems, Victoria, Australia) within the field of microscope view and in a region of the vessel demonstrating typical vasoreactivity.

**Intracellular Ca$^{2+}$ measurements**

In experiments requiring measurement of changes in $[\text{Ca}^{2+}]_{\text{cyt}}$, Fura 2-AM (MW, 1002; an acetoxymethyl [AM] form of Fura 2) was used as it is a common dye of choice for ratio-imaging microscopy. An important advantage of using Fura 2 is that it allows measurement of a single emission wavelength after excitation at two wavelengths (Moore, Becker et al. 1990). The absorption wavelengths for Ca$^{2+}$ bound and Ca$^{2+}$ free form of Fura 2-AM are 340 nm and 380 nm respectively. The peak emission on excitation occurs at ~ 510 nm for either wavelength. This cancels the parallel changes in the intensities of the two emitted light signals which are induced by moving artifacts (Meininger, Zawieja et al. 1991). As dye leakage, photo bleaching or movement artifacts can all affect the results of Ca$^{2+}$ measurements, a ratiometric indicator such as Fura-2 gives a more stable result as absorption at 340 and 380 is equally affected (Kanaide 1999). Fura 2-AM was chosen over Fura 2, as esterification of carboxylic acid group of
Fura 2 makes it lipophilic and plasma membrane permeant. The cell contains non-specific esterases to cleave the ester moiety and hence trapping the dye inside the cell (Karaki, Ozaki et al. 1997).

**Dye loading and preparation**

Vessels were loaded with Fura 2-AM after developing tone and demonstrating viability. 50 µl of Fura 2-AM (Molecular Probes, OR, USA) was dissolved in 50 ml of dimethyl sulphoxide (DMSO), 25 µl of this solution was dissolved in 10 ml of Krebs-bicarbonate buffer solution containing 0.1 mg/mL Pluronic F-127 (0.01% w/v: Molecular Probes, OR, USA). Pluronic F-127 is a non-anionic detergent, which increases solubility of esterified indicators in the physiological media. The remaining volumes of Fura 2-AM /DMSO were kept in aliquots at -20º C for future use. Vessels were incubated in the loading solution for one hour (room temperature); dye loading from the abluminal surface of the vessel ensured that loading of Fura 2-AM was restricted to the VSM layer while minimizing endothelial cell uptake (Meininger et al., 1991; Zou et al., 1995). After the dye-loading procedure, excess Fura 2-AM solution was removed and the preparation washed for 30 minutes in fresh Krebs-bicarbonate buffer solution.

**Western blotting**

Vessel segments were dissected as previously described in this chapter and 2-3 long segments (approximately 5-10 mm each) were pooled and stored at -80º C prior to use. Vessel segments were lysed in 100 µl ice cold homogenizing buffer (Tris-HCl, 25 mM (pH 6.8); SDS, 1% (w/v); EGTA, 5 mM; NaF, 50 mM; sodium vanadate, 1 mM; glycerol, 10% (v/v) and protease inhibitor cocktail; Sigma). The homogenate was then vortexed, sonicated and finally clarified by centrifugation. The supernatant was aspirated and
protein content of all fractions was determined using Biorad’s protein assay kit. Samples were then diluted with sample buffer (Tris-HCl, 250 M (pH 6.8); SDS, 2% (w/v); glycerol, 10% (v/v); DTT, 10 mM; (3-mercapto-ethanol, 2% (v/v) and bromophenol blue, 0.01% (w/v) in preparation for electrophoretic separation) and the samples were heated at 56°C for 3 minutes and put on ice to cool down ready to load.

8% gels were cast, mounted in an electrophoresis tank (BioRad Mini-gel) and 25 µg of protein was loaded in each lane and resolved by SDS-PAGE and transferred on to nitrocellulose membranes using a semi-dry blotting system. To reduce non-specific binding the membrane was blocked with 3% bovine serum albumin (BSA) in preparation for exposure to antibodies. The membrane(s) were probed with primary antibody against NCX (anti NCX IgG, R3F1, 1:1,000; Swant) then washed in TBST followed by incubation with appropriate secondary antibody and precision protein StrepTactin-HRP (horse radish peroxidase, Biorad) conjugate in TBST (Tris buffer solution with Tween).

Membrane(s) were then washed in TBST to remove the excess secondary antibody. Chemiluminescent substrate (ECL: Perkin Elmer, Boston, MA, USA) was added and protein bands imaged and quantified. An internal standard of rat aorta homogenate was included in each electrophoretic separation. To remove the NCX antibody the membrane(s) were placed in a stripping buffer (Restore™) to be used again for probing with an α actin antibody. The NCX results are expressed as a ratio of the actin band to account for variations in sample loading.

Reverse-transcriptase polymerase chain reaction (PCR)
Cremaster arterioles were dissected as described previously in this chapter. Total RNA was extracted by using RNeasy Micro kit (Qiagen) as per the manufacturer's instructions. Tissues including arterioles, liver, heart, aorta and skeletal muscle were collected for extraction of total RNA and RNA content and purity was established by measuring absorbance readings at 260 and 280 nm.

Samples were reverse transcribed at a final concentration of 10 ng/µl and stored at -20°C until further analysis. PCR was performed for amplification and data collection and analysis. Real-time PCR allows for the characterization of mRNA by the cycle number at which PCR product accumulation is first detected by fluorescence. This method is preferred to conventional PCR; which determines the amount of PCR product accumulated after a fixed number of cycles. The elimination of post-PCR procedures reduces the risk of contamination and errors, leading to reproducible and quantitative results. To check for purity of the product formed, melt-curve analysis was also performed at the completion of each experiment (see Chapter 4).

**Arteriolar RP**

Arterioles were dissected and transferred to a plastic slide in which narrow channels had been machined, kept on ice and exposed to a series of four solutions. Solution 1 was added to the channel, making sure that the arteriole was submerged in the solution for 60 minutes, followed by solutions 2, 3 and 4 (*Table 2-1, 2, 3 and 4*). In solution 2 was dissolved either antisense/sense (10⁻⁶M; experimental groups) or no ODNs (control group). Solution 4 was gradually warmed to 22°C and CaCl₂ was added at 10 minute intervals to reach the following concentrations: 0.001, 0.01, 0.1 and 1.6 mM/l (Morgan and Morgan 1982; Rembold and Murphy 1986; Richards, Davis et al. 2003). Arterioles
were incubated for 24 hours in fresh Krebs-bicarbonate buffer solution with antisense/sense (10^{-6}M) or no ODNs containing 1% dilution of penicillin-streptomycin solution (Sigma, St. Louis, MO).

At the end of the 24 hour period, arterioles were cannulated, as described earlier in this chapter (General Methods). Vessels were checked for any leaks and gradually pressurized to 70 mmHg for the vessel to develop tone for 1 hour. This was followed by functional studies to determine the involvement of NCX in myogenic response and also to confirm the viability of the vessels.

**NCX Oligonucleotides**

The antisense and sense ODNs were synthesized by Sigma Genosys (Castle Hill, NSW, Australia). Antisense ODNs were targeted against 20 nucleotides to the region upstream (5') to, and encompassing the ATG start codon of the NCX1 mRNA (Table 2-3). Sense ODNs were used as controls to check for any non-specific effects of antisense. The sequences showed homology only to NCX DNA in the Gen Bank. The antisense ODN is complementary to a region near the 3' end of the canine heart exchanger mRNA transcript (nucleotides 2638–2657) (Takahashi, Bland et al. 1995; Takahashi, Azuma et al. 1999). This peptide sequence is totally conserved in all three isoforms of NCX and is presumed to be located in the 9th transmembrane domain of the exchanger (Nicoll, Longoni et al. 1990; Li, Matsuoka et al. 1994; Nicoll, Quednau et al. 1996a). Phosphodiester forms were used, as phosphorothioate forms are known to be associated with significant toxicities (Ranciat-McComb, Bland et al. 2000). Sequences of chimeric antisense and sense ODNs targeted to the start codon of NCX1 are as follows:
NCX sense sequence (5’-3’): AACGCGTGAACGTGTTCCT
NCX antisense sequence (5’-3’): AGGAACACGTTCACGGCGTT

Drugs and Chemicals

Sodium Thiopentone (Pentathal, Abbotts, Australasia) was used at a concentration of 0.1 g/ml in milliQ water. Acetylcholine (BDH chemicals) and adenosine (Sigma) stocks were made as a 10⁻²M stock solution with Krebs-bicarbonate buffer solution and then diluted in Krebs-bicarbonate buffer solution to obtain required concentration.

100 mM stock was made of KB-R7943 mesylate (Tocris™) by dissolving the drug in dimethyl sulphoxide (DMSO: BDH chemicals, Kilysyth, Victoria, Australia.) and diluting it further in Krebs-bicarbonate buffer solution for the required concentration. SEA0400 was synthesized by Taisho Pharmaceutical Company Limited (Saito, Japan); a 100 mM stock was made by dissolving the drug in DMSO and then diluted in Krebs-bicarbonate buffer solution to make the required concentration. All the drugs and superfusates were made fresh on the day of experiment and were kept on ice until they were used. Monoclonal NCX antibody was obtained from Swant (Switzerland) and monoclonal actin antibody was obtained from DakoCytomation (Denmark).

Statistical and data analysis

A minimum of 4-6 experiments were required for all the study groups to enable meaningful statistical comparisons. Statistical analysis was performed on raw data using GraphPad Prism 4.0 (GraphPad software, USA). For comparisons between multiple data sets at multiple time points, one-way or two-way analysis of variance (ANOVA) was performed, as appropriate. For comparisons between normally distributed data,
comparisons of the means and standard error (SE) were performed using a Student’s t-test. In all cases P<0.05 was taken as the level of significance. GraphPad Prism software was used for calculations, statistical analysis, graphs and fitting curves.
### Table 2-5. Solution 1

<table>
<thead>
<tr>
<th>Substance</th>
<th>mM/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGTA</td>
<td>10</td>
</tr>
<tr>
<td>KCl</td>
<td>120</td>
</tr>
<tr>
<td>ATP</td>
<td>5</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>2</td>
</tr>
<tr>
<td>N-Tris [hydroxymethyl] methyl-2-amino-ethane sulphonic acid (TES)</td>
<td>20; (pH 6.8 at 2° C for 30 minutes)</td>
</tr>
</tbody>
</table>

### Table 2-6. Solution 2

<table>
<thead>
<tr>
<th>Substance</th>
<th>mM/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGTA</td>
<td>0.1</td>
</tr>
<tr>
<td>KCl</td>
<td>120</td>
</tr>
<tr>
<td>ATP</td>
<td>5</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>2</td>
</tr>
<tr>
<td>N-Tris [hydroxymethyl] methyl-2-amino-ethane sulphonic acid (TES)</td>
<td>20; (pH 6.8 at 2° C for 30 minutes)</td>
</tr>
</tbody>
</table>
### Table 2-7. Solution 3

<table>
<thead>
<tr>
<th>Substance</th>
<th>mM/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGTA</td>
<td>0.1</td>
</tr>
<tr>
<td>KCl</td>
<td>120</td>
</tr>
<tr>
<td>ATP</td>
<td>5</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>10</td>
</tr>
<tr>
<td>N-Tris [hydroxymethyl] methyl-2-amino-ethane sulphonic acid (TES)</td>
<td>20; (pH 6.8 at 2° C for 30 minutes)</td>
</tr>
</tbody>
</table>

### Table 2-8. Solution 4

<table>
<thead>
<tr>
<th>Substance</th>
<th>mM/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>140</td>
</tr>
<tr>
<td>KCl</td>
<td>5</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>10</td>
</tr>
<tr>
<td>MOPS</td>
<td>2; (pH 7.1 AT 22° C for 30 minutes)</td>
</tr>
</tbody>
</table>
CHAPTER 3

FUNCTIONAL SIGNIFICANCE OF THE SODIUM CALCIUM EXCHANGER AND RELEVANCE TO MYOGENIC TONE
INTRODUCTION

The Na⁺/Ca²⁺ exchange (NCX) system plays an important role in regulating [Ca²⁺]_{cyt} concentration in smooth muscle cells, cardiomyocytes, neuronal cells, kidney and a variety of other cells (Iwamoto 2004). As such, its role in arteriolar contractile responses is important to consider.

The myogenic response, as described earlier, is the ability of VSM to respond to a mechanical force such as stretch/tension which results in the activation of contractile machinery and as in other types of contractile activation Ca²⁺ is considered to play an important role. Myogenic tone and stretch activation have been shown to be dependent on an extracellular Ca²⁺ source initially by Uchida and Bohr (Uchida and Bohr 1969) and subsequently by numerous investigators (Nakayama 1982; Harder 1984; Hwa and Bevan 1986; Laher and Bevan 1989). Further in support for the role of Ca²⁺ it has been shown that stretch of myogenically active facial veins causes increased influx of Ca²⁺ (Nakayama 1982).

Pressure-induced contraction involves Ca²⁺/CAM myosin light chain kinase mediated phosphorylation of the regulatory light chains with subsequent interaction of actin and myosin (Zou, Ratz et al. 1995; Zou, Ratz et al. 2000). However, it is now evident that changes in intracellular Ca²⁺ sub-serve functions other than contraction per se (for example modulation of membrane ion channels and Ca²⁺ release from intracellular stores) and that spatio-temporal aspects of Ca²⁺ signalling play a key role in the control

Despite the pivotal role of VGCCs it is clear that such Ca$^{2+}$ entry occurs distal to a series of events initiated by the mechanical stimulus and leading to membrane depolarization. It is generally accepted that an increase in intra luminal pressure produces depolarization of smooth muscle cell membranes (Harder 1984; Jaggar, Wellman et al. 1998) with subsequent depolarization induced Ca$^{2+}$ entry being necessary for myogenic contraction (Davis and Hill 1999). Thus, pressure-induced changes in smooth muscle $E_m$ was not altered by exposure of cannulated (and pressurized) arterioles to nifedipine (Knot and Nelson 1998; Kotecha and Hill 2005).

Current evidence would suggest that depolarization occurs secondarily to Na$^+$ entry through non-selective cation channels, such as SACC. Thus activation of SACCs (Hwa and Bevan 1986; Kirber, Walsh et al. 1988; Meininger and Davis 1992) and depolarization-induced activation of L-type VGCC (Nakayama, Tanaka et al. 1989; Nordlander 1989) are the possible mechanisms coupling the pressure or stretch stimulus to Ca$^{2+}$ entry.

An additional consequence of Na$^+$ entry via the above mechanism is intracellular Na$^+$ accumulation, particularly in the micro-domain formed by close apposition between the plasma membrane and the superficial sarcoplasmic reticulum (Moore, Etter et al. 1993; Arnon, Hamlyn et al. 2000a; Arnon, Hamlyn et al. 2000b). In this situation NCX operates in the reverse mode resulting in Ca$^{2+}$ entry into VSM cells of the arteriolar wall (Iwamoto, Kita et al. 2005). While this may seem paradoxical given that under resting conditions
the NCX is believed to favor Ca\textsuperscript{2+} extrusion it must be emphasized that arterioles pressurized to physiological levels are in an active state with E\textsubscript{m}s of greater than -40 mV and that the signaling events may be occurring within restricted domains that may not reflect global cellular ion levels. Further, the rheogenic properties of the exchanger are postulated to favor Ca\textsuperscript{2+} entry at E\textsubscript{m}s more positive to approximately -40 mV (Blaustein and Lederer 1999).

A number of studies on vascular and non VSM have shown that reduction in the Na\textsuperscript{+} gradient across the cell membrane increases Ca\textsuperscript{2+} entry, raising [Ca\textsuperscript{2+}]\textsubscript{cyt} and thereby inducing tonic contractions in otherwise un-stimulated smooth muscles. Further, reduction in extracellular Na\textsuperscript{+} also enhances contractions caused by agonists (Pritchard and Ashley 1986; Smith and Smith 1987; Blaustein 1988; Woolfson, Hilton et al. 1990; Sato and Aoki 1991). When the Na\textsuperscript{+} gradient is reduced, Ca\textsuperscript{2+} extrusion is inhibited, thereby augmenting contractions (Petersen and Mulvany 1984; Ashida and Blaustein 1987; Smith and Smith 1987; Smith, Zheng et al. 1989; Bova, Goldman et al. 1990).

In addition to functional evidence NCX mRNA and protein have been demonstrated in primary cultured VSM cells (Juhaszova, Ambesi et al. 1994) and it was shown that in smooth muscle, the exchanger is present in plasma membrane regions that are in close proximity to junctional sarcoplasmic reticulum (Moore, Etter et al. 1993; Juhaszova, Ambesi et al. 1994). This location of NCX implies that NCX may indirectly modulate the Ca\textsuperscript{2+} content of the sarcoplasmic reticulum stores in smooth muscles (Bova, Goldman et al. 1990; Blaustein 1993) and can thereby influence Ca\textsuperscript{2+} signalling and contraction of VSM.
Chapter 3  
Significance of NCX in myogenic tone

NCX inhibitors potentially play an important role in predicting the physiological and patho-physiological roles of NCX. Although VSM cells express NCX proteins, their functional role has not been clear, mainly due to the lack of specific inhibitors of NCX and relatively low levels of expression of NCX (Takai, Yamada et al. 2004). As NCX can move ions in both the directions, it may be of high therapeutic benefit if mode specific inhibition of NCX was available. Specific reverse mode blockers of NCX will cause inhibition of Ca$^{2+}$ entry thereby reducing Ca$^{2+}$ overload for e.g. in hypertension. In 1996 the benzyloxyphenyl derivative KB-R7943 was developed as a selective NCX inhibitor (Watano, Kimura et al. 1996; Iwamoto, Watano et al. 1996a; Iwamoto and Kita 2004b) preferentially inhibiting the reverse mode of the exchanger. More recently, Iwamoto et al. identified the role of NCX1 in salt-sensitive hypertension using the more selective NCX inhibitor, SEA0400. SEA0400 lowered the arterial blood pressure in salt-dependent hypertensive rat models, but not in other types of hypertensive rats or in normotensive rats. They concluded that salt-sensitive hypertension is triggered by Ca$^{2+}$ entry through NCX1 in arterial smooth muscle and suggested that NCX1 inhibitors might be useful therapeutically (Iwamoto, Kita et al. 2004d).

The following studies were designed to determine the involvement of NCX in the arteriolar myogenic response with specific reference to effects on global smooth muscle [Ca$^{2+}$]$_{cyt}$ and $E_{m}$. Further, the effects of the NCX inhibitors KB-R7943 and SEA0400 were determined on myogenic tone and the response of cremaster muscle arterioles to reduced extracellular [Na$^{+}$].
MATERIALS AND METHODS

Experimental protocols

Relationship of intra luminal pressure and arteriolar diameter:
Following equilibration at 70 mmHg, a pressure-diameter response curve was obtained in each arteriole. Intra luminal pressure was reduced to 30 mmHg, followed by sequential step increases in intra luminal pressure to 60, 90, 120 and 150 mmHg. Vessels were maintained at each pressure for 5 minutes, and steady-state intra luminal diameters were recorded. The intra luminal pressure of the vessels were returned to 70 mmHg and after superfusing the arteriole with Ca$^{2+}$ free Krebs-bicarbonate buffer solution containing 2 mM EGTA for 15 minutes, a passive diameter was recorded. Diameters for the pressure-diameter response curve have been normalized as a ratio of the diameter under a given experimental condition to that at 70 mmHg in Ca$^{2+}$ free Krebs-bicarbonate buffer solution.

Effect of extracellular manipulation of Na$^+$ on vessel diameter and [Ca$^{2+}$]$_{cyt}$:
Isolated, intact arterioles were equilibrated at 70 mmHg in Krebs-bicarbonate buffer solution with a Na$^+$ concentration of 137 mM. Arteriolar smooth muscle was then loaded with Fura-2 AM as described in Chapter 2 (General Methods). Diameter and [Ca$^{2+}$]$_{cyt}$ were then recorded at extracellular Na$^+$ concentrations of 137, 100, 75 and 25 mM. Arterioles were superfused at each [Na$^+$] for at least 5 minutes prior to taking steady-state measurements. The Na$^+$ in the buffer was replaced by choline chloride to maintain
the osmolarity of the solution. Appropriate controls were conducted to exclude an effect due to the time taken to study the four different Na\(^+\) concentrations.

**Effect of NCX inhibitors on vessel diameter:**

Vessels were allowed to develop spontaneous myogenic tone at 70 mmHg. Experiments were performed on de-endothelialized vessels to show that the obtained effects are solely due to smooth muscle and not endothelium (see Chapter 2, General Methods). The arterioles were then superfused with varying concentrations of the NCX inhibitors to obtain a dose-response relationship. KB-R7943 (10\(^{-7}\)M-10\(^{-4}\)M), an NCX inhibitor, which has a relative selectivity for the 'reverse' mode of the NCX transport (Ca\(^{2+}\) entry) and SEA0400 (10\(^{-9}\)M-3\(\times\)10\(^{-6}\)M), a potent and selective blocker of NCX, were used in these studies.

From the dose-response curves for KB-R7943 and SEA0400, concentration of inhibitors causing 70% dilatation, KB-R7943 (10\(^{-5}\)M) and SEA0400 (10\(^{-6}\)M), were chosen. To force the NCX into reverse mode, extracellular [Na\(^+\)] was reduced from 137 to 100, 75 and 25 mM. Arterioles were superfused with, KB-R7943 (10\(^{-5}\)M) dissolved in Krebs-bicarbonate buffer solution in decreasing extracellular [Na\(^+\)]. The arterioles were exposed to each concentration of extracellular [Na\(^+\)] for 15 minute period to obtain each diameter measurement. The diameters were measured for each extracellular [Na\(^+\)] in all the arterioles. Vehicle controls were performed by dissolving DMSO (0.5%) in Krebs-bicarbonate buffer solution and superfusing the arteriole, whilst measuring the diameters. The arterioles were washed with 137 mM Na\(^+\) buffer after 25 mM extracellular [Na\(^+\)] and control experiments were performed by superfusing the arteriole with reduced extracellular [Na\(^+\)] without any inhibitors. At the end of the experiment the
arterioles were superfused with Ca$^{2+}$ free Krebs-bicarbonate buffer solution to allow measurement of passive diameters. All diameters were normalized relative to diameters in Ca$^{2+}$ free Krebs-bicarbonate buffer solution. Similar experiments were performed in a separate set of arterioles with NCX inhibitor SEA0400 ($10^{-6}$M).

To examine whether the inability of the vessels to constrict in the presence of the inhibitors to decreasing extracellular [Na$^{+}$] is specific to involvement of NCX, another set of experiments were designed using unrelated vasodilators. After regaining tone at 70 mmHg the arterioles were superfused with nifedipine ($10^{-6}$M) for 15 minutes with each extracellular [Na$^{+}$], i.e. 137 to 100, 75 and 25 mM in Krebs-bicarbonate buffer solution. The diameters were measured for each extracellular [Na$^{+}$] in all the arterioles. The arterioles were washed with 137 mM Na$^{+}$ buffer after 25 mM extracellular [Na$^{+}$] and control experiments were performed on the same arteriole by superfusing the arteriole with each extracellular [Na$^{+}$] in the absence of inhibitors. At the end of the experiment the arteriole was superfused with Ca$^{2+}$ free Krebs-bicarbonate buffer solution containing 2 mM EGTA. All diameters were represented relative to diameters in Ca$^{2+}$ free Krebs-bicarbonate buffer solution.

**Effect of NCX inhibitor KB-R7943 and VGCC blocker nifedipine on vessel diameter and $E_m$ of cremaster arterioles:**

Vessels were allowed to develop spontaneous myogenic tone at 70 mmHg. The arterioles were de-endothelialized and intracellular recordings of $E_m$ were performed using glass microelectrodes as described in Chapter 2 (General Methods). After the vessel developed tone the arterioles were superfused with NCX inhibitor KB-R7943
(3×10^{-5}M) or nifedipine (10^{-6}M). Arteriolar diameters and $E_m$ was recorded and compared to controls (arterioles not superfused with KB-R7943 or nifedipine).

**Effect of decreased extracellular [Na$^+$] on acute myogenic reactivity and myogenic index (MI) in endothelium-intact and endothelium-denuded cremaster arterioles:**

Vessels were allowed to develop spontaneous myogenic tone at 70 mmHg and de-endothelialized as described in Chapter 2 (General Methods). To assess acute myogenic reactivity, vessels were subjected to a pressure step from 50 to 120 mmHg, both in endothelium-intact and endothelium-denuded vessels. To see the involvement of NCX in the myogenic response, diameters were recorded in Krebs-bicarbonate buffer solution with decreasing extracellular [Na$^+$] from 137 mM to 100, 75 and 25 mM. Arterioles were superfused in Krebs-bicarbonate buffer at each of the extracellular [Na$^+$] for at least 15 minutes. At the end of 15 minutes, a pressure step was applied and arteriolar diameters were recorded at 50 versus 120 mmHg after keeping the arteriole at each intra luminal pressure for 5 minutes. As in previous experiments Na$^+$ in the buffer was replaced by choline chloride to maintain the osmolarity of the superfusate. Time controls were performed, where arterioles were maintained at an extracellular [Na$^+$] of 137 mM through the course of experiment.

Myogenic index, a measure of myogenic responsiveness of an arteriole, was calculated as shown below (Halpern, Mongeon et al. 1984; Jackson and Duling 1989):

\[
\text{Myogenic Index (MI)} = 100 \frac{\Delta D/D_0}{\Delta P}
\]
\[ \Delta D = \text{Diameter (Final – Initial)} \]

\[ D_i = \text{Initial Diameter} \]

\[ \Delta P = \text{Pressure (Final - Initial)} \]

This equation represents the normalized slope of the pressure step graph at each pressure point. A negative value indicates the presence of a myogenic response by showing that an increase in intra luminal pressure is associated with a corresponding vasoconstriction (Jackson and Duling 1989).
**RESULTS**

To establish the myogenic responsiveness of rat cremaster vessels, pressure-diameter curves were performed as shown in Fig 3-1. As described earlier, intra luminal pressure was increased in steps from 30, to 60, 90, 120 and 150 mmHg. With an increase in the intra luminal pressure from 30 to 150 mmHg a significant reduction in internal diameter (62.3 ± .9% to 36.5 ± .5% of passive diameter) of the arterioles was observed (P<0.0001, one-way ANOVA with a post hoc Bonferroni’s multiple comparison test, n = 6).

**Effects of extracellular [Na⁺] on arteriolar diameter and [Ca²⁺]_{cyt} of rat cremaster arterioles.**

To determine the effects of NCX on arteriolar diameter and [Ca²⁺]_{cyt} extracellular [Na⁺] was decreased to potentiate the reverse mode activity of NCX. This resulted in vasoconstriction (50.2 ± 1.7% to 36.8 ± 1.3% passive diameter) with a resultant significant increase in the level of tone (Fig 3-2; P<0.0001, one-way ANOVA with a post hoc Bonferroni’s multiple comparison test, n = 18).

Decreasing extracellular [Na⁺] caused an increase in the [Ca²⁺]_{cyt} by 9% in smooth muscle of the cannulated arterioles. With a decrease in the extracellular [Na⁺] from 137 to 25 mMol, 340:380 increased from .59 ± 0.02 to .65 ± 0.03 (intra luminal pressure was maintained at 70 mmHg throughout the experiment). For [Ca²⁺]_{cyt} measurements the values of R340/380 are expressed as a ratio to R340/380 under 137 mM extracellular...
Concentration-dependent effects of NCX inhibitors KB-R7943 and SEA0400 on the diameter of cremaster arterioles.

NCX inhibitors are potential tools to predict the physiological and patho-physiological roles of NCX. Thus to substantiate the role of NCX in myogenic responses of the cremaster arterioles, NCX inhibitors, KB-R7943 and SEA0400 were initially examined for concentration-dependent effects. A concentration-dependent vasodilatation (P<0.0001, one-way ANOVA with a post hoc Bonferroni's multiple comparison test, n = 8) was observed for KB-R7943 over the range $10^{-7}$-3×$10^{-4}$M (Fig 3-4). Similarly SEA0400 ($10^{-9}$-3×$10^{-6}$M) caused concentration-dependent vasodilatation (P<0.0001, one-way ANOVA with a post hoc Bonferroni's multiple comparison test, n = 8, Fig 3-4). The IC$_{50}$ values of dose-response curves of KB-R7943 and SEA0400 were 5.4 ± 0.07 and 6.7 ± 0.1 respectively. SEA0400 was ~ 100 times more potent than KB-R7943 in causing vasodilatation (P<0.0001, unpaired student t-test, n = 8) in rat cremaster arterioles.

Fig 3-6 shows the effects of NCX inhibitor, KB-R7943 ($10^{-5}$M) on the diameter of deendothelialized cremaster arterioles in decreasing extracellular [Na$^+$]. KB-R7943 significantly attenuated the vasoconstriction (73.6 ± 2.5% to 98 ± 3.5%), caused by decreasing extracellular [Na$^+$] from 137 mM to 25 mM (P<0.0001, two-way ANOVA with a post hoc Bonferroni’s multiple comparison test, n = 9). Diameters shown in the graph have been normalized as a percentage of diameter in 137 mM extracellular [Na$^+$]. Fig 3-7 shows that KB-R7943 ($10^{-5}$M) causes significant vasodilatation (P<0.0001, unpaired
student t-test, n = 8) of arterioles in 137 mM extracellular [Na\(^+\)] as compared to control vessels (no change in extracellular [Na\(^+\)]) (50.2 ± 8.9% to 80.4 ± 2.9%).

Similar results were observed for the NCX inhibitor, SEA0400 (10\(^{-6}\)M) (Fig 3-8). SEA0400 significantly attenuated the vasoconstriction (73.3 ± 2.1% to 92.4 ± 2.5%) caused by lowering extracellular [Na\(^+\)] from 137 mM to 25 mM (P<0.0001, two-way ANOVA with a post hoc Bonferroni's multiple comparison test, n = 9). Diameters shown in the graph have been normalized as a percentage of diameters in 137 mM extracellular Na\(^+\). As with KB-R7943, SEA0400 also caused a significant vasodilatation of the arterioles in 137 mM extracellular [Na\(^+\)] as compared to controls (51.9 ± 7.2% to 85.3 ± 2.4%) (Fig 3-9).

**Effects of the L-type VGCC blocker, nifedipine (10\(^{-6}\)M), on the diameter of de-endothelialized cremaster arterioles during decreasing extracellular [Na\(^+\)].**

To examine whether the effects of KB-R7943 on responses to decreasing extracellular [Na\(^+\)] were related to NCX inhibition as opposed to a non-specific effect of vasodilatation, an additional set of experiments were implemented using the L-type VGCC inhibitor, nifedipine (10\(^{-6}\)M). Fig 3-10 shows the effects of nifedipine (10\(^{-5}\)M) on the diameter of de-endothelialized cremaster arterioles in decreasing extracellular [Na\(^+\)]. Unlike NCX inhibitors KB-R7943 and SEA0400, nifedipine did not cause attenuation of vasoconstriction (66 ± 3.7% to 60.2 ± 5.70%) caused by decreasing the extracellular [Na\(^+\)] from 137 mM to 25 mM. Fig 3-11 shows that nifedipine (10\(^{-6}\)M) did, however, cause a significant vasodilatation of the cremaster arterioles in 137 mM extracellular [Na\(^+\)] as compared to control vessels (52.4 ± 1.3% to 76.7 ± 4.3%).
Effects of NCX inhibitor KB-R7943 and VGCC blocker nifedipine on arteriolar diameter and membrane potential.

Arterioles were superfused with KB-R7943 (3×10^{-5}M) or nifedipine (10^{-6}M) and arteriolar diameter and $E_m$ of the VSM cells recorded. As shown in Fig 3-12 and Fig 3-13, there was a significant increase in the arteriolar diameter (46.8 ± 8.2 µm to 131 ± 10.2 µm) and hyperpolarization (34.2 ± 0.9 mV to 42.8 ± 1.9 mV) of the VSM cells after the vessels were treated with KB-R7943. Nifedipine caused a significant increase (74.4 ± 5.2 µm to 108.9 ± 9.9 µm) in the arteriolar diameter (Fig 3-14) but it was not associated with significant hyperpolarization (38.1 ± 1.3 mV to 39.6 ± 1.3 mV), (Fig 3-15).

Effect of decreasing extracellular [Na$^+$] on acute myogenic reactivity and myogenic index in endothelium-intact and endothelium-denuded cremaster arterioles.

Vessels were subjected to a pressure step from 50 to 120 mmHg to assess the myogenic reactivity. To examine the involvement of NCX in the response to the pressure step this protocol was repeated at decreasing extracellular Na$^+$ concentrations as used above (Fig 3-16). Time controls were also performed (Fig 3-17), where arterioles were not subjected to an extracellular change in [Na$^+$]. In another set of experiments vessels were denuded of endothelium (Fig 3-18 and Fig 3-19), and the above protocol was applied. There was a significant decrease in the myogenic reactivity as seen by the decrease in the slopes of both, endothelium-intact (P<0.0001, one way repeated-measures ANOVA with a post hoc Bonferroni’s multiple comparison test, n=6) and endothelium-denuded (P<0.0001, one way repeated-measures ANOVA with a post hoc Bonferroni’s multiple comparison test, n = 6) vessels as compared to the control vessels.
(Table 3-1 and Table 3-2). There were no apparent effects of time on myogenic reactivity as shown by control experiments.

Myogenic index was calculated as a measure of myogenic responsiveness for both in endothelium-intact and endothelium-denuded arterioles as shown in Fig 3-20. Myogenic index was significantly decreased with decreasing extracellular [Na⁺] (P<0.0001, two-way repeated-measures ANOVA with a post hoc Bonferroni’s multiple comparison test). There were no significant differences detected between endothelium-intact and endothelium-denuded vessels.
Fig 3-1.
Fig 3-21. Pressure-diameter curve for rat cremaster arterioles.
Intra luminal pressure of the vessels increased from 30 to 150 mmHg through the course of the experiment, with vessels being maintained at each pressure for 5 minutes. Vessel diameters are shown as a fraction of diameters under passive conditions (without Ca^{2+} with 2 mM EGTA). Data are shown as mean ± SEM. Analysis shows a significant reduction (P<0.0001, one-way ANOVA with a post hoc Bonferroni's multiple comparison test, n = 6) in diameter of the arterioles with an increase in the intra luminal pressure.
Chapter 3  
Significance of NCX in myogenic tone

Fig 3-2.

![Graph showing D/Dmax vs. Extracellular [Na⁺, mM]](image)

Fig 3-3.

![Graph showing Normalized 340/380 vs. Extracellular [Na⁺]](image)
Fig 3-22. Effect of extracellular [Na\(^+\)] on arteriolar diameter of rat cremaster arterioles.

The extracellular [Na\(^+\)] was reduced from 137 mM to 100, 75 and 25 mM. Intra luminal pressure of the vessels was maintained at 70 mmHg throughout the experiment. Vessel diameters are shown as a fraction of diameters under passive conditions (without Ca\(^{2+}\) with 2 mM EGTA). Data are shown as mean ± SEM. Analysis shows a significant reduction (P<0.0001, one-way ANOVA with a post hoc Bonferroni’s multiple comparison test, n = 18) in diameter of the arterioles with reduction in extracellular [Na\(^+\)].

Fig 3-23. Effects of extracellular [Na\(^+\)] on [Ca\(^{2+}\)]\(_{\text{cyt}}\) in rat cremaster arterioles.

Arterioles were incubated with 2.5 µM Fura-2AM for 60 minutes and [Ca\(^{2+}\)]\(_{\text{cyt}}\) was measured as the fluorescence ratio (R340/R380). The extracellular [Na\(^+\)] was reduced from 137 mM to 100, 75 and 25 mM. Intra luminal pressure of the vessels was maintained at 70 mmHg throughout the experiment. The values of R340/380 are expressed as a ratio to R340/380 under 137 mM extracellular [Na\(^+\)]. Data are shown as mean ± SEM. Analysis shows a significant increase (P<0.0001, one-way ANOVA with a post hoc Bonferroni’s multiple comparison test, n = 18) in [Ca\(^{2+}\)]\(_{\text{cyt}}\) of the vessel with reduction in extracellular [Na\(^+\)].
Fig 3-4.

-Log IC₅₀ = 5.4 ± 0.07

Fig 3-5.

-Log IC₅₀ = 6.7 ± 0.1
Fig 3-24. Dose-response curve for NCX inhibitor KB-R7943 ($10^{-7}$-$3\times10^{-4}$ M) on the diameter of de-endothelialized arterioles.

KB-R7943, which has a relative selectivity for the ‘reverse’ mode of the exchanger, caused a concentration-dependent dilatation of the arterioles. Intra luminal pressure of the vessels was maintained at 70 mmHg throughout the experiment. Vessel diameters were shown as a fraction of diameters under passive conditions (without Ca$^{2+}$ with 2 mM EGTA) at 70 mm Hg. Data are shown as mean ± SEM. Analysis shows a concentration-dependent (P<0.0001, one-way ANOVA with a post hoc Bonferroni’s multiple comparison test, n = 5) increase in diameter of the arterioles with NCX inhibitor KB-R7943.

Fig 3-25. Dose-response curve for NCX inhibitor SEA0400 ($10^{-9}$-$3\times10^{-6}$M) on the diameter of de-endothelialized arterioles.

SEA0400, which is reportedly a selective NCX inhibitor, caused a concentration-dependent dilatation of the arterioles. Intra luminal pressure of the vessels was maintained at 70 mmHg throughout the experiment. Vessel diameters were shown as a fraction of diameters under passive conditions (without Ca$^{2+}$ with 2 mM EGTA) at 70 mm Hg. Data are shown as mean ± SEM. Analysis shows a concentration-dependent (P<0.0001, one-way ANOVA with a post hoc Bonferroni’s multiple comparison test, n = 8) increase in diameter of the arterioles with NCX inhibitor SEA0400.
**Fig 3-6.**

**Fig 3-7.**
Fig 3-26. Effects of NCX inhibitor, KB-R7943 (10^{-5}M) on the diameter of the de-endothelialized cremaster arterioles in decreasing extracellular [Na^+].

KB-R7943 which has a relative selectivity for the ‘reverse’ mode of the exchanger attenuated the vasoconstriction induced by lowering of extracellular [Na^+] from 137 mM to 100, 75 and 25 mM. Intra luminal pressure of the vessels was maintained at 70 mmHg throughout the experiment. Diameter has been normalized as a percentage of diameter in 137 mM extracellular [Na^+] in both, controls and KB-R7943 treated groups. Data are shown as mean ± SEM. Analysis shows a significant attenuation (P<0.0001, two-way ANOVA with a post hoc Bonferroni’s multiple comparison test, n = 9) of vasoconstriction after treating the arterioles with KB-R7943 as compared to controls.

Fig 3-27. Effects of NCX inhibitor, KB-R7943 (10^{-5}M) on the diameter of the de-endothelialized cremaster arterioles in 137 mM extracellular [Na^+].

KB-R7943, which has a relative selectivity for the ‘reverse’ mode of the exchanger, dilates the arterioles. Intra luminal pressure of the vessels was maintained at 70 mmHg throughout the experiment. Vessel diameters are shown as a fraction of diameters under passive conditions (without Ca^{2+} with 2 mM EGTA) at 70 mm Hg. Data are shown as mean ± SEM. Analysis shows a significant increase (P<0.0001, unpaired t-test, n = 9) in the diameter of the arterioles treated with KB-R7943 as compared to controls.
Fig 3-8.

Fig 3-9.
Fig 3-28. Effects of NCX inhibitor, SEA0400 (10⁻⁶M) on the diameter of deendothelialized cremaster arterioles in decreasing extracellular [Na⁺].

SEA0400, which is a selective NCX inhibitor, attenuated vasoconstriction induced by lowering of extracellular [Na⁺] from 137 mM to 100, 75 and 25 mM. Intra luminal pressure of the vessels was maintained at 70 mmHg throughout the experiment. Diameter has been normalized as a percentage of diameter in 137 mM extracellular [Na⁺] in both, control and SEA0400 treated groups. Data are shown as mean ± SEM, n = 9. Analysis shows a significant attenuation (P<0.0001, two-way ANOVA with a post hoc Bonferroni’s multiple comparison test, n = 9) of vasoconstriction after treating the arterioles with SEA0400 as compared to controls.

Fig 3-29. Effects of NCX inhibitor, SEA0400 (10⁻⁶M) on the diameter of the deendothelialized cremaster arterioles in 137 mM extracellular [Na⁺].

SEA0400, which is a selective NCX inhibitor, dilates the arteriole. Intra luminal pressure of the vessels was maintained at 70 mmHg throughout the experiment. Vessel diameters are shown as a fraction of diameters under passive conditions (without Ca²⁺ with 2 mM EGTA) at 70 mm Hg. Data are shown as mean ± SEM. Analysis shows a significant increase (P<0.0001, unpaired t-test, n = 9) in the diameter of the arterioles treated with SEA0400 as compared to controls.
Chapter 3  
Significance of NCX in myogenic tone

Fig 3-10.

Fig 3-11.
Fig 3-30. Effects of Nifedipine (10^{-6}M) on the diameter of de-endothelialized cremaster arterioles in decreasing extracellular [Na^+].

Nifedipine, which is a L-type VGCC blocker, did not attenuate vasoconstriction induced by decreasing extracellular [Na^+] from 137 mM to 100, 75 and 25 mM. Intra luminal pressure of the vessels was maintained at 70 mmHg throughout the experiment. Diameter has been normalized as a percentage of diameter in 137 mM extracellular [Na^+] in both, control and nifedipine treated groups. Data are shown as mean ± SEM, (n = 4). Analysis shows that there is no significant attenuation of vasoconstriction after treating the arterioles with nifedipine as compared to controls.

Fig 3-31. Effects of Nifedipine (10^{-6}M) on the diameter of the de-endothelialized cremaster arterioles in 137 mM extracellular [Na^+].

Intra luminal pressure of the vessels was maintained at 70 mmHg throughout the experiment. Vessel diameters are shown as a fraction of diameters under passive conditions (without Ca^{2+} with 2 mM EGTA) at 70 mm Hg. Data are shown as mean ± SEM. Analysis shows a significant increase in the diameter of the arterioles treated with nifedipine (P<0.0001, unpaired t-test, n = 4) as compared to controls.
Chapter 3

Significance of NCX in myogenic tone

Fig 3-12.

Fig 3-13.
Fig 3-32. Effects of NCX inhibition on arteriolar diameter (3×10⁻⁵M of KB-R7943).

KB-R7943 which has a relative selectivity for the ‘reverse’ mode of the exchanger caused vasodilatation of the arterioles. Intra luminal pressure of the vessels was maintained at 70 mmHg throughout the experiment. Vessel diameters are shown as actual internal diameters (μm). Data are shown as means ± SEM. Analysis shows a significant (P<0.05, unpaired t-test with a post hoc Bonferroni’s multiple comparison test, n = 4) vasodilatation in arterioles treated with KB-R7943 as compared to controls.

Fig 3-33. Effects of NCX inhibition on arteriolar smooth muscle Eₘ (3×10⁻⁵M of KB-R7943).

KB-R7943 which has a relative selectivity for the ‘reverse’ mode of the exchanger caused hyperpolarization of the VSM cells. Intra luminal pressure of the vessels was maintained at 70 mmHg throughout the experiment. Vessel diameters are shown as a percentage of diameters under passive conditions (without Ca²⁺ with 2 mM EGTA). Intracellular recordings of Eₘ were performed using glass microelectrodes and an amplifier in pressurized arteriole preparations. Data are shown as mean ± SEM. Analysis shows that the arterioles treated with KB-R7943 are significantly hyperpolarized (P<0.05, unpaired t-test with a post hoc Bonferroni’s multiple comparison test, n = 4) as compared to the controls.
Fig 3-14.

Fig 3-15.
Fig 3-34. Effect of nifedipine \((10^{-6} \text{M})\) on arteriolar diameter.

The arterioles were superfused with nifedipine, (L-type Ca\(^{2+}\) channel blocker), which caused vasodilatation. Intra luminal pressure of the vessels was maintained at 70 mmHg throughout the experiment. Vessel diameters are shown as actual internal diameters (\(\mu\text{m}\)). Data are shown as means ± SEM. Analysis shows a significant but partial loss of tone \((P<0.05, \text{unpaired } t\text{-test with a post hoc Bonferroni’s multiple comparison test, } n = 13)\) in arterioles treated with nifedipine as compared controls.

Fig 3-35. Effects of nifedipine \((10^{-6} \text{M})\) on arteriolar smooth muscle \(E_m\).

The arterioles superfused with nifedipine, (L-type Ca\(^{2+}\) channel blocker), caused hyperpolarization of the VSM cells. Intra luminal pressure of the vessels was maintained at 70 mmHg throughout the experiment. Intracellular recordings of \(E_m\), was done using glass microelectrodes and an amplifier, in pressurized arteriole preparations. Data are shown as means ± SEM. Analysis shows that there was no significant change \((P>0.05, \text{unpaired } t\text{-test with a post hoc Bonferroni’s multiple comparison test, } n = 13)\) in the hyperpolarization caused by nifedipine as compared to controls.
Chapter 3

Significance of NCX in myogenic tone

Fig 3-16.

Fig 3-17.
Fig 3-36. Effect of decreasing extracellular [Na\(^+\)] on acute myogenic reactivity in endothelium-intact cremaster arterioles.

Myogenic reactivity was assessed by performing pressure step. The effect of decreasing extracellular [Na\(^+\)] was seen on the myogenic reactivity in arterioles pressurized to 50 versus 120 mmHg. Vessel diameters are shown as a fraction of diameters under passive conditions (without Ca\(^{2+}\) with 2 mM EGTA). Data are shown as mean ± SEM. Analysis shows a significant increase (P<0.0001, two-way repeated-measures ANOVA with a post hoc Bonferroni’s multiple comparison test, n = 6) in myogenic reactivity with increase in the extracellular [Na\(^+\)] from 25 to 137 mM.

Fig 3-37. Effect of time on myogenic reactivity in endothelium-intact cremaster arterioles.

Time controls were performed; where arterioles were not subjected to an extracellular change in [Na\(^+\)] from 137 mM to 100, 75 and 25 mM. There was no significant effect of time on the present set of experiments (n = 6).
Fig 3-18.

Fig 3-19.
Fig 3-38. Effect of decreasing extracellular [Na⁺] on acute myogenic reactivity in endothelium-denuded cremaster arterioles.

Myogenic reactivity was assessed by performing pressure step. The effect of decreasing extracellular [Na⁺] was seen on the myogenic reactivity in arterioles pressurized to 50 versus 120 mmHg. Vessel diameters are shown as a fraction of diameters under passive conditions (without Ca²⁺ with 2 mM EGTA). Data are shown as mean ± SEM. Analysis shows that there was a significant increase (P<0.0001, two-way repeated-measures ANOVA with a post hoc Bonferroni’s multiple comparison test, n = 6) in myogenic reactivity with increase in the extracellular [Na⁺] from 25 to 137 mM.

Fig 3-39. Effect of time on myogenic reactivity in endothelium-denuded cremaster arterioles.

Time controls were performed; where arterioles were not subjected to an extracellular change in [Na⁺] from 137 mM to 100, 75 and 25 mM. There was no significant effect of time on the present set of experiments (n = 6).
Fig 3-20.
Fig 3-40. Effect of decreasing extracellular [Na⁺] on acute myogenic index in endothelium-intact and denuded cremaster arterioles.

The effect of decreasing extracellular [Na⁺] was seen on MI both in endothelium-intact and denuded cremaster arterioles. MI was calculated by the formula described in materials and methods of this chapter. Data are shown as means ± SEM. Analysis shows that there was a significant increase in myogenic index with increase in the extracellular [Na⁺] from 25 to 137 mM both in endothelium-intact (P<0.0001, two-way repeated-measures ANOVA with a post hoc Bonferroni’s multiple comparison test, n=6) and endothelium-denuded (P<0.0001, Two-way ANOVA with a post hoc Bonferroni’s multiple comparison test, n = 6) arterioles. There was no significant difference in endothelium-intact and denuded arterioles.
### Table 3-3. Myogenic indice of endothelium-intact and endothelium-denuded vessels in experimental group.

<table>
<thead>
<tr>
<th>Extracellular [Na⁺]</th>
<th>Endothelium-intact</th>
<th>Endothelium-denuded</th>
</tr>
</thead>
<tbody>
<tr>
<td>137</td>
<td>-0.30 ± 0.03</td>
<td>-0.31 ± 0.02</td>
</tr>
<tr>
<td>100</td>
<td>-0.12 ± 0.03</td>
<td>-0.13 ± 0.03</td>
</tr>
<tr>
<td>75</td>
<td>-0.08 ± 0.02</td>
<td>-0.07 ± 0.01</td>
</tr>
<tr>
<td>25</td>
<td>-0.06 ± 0.03</td>
<td>-0.07 ± 0.01</td>
</tr>
</tbody>
</table>

### Table 3-4. Myogenic indice of endothelium-intact and endothelium-denuded vessels in control group.

<table>
<thead>
<tr>
<th>Time points</th>
<th>Endothelium-intact</th>
<th>Endothelium-denuded</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-0.24 ± 0.01</td>
<td>-0.23 ± 0.01</td>
</tr>
<tr>
<td>2</td>
<td>-0.23 ± 0.02</td>
<td>-0.17 ± 0.02</td>
</tr>
<tr>
<td>3</td>
<td>-0.22 ± 0.02</td>
<td>-0.19 ± 0.04</td>
</tr>
<tr>
<td>4</td>
<td>-0.24 ± 0.01</td>
<td>-0.20 ± 0.03</td>
</tr>
</tbody>
</table>
Values are mean ± SE. Linear regression was used to calculate slopes utilizing the equation $y = mx + c$.

Where $y$ is the diameter, $x$ is the intra luminal pressure and $m$ is the slope of the fitted line. There was a significant difference between slopes of experimental (Table 3-1) and control (Table 3-2) groups of both the endothelium-intact and denuded groups.

For Table 3-1 refer Fig. 3-16 and 3-18

For Table 3-2 refer Fig. 3-17 and 3-19
DISCUSSION

The Na\(^+\)/Ca\(^{2+}\) exchange system plays an important role in regulating intracellular Ca\(^{2+}\) concentration in smooth muscle cells, cardiomyocytes, neuronal cells, kidney and a variety of other cells (Iwamoto 2004). NCX can operate in forward mode (remove Ca\(^{2+}\) from the cells in exchange for Na\(^+\)) or reverse mode (ion flux is reversed) in stoichiometry of 3:1 for Na\(^+\) to Ca\(^{2+}\) (Blaustein and Lederer 1999). This exchange is dependent on $E_m$ and Na\(^+\) and Ca\(^{2+}\) gradients across the plasma membrane (Shigekawa and Iwamoto 2001; Philipson, Nicoll et al. 2002).

Lowering extracellular [Na\(^+\)] is commonly used for activation of NCX in the reverse mode and typically produces a rise in [Ca\(^{2+}\)]\(_{cyt}\). Our experiments using a rapid change to low extracellular [Na\(^+\)] solution (to inhibit forward mode NCX) caused vasoconstriction and an increase in [Ca\(^{2+}\)]\(_{cyt}\) proportional to the magnitude of extracellular [Na\(^+\)] decrease. Henrion et al. similarly suggested that the myogenic tone in the rabbit posterior cerebral artery and rabbit facial vein is modulated by changes in extracellular [Na\(^+\)] (Henrion, Laher et al. 1994; Henrion, Laher et al. 1997). Many reports have indicated that in VSM, reducing extracellular Na\(^+\) promotes Ca\(^{2+}\) entry (Takai, Yamada et al. 2004) by activating large capacity Na\(^+\)/Ca\(^{2+}\) exchange system in reverse mode (Ashida and Blaustein 1987; Johansson and Hellstrand 1987; Lee, Poburko et al. 2001; Rebolledo, Speroni et al. 2006). However studies, particularly in isolated arterioles, to show the effects of NCX inhibitors on the VSM cells are limited.
An obvious concern is that experimentally decreasing the Na\(^+\) gradient across the cell membrane could result in a wide range of effects other than modulation of NCX. Apart from the NCX it can also influence the Na\(^+\)/H\(^+\) exchanger and the Na\(^+\)/K\(^+\) ATPase (Bova, Goldman et al. 1990). Thus using decreased Na\(^+\) concentrations alone is not a sufficiently robust method to analyze the physiological role of NCX; unless combined with other approaches for modulating NCX activity such as utilizing NCX inhibitors.

To elucidate a possible contribution of the reverse mode of NCX on the Ca\(^{2+}\) response to decreased extracellular Na\(^+\), NCX inhibitors were utilized. KB-R7943 an isothiourea derivative that appears to exhibit selectivity for Ca\(^{2+}\) influx mode (reverse mode of NCX activity) (Watano, Kimura et al. 1996; Iwamoto, Watano et al. 1996a; Satoh, Ginsburg et al. 2000; Elias, Lukas et al. 2001) and SEA0400 the most potent NCX inhibitor (Matsuda, Arakawa et al. 2001; Iwamoto, Kita et al. 2004c) which also appeared to selectively block the outward current (i.e. reverse mode) (Iwamoto, Kita et al. 2004c), were used.

In the present study it was observed that in cremaster arterioles SEA0400 is the more potent inhibitor of NCX, IC\(_{50}\) value being 100 times that of KB-R7943, observed by comparing the two dose-response curves. Both the inhibitors caused a dose-dependent vasodilatation which can be attributed to their inhibitory effects on the Ca\(^{2+}\) uptake (Iwamoto, Kita et al. 2004c) in the VSM cells. These findings are consistent with some recent studies showing that SEA0400 strongly inhibited NCX activity as compared to KB-R7943. Matsuda et al. observed that in cultured neurons, astrocytes and microglia, IC\(_{50}\)s of SEA0400 and KB-R7943 were 5-33 nM and 2-4 µM respectively (Matsuda, Arakawa et al. 2001; Takahashi, Takahashi et al. 2003). In another study, SEA0400
potently inhibited Na\(^+\) dependent \(^{45}\)Ca\(^{2+}\) uptake in canine cardiac sarcolemmal vesicles and rat cardiomyocytes with an IC\(_{50}\) of 90 and 92 nM, compared with KB-R7943 7.0 and 9.5 \(\mu\)M respectively (Takahashi, Takahashi et al. 2003).

Further evidence for the functional existence of NCX was obtained by utilizing KB-R7943 and SEA0400 in protocols where extracellular [Na\(^+\)] was reduced to force the reverse-mode function of the NCX. Decreasing extracellular [Na\(^+\)] from 137 mM to 25 mM drives the NCX in reverse mode (Ca\(^{2+}\) influx), thereby causing Ca\(^{2+}\) entry in the VSM cells and hence vasoconstriction. KB-R7943 (10\(^{-5}\)M) significantly attenuated the vasoconstriction induced by decreasing extracellular [Na\(^+\)] thereby suggesting a role of NCX in regulating myogenic tone in rat cremaster arterioles. Similarly the NCX inhibitor SEA0400 (10\(^{-6}\)M) significantly attenuated the vasoconstriction induced by lowering of extracellular [Na\(^+\)]. As already shown, KB-R7943 and SEA0400 both caused significant vasodilatation of the de-endothelialized cremaster arterioles in 137 mM extracellular [Na\(^+\)] as compared to control vessels.

Na\(^+\) deficiency in the extracellular compartment can assist the opening of VGCC and hence Ca\(^{2+}\) entry independent of NCX entry (Blaustein and Lederer 1999). To show that the vasoconstriction caused by decreasing extracellular [Na\(^+\)] is due to Ca\(^{2+}\) entry via NCX, Nifedipine (a specific VGCC blocker; 10\(^{-6}\)M) was utilized. We observed that extracellular application of nifedipine does not have any significant effects on contraction driven by the reverse mode of the NCX in rat cremaster arterioles. In recent studies D-600 (VGCC blocker) (Takai, Yamada et al. 2004) and nifedipine (10\(^{-5}\)M) (Zhang, Yuan et al. 2005; Rebolledo, Speroni et al. 2006) also had no effect on [Ca\(^{2+}\)]\(_{cyt}\) elevation as a result of decreasing extracellular [Na\(^+\)]. Nifedipine (10\(^{-6}\)M and 10\(^{-5}\)M) did not induce any
change in the basal tone and did not modify contractions induced by NCX driven in reverse mode (Rebolledo, Speroni et al. 2006).

$E_m$ can influence exchange processes such as NCX via the electrochemical driving forces on $Na^+$ and $Ca^{2+}$. On the other hand NCX itself contributes to the cardiac action potential (Weidmann 1993; Benardeau, Hatem et al. 1996; Janvier and Boyett 1996; Janvier, Harrison et al. 1997), although little work has been done in smooth muscle. The rheogenic properties of the exchanger are postulated to favor $Ca^{2+}$ entry, thus NCX is likely to operate in reverse mode at potentials usually seen in arteriolar VSM cells (Blaustein and Lederer 1999). Therefore we examined relationships between $E_m$ and probable involvement of NCX in regulating myogenic tone in cannulated skeletal muscle arterioles utilizing KB-R7943 and nifedipine. Findings from this group of data supported our hypothesis that NCX may be involved in regulation of myogenic tone, as KB-R7943 caused a vasodilatation of cannulated arterioles that was associated with significant membrane hyperpolarization. It is unlikely that these data can be simply explained by a non-specific effect on VGCCs as while nifedipine could cause vasodilatation but was not associated with hyperpolarization of cannulated arterioles.

To further observe the role of NCX in acute myogenic reactivity of an arteriole, NCX was driven in reverse mode by decreasing the extracellular $[Na^+]$ levels and a pressure step performed at each $Na^+$ concentration. In response to the pressure step, internal diameter was recorded and compared. In the present study, we demonstrated that in response to step increases in intra luminal pressure (from 50 to 120 mmHg), the diameter of isolated skeletal muscle arterioles was significantly reduced by reducing extracellular $[Na^+]$, whereas in control experiments (without a change in extracellular $[Na^+]$) diameters were
not significantly different between the groups. These findings indicate that myogenic reactivity is greater in 137 mM extracellular [Na⁺].

Finally, myogenic indexes were calculated to assess the effect of changing extracellular [Na⁺] on pressure-sensitive behavior of skeletal muscle arterioles. There was a significant decrease in myogenic index with a decrease in the extracellular [Na⁺]. The endothelium played no role as there was no difference in the myogenic reactivity of endothelium-intact and endothelium-denuded vessels. Thus these data are consistent with a role for the NCX in pressure-sensitive acute myogenic constriction.

Collectively the functional data obtained in this section of our studies suggests that NCX plays an important role in regulating myogenic tone in rat cremaster arterioles, possibly via the reverse mode of NCX. Therefore a better understanding of the exact role of NCX is required to both understand its role in local microcirculatory control and ultimately for the development of novel therapeutic approaches for treatment of vascular diseases including arterial hypertension. The following sections of this thesis, therefore, used cellular and molecular approaches to better understand the nature of the NCX in arterioles.
CHAPTER 4

IDENTIFICATION OF THE SODIUM CALCIUM EXCHANGER IN THE ARTERIOLAR WALL USING CELLULAR APPROACHES
INTRODUCTION

The interdependence of Na\(^+\) and Ca\(^{2+}\) was known prior to 1968 and it was reported that heart contractility was dependent on the ratio between Na\(^+\) and Ca\(^{2+}\) (Luttgau and Niedergerke 1958). The Na\(^+\)/Ca\(^{2+}\) exchanger was first identified in squid giant axon and heart muscle (Reuter and Seitz 1968; Baker, Blaustein et al. 1969). NCX was subsequently cloned and sequenced in 1990 (Nicoll, Longoni et al. 1990), this milestone study allowing examination of NCX using sophisticated molecular biological approaches. This has further led to the discovery of several NCX isoforms, in different tissues and species (Aceto, Condrescu et al. 1992; Kofuji, Hadley et al. 1992; Komuro, Wenninger et al. 1992; Hryshko, Matsuoka et al. 1996).

There are two families of plasma membrane exchanger proteins in mammalian tissues (Blaustein and Lederer 1999; Lytton, Li et al. 2002). One with a stoichiometry of 3 Na\(^+\) : 1 Ca\(^{2+}\) (Barcenas-Ruiz, Beuckelmann et al. 1987; Kimura, Miyamae et al. 1987; Lauger 1987; Crespo, Grantham et al. 1990) is present in mostly all tissues. This uses the electrochemical Na\(^+\) gradient and thus causes uphill transport of Ca\(^{2+}\). The second type has a stoichiometry of 4 Na\(^+\) : 1 Ca\(^{2+}\), 1 K\(^+\) and is present in photoreceptor cells and neural tissue, termed NCKXs (Reilander, Achilles et al. 1992; Tsoi, Rhee et al. 1998; Poon, Leach et al. 2000). Both NCX and NCKX can operate in a forward or reverse mode, depending on the Na\(^+\), Ca\(^{2+}\) (K\(^+\)) gradients and also on the \(E_m\) (Blaustein and Lederer 1999). Although there is a very little structural similarity between the two families but they both serve as a Ca\(^{2+}\) transporters (Hryshko 2002). For a constant extracellular [Ca\(^{2+}\)] (1.8–2 mM) and [Na\(^+\)] (140 mM), [Ca\(^{2+}\)]\(_{cyt}\) is a cubic function of intracellular [Na\(^+\)]
Chapter 4
Identification of NCX in arteriolar wall

and an exponential function of $E_m$. Therefore, a small change in intracellular $[Na^+]$ or $E_m$ can cause a large change in $[Ca^{2+}]_{cyt}$ (Zhang, Yuan et al. 2005).

Evidence of NCX in smooth muscle is available in various smooth muscle tissues e.g. intestinal (Mandel and Murphy 1984; Raeymaekers and Casteels 1984; Scheid and Fay 1984), urinary tract (Aickin, Brading et al. 1984), uterine (Matsuzawa, Masahashi et al. 1987; Tomita 1992) and tracheal/bronchial (Tsuchiya, Hosokawa et al. 1990; Tsuchiya, Hosokawa et al. 1990a). On the basis of $^{45}Ca^{2+}$ fluxes and contraction experiments NCX was identified by Reuter et al. in VSM cells (Reuter, Blaustein et al. 1973). It is thus known that NCX is present in the plasma membrane of VSM apart from being present in other organelles, including mitochondria (Crompton and Roos 1985; Carafoli 1987; Gunter and Pfeiffer 1990; Lee, Miles et al. 2003) and secretory vesicles (Saermark and Gratzi 1986; Jan and Schneider 1992a). Na$^+$/Ca$^{2+}$ exchange can play an important role in modulating the cytosolic free concentration of Ca$^{2+}$ as well as the intracellular stores of Ca$^{2+}$ (Bova, Goldman et al. 1990; Missiaen, De Smedt et al. 1992; Blaustein 1993; Nakasaki, Iwamoto et al. 1993; Borin, Tribe et al. 1994). It is thus been suggested that NCX may play a role in the myogenic response of arterioles (Mulvany 1985). Nevertheless the exact physiological role of NCX in the VSM cells remains controversial.

Cloning of cDNA for NCX1 from canine cardiac tissue by screening an expression cDNA library with polyclonal antibodies (Nicoll, Longoni et al. 1990), was soon followed by cloning of cDNA for NCX2 (Li, Matsuoka et al. 1994) and NCX3 (Nicoll, Quednau et al. 1996a). NCX1 has been isolated in almost every tissue including rabbit kidney, rat brain, rat aortic smooth muscle, human, bovine and dog hearts (Nicoll, Longoni et al. 1990; Aceto, Condrescu et al. 1992; Komuro, Wenninger et al. 1992; Furman, Cook et al. 1994).
Chapter 4  Identification of NCX in arteriolar wall

1993; Kofuji, Lederer et al. 1993; Low, Kasir et al. 1993; Nakasaki, Iwamoto et al. 1993; Kofuji, Lederer et al. 1994). The human NCX1 gene was mapped to human chromosome 2 (Shieh, Xia et al. 1992) and mouse chromosome 17 (Shi, Chang et al. 1998). Out of the two transcripts identified for NCX1, the 7 kilobase (kb) transcript is expressed in many tissues such as heart, brain, kidney, lung, smooth muscle and skeletal muscle (Nicoll, Longoni et al. 1990; Kofuji, Hadley et al. 1992; Komuro, Wenninger et al. 1992; Juhaszova, Ambesi et al. 1994), whereas the 14 kb transcript has only been detected in brain tissue (Kofuji, Hadley et al. 1992; Marlier, Zheng et al. 1993).

NCX2 and NCX3 were both found to be expressed in the brain and skeletal muscle (Li, Matsuoka et al. 1994; Nicoll, Quednau et al. 1996a). NCX2 has been cloned from rat brain; the gene is mapped to mouse chromosome 7 and human chromosome 14. NCX1 and NCX2 are 61 and 65% identical at the nucleotide and amino acid levels, respectively. NCX2 transcripts are present in brain and skeletal muscle and are 5 kb in size (Li, Matsuoka et al. 1994). Recently it was shown that NCX2 was the predominant NCX isoform expressed in fundus of the stomach (Sakai, Kinoshita et al. 2005). The third isoform, NCX3 has been cloned from rat brain; the gene is mapped to human chromosome 14 and mouse chromosome 12. NCX3 shares 73% identity with NCX1 and 75% identity with NCX2 at the amino acid level and NCX3 transcripts are 6 kb in size (Nicoll, Quednau et al. 1996a). The three isoforms are products of separate genes (Nicoll, Quednau et al. 1996a).

It is already known that NCX is involved in regulation of Ca²⁺ homeostasis of blood vessels (Juhaszova, Ambesi et al. 1994; Slodzinski, Juhaszova et al. 1995; Blaustein...
and Lederer 1999; Arnon, Hamlyn et al. 2000a). This functional evidence of the presence of NCX is also supported by the demonstration of NCX in VSM cells (Juhaszova, Ambesi et al. 1994). However in all these studies, cultured arterial myocytes were used and not an intact vessel. Thus in the present study we attempted to obtain information about the NCX in vascular tissue at the cellular and molecular tissue by utilizing rat cremaster muscle arterioles. To accomplish this we used antibodies raised against canine cardiac exchanger. These antibodies cross-react with rat brain, heart and membrane protein from Xenopus neurons (Luther, Yip et al. 1992) suggesting that cardiac NCX is highly conserved in different tissues. In addition real-time PCR was used to determine the relative abundance of NCX isoforms (NCX 1, 2 and 3) in the cremaster muscle arterioles.
MATERIALS AND METHODS

Vessel segments were dissected as previously described in Chapter 2 (General Methods) and 2-3 long (~ 10 mm) segments were pooled and stored at -80º C.

Western blotting
Vessel segments were lysed in ice-cold homogenizing buffer (100 µl) consisting of Tris-HCl, 25 mM (pH 6.8); SDS, 1% (w/v); EGTA, 5 mM; NaF, 50 mM; sodium vanadate, 1 mM; glycerol, 10% (v/v) and protease inhibitor cocktail (Sigma). The homogenate was then vortexed, sonicated and finally clarified by centrifuging at 7000 g (6 minutes). The supernatant was aspirated and protein content of all fractions was determined by Lowry’s method. Samples were then diluted with sample buffer (dilution, 1:6) consisting of Tris-HCl, 250 mM (pH 6.8); SDS, 2% (w/v); glycerol, 10% (v/v); DTT, 10 mM; (3-mercaptopo-ethanol, 2% (v/v) and bromophenol blue, 0.01% (w/v) in preparation for electrophoretic separation. The samples were heated at 56º C for 3 minutes and put on ice to cool before loading on electrophoretic gels.

The gels were cast and then mounted using a BioRad Mini PROTEAN® II cell and the tank chamber was filled with running buffer (Tris base, 0.025 M; glycine, 0.025 M and SDS, 0.01% w/v; pH 8.3). 25 µg of protein was loaded in each lane and resolved by 8% SDS-PAGE along with molecular weight markers (5 µL; BioRad; Cat No. 161-0363) and transferred using a semi-dry blotting system (BioRad, Sydney). Separations were performed at 120 V DC for 15 minutes then 150 V DC for 45 minutes. The gels were removed from the electrophoretic tank and placed on the Hybond nitrocellulose
membranes (Amersham Chemical Company, Amersham, UK, 0.45 microns pore size) cut to size. Transfer of protein from gel to the membrane(s) was completed using a semi-dry transfer cell (BioRad TRANS-BLOT® SD) at 20 V DC for 75 minutes (transfer current was less than 350 mA).

Following transfer, the membranes were rinsed in fresh Tris-buffered saline with Tween® 20, 0.1% (v/v) (TBST). Protein bands were visualized after soaking the membrane(s) in Ponceau S dye, 1% w/v (1-2 minutes). Membranes were then washed in TBST (3×10 minutes, on a rocker) to remove the Ponceau S dye. To reduce non-specific binding the membrane was blocked with 3% bovine serum albumin (BSA), overnight at 4° C in preparation for exposure to antibodies.

The following day membrane(s) were probed with primary antibody (anti NCX IgG, R3F1, 1:1,000; Swant) for 1 hour at room temperature, followed by a wash in TBST (5×10 minutes). The monoclonal antibody against the canine NCX recognizes two neighboring but non-overlapping sequences of the hydrophilic region of the exchanger, connecting the putative transmembrane segments 5 and 6 (Porzig, Li et al. 1993). Membrane(s) were then incubated with antimouse IgG-HRP conjugated secondary antibody (1:2,000; New England BioLab) and precision protein StrepTactin-HRP conjugate (1:50,000, Biorad; Cat. No. 161-0380) for 1 hour at room temperature. Both the primary and the secondary antibodies were prepared in 1% bovine serum albumin (BSA) in TBST. Membrane(s) were then washed in TBST (5×10 minutes) to remove the excess secondary antibody. Chemiluminescent (ECL: Perkin Elmer, Boston, MA, USA) substrate was added and protein bands imaged and quantified on a Chemidoc EQ.
system with Quantity One software (BioRad Laboratories, Hercules, CA, USA). An internal standard of rat aorta homogenate was included in each electrophoresis.

The membrane(s) were washed of the excess ECL solutions with TBST for 10 minutes before the membrane was used again for probing with α actin antibody. To remove the NCX antibody the membrane(s) were placed in a stripping buffer (Restore™ Western Blot Stripping Buffer, Cat. No. 21059) and incubated for 10 minutes at room temperature on a slow moving rocker. After 10 minutes the membrane(s) were removed from stripping buffer and washed in TBST (2×10 minutes). We tested for the complete removal of the HRP label (i.e. secondary antibody) by incubating the membrane with ECL substrate, which was shown by a complete absence of signal after 5 minutes of exposure. Further, to check for the complete removal of the primary antibody membrane(s) were incubated with secondary antibody, followed by the above-mentioned steps. A complete absence of signal after 5 minutes exposure to ECL substrate was considered as removal of primary antibody from the membranes.

After the membrane(s) were properly stripped, the second immunostaining was performed with actin (anti Actin IgG, 1:2,000; Dako cytometry), overnight at 4° C. Next morning membrane(s) were washed in TBST (5×10 minutes) followed by incubation with antimouse IgG-HRP conjugated secondary antibody (1:4,000; New England BioLab) and precision protein StrepTactin-HRP conjugate for 1 hour at room temperature. Both the primary and the secondary antibodies were prepared in 1% bovine serum albumin (BSA). Membrane(s) were then washed in TBST (5×10 minutes) to remove the excess secondary antibody. Chemiluminescent (ECL: Perkin Elmer, Boston, MA, USA) substrate was added and protein bands imaged and quantified as described earlier in
this section. The NCX signal was expressed as an intensity ratio relative to the actin band to account for variations a sample loading.

**Reverse-transcriptase polymerase chain reaction (PCR)**

*mRNA preparation and cDNA synthesis:*

Rat cremaster arterioles were dissected as described previously in Chapter 2 (General Methods). Tissues including liver, heart, aorta and skeletal muscle were also collected for extraction of total RNA using the modified acid Guanidinium thiocyanate-phenol-chloroform extraction of Chomczynski and Sacchi (Chomczynski and Sacchi 1987). For the arterioles total RNA was extracted by using RNeasy micro kit (Qiagen) as per the manufacturer’s instructions. The DNase I stock solution provided with the kit was used to remove DNA from the samples. RNA content and purity was established by measuring absorbance at 260 and 280 nm. 1 µg of each RNA sample was reverse transcribed using Taqman Reverse Transcription reagents (Applied Biosystems, Foster City, CA, USA) at a final concentration of 10 ng/µl. Blanks and reverse transcriptase negative samples were also analyzed where all the above reagents are added to the RNA samples except for cDNA or multiscribe reverse transcriptase respectively. The reverse transcription reactions were performed using a GeneAmp PCR system 9600 (Applied Biosystems) with conditions at 30° C for 10 minutes 42° C for 60 minutes and 95° C for 5 minutes. 2 µl of 0.5 M EDTA (pH 8.0) was added to each sample and stored at -20° C until further analysis.

**Real-time RT-PCR:**

To determine the level of expression of various genes, Polymerase chain reaction (PCR) was performed on the iCycler iQ® Real-Time PCR (Bio-Rad) for amplification, data
collection and analysis. Real-time PCR allows for the characterization of mRNA by the cycle number at which PCR product accumulation is first detected by fluorescence. This method is preferred to conventional PCR; which determines the amount of PCR product accumulated after a fixed number of cycles. The elimination of post-PCR procedures reduces the risk of contamination and other errors, thus leading to reproducible and quantitative results. To check for purity of the product formed, melt-curve data collection and analysis was also performed at the completion of each experiment. Real-time reactions were performed in 25 µl volumes that contained master mix (×1), forward or reverse primers (900 nM), sample cDNA (1 µl), and Sybr Green (Sigma, SYBR® Green JumpStart™ Taq ReadyMix™) and made to volume with H₂O (0.5% DEPC treated) in a 96-well, thin wall PCR plate (Applied Biosystems) that was sealed with optical quality tube caps. SYBR Green I, a commonly used fluorescent DNA binding dye, binds all double stranded DNA and detection is monitored by measuring the increase in fluorescence throughout the cycle. SYBR Green I has an excitation and emission maxima of 494 nm and 521 nm, respectively.

Primers were designed using Primer Express software (Applied Biosystems) to appropriate specifications. 18S mRNA (Applied Biosystems) was used as a constitutively expressed “house-keeping” gene. Thermal cycling conditions were 2 minutes at 50° C and 10 minutes at 95° C, followed by 50 cycles of denaturing (30 seconds at 95° C), annealing (30 seconds at 60° C), and extending (60 seconds at 72° C) (Seiler, Stypmann et al. 2004).

The forward and the reverse primers primers for NCX are listed in Table 4-1. PCR for NCX1 was performed using primers designed to mammalian NCX1 gene. These primers
would yield a 96 base pair product corresponding to nucleotides 793-888 of the published NCX1 gene (Nicoll, Longoni et al. 1990; Li, Matsuoka et al. 1994). PCR for NCX2 was performed using primers designed to mammalian NCX2 gene. These primers would yield a 100 base pair product corresponding to nucleotides 1714-1813 of the published NCX2 gene (Li, Matsuoka et al. 1994). Primers for NCX3 would yield a product of 83 base pairs, corresponding to nucleotides 3554-3636 of the published NCX3 gene.

A melting curve analysis was added at the end of final PCR run to evaluate the presence of non-specific PCR product and formation of primer dimers. The Threshold cycle (Ct) is the PCR cycle at which an increase in SYBR Green fluorescence is first detected above a baseline signal. Relative differences in mRNA were standardized by normalizing the Ct values of sample gene to the internal standard (18S) called Δ Ct. Results were calculated and reported as fold changes, using the comparative 2^ΔΔCt method (Livak and Schmittgen 2001).
### Table 4-2. Primer sequences

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Gen Bank</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCX1</td>
<td>NM_019268</td>
<td>Forward primer: 5'</td>
<td>AGCAAGGCVGGCTTCTTTT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse primer: 5'</td>
<td>GCTGGTCTGTCTCCTTCATGT</td>
</tr>
<tr>
<td>NCX2</td>
<td>NM_078619</td>
<td>Forward primer: 5'</td>
<td>CACTACGAGGATGCTTGTGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse primer: 5'</td>
<td>CTTCTTCTCATACTCTTCTCGT</td>
</tr>
<tr>
<td>NCX3</td>
<td>NM_078260</td>
<td>Forward primer: 5'</td>
<td>CCTGTGGCTCCTCTACGTACTCTT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse primer: 5'</td>
<td>GAGGTCTTGTTCGTTGTTCA</td>
</tr>
</tbody>
</table>
RESULTS

Identification of the NCX protein in rat cremaster arterioles by Western blotting.

There is evidence that NCX in arterial smooth muscle is similar to cardiac and neuronal NCX (Juhaszova, Ambesi et al. 1994), thus we used antibodies raised against purified canine cardiac NCX to determine the presence of NCX in rat cremaster arterioles and developed by using horseradish peroxides-conjugated mouse anti-rabbit IgG followed by enhanced chemiluminescence. **Fig 4-1a** shows immunoblots of aorta (lane 1) and rat cremaster arterioles (lanes 2 and 3). Anti NCX monoclonal (R3F1) antibody was used which specifically reacted with protein bands of 120 (molecular mass of mature glycosylated NCX) and 70 kDa (believed to be an N terminal proteolytic fragment) (Nicoll, Longoni et al. 1990; Juhaszova, Ambesi et al. 1994). Due to the small size of arterioles, and comparatively low protein yields, approximately “2-4” vessel segments were pooled to ensure a result. This made it nearly impossible to denude the arterioles of endothelium. As endothelium also expresses the exchanger therefore it is possible that the results may also have a component due to the exchanger from endothelium. The bottom panel shows the actin band at 42 kDa.

**Fig 4-1b** shows the Western blots analysis of NCX1 in rat arterial smooth muscle (n=6) and rat aorta (n = 12). The protein was probed with NCX1-specific antibody (R3F1) and the secondary antibody conjugated with HRP. The results are expressed as a ratio of the actin band to account for variations in sample loading. As seen from the blot there is increased density of the aortic band as compared to the vessel, although the amount of protein loaded is similar in both the cases.
Detection of NCX isoforms by real-time PCR.

In order to determine which isoforms of NCX are present in rat cremaster arterioles, gene specific primers were designed to NCX isoforms 1, 2 and 3. RNA from liver, heart, aorta, rat cremaster arteriole and skeletal muscle was isolated. Since SYBR Green can bind in a sequence independent way to all double-stranded DNA, it is possible that other products are formed. There was no non-specific amplification seen. Purity of the product generated by gene-specific primers was seen by melting curve analysis and 2% agarose gel electrophoresis to confirm successful amplification and crude variability in length of the final product so that only one gene specific product is formed.

Real-time PCR demonstrated the expression of three different isoforms of NCX. In VSM the most abundant isoform of NCX is NCX1 and the least abundant is NCX3 (Fig 4-2a, b and c). NCX1 was also found in abundance in other tissues like aorta and heart, and in lesser quantities in skeletal muscle and liver. NCX2 and NCX3 were present mainly in skeletal muscle.

Fig 4-3 shows an amplification graph after real-time PCR amplification. Fig 4-4 and Fig 4-5 show the melting curve analysis which was done at the end of real-time PCR amplification. The melting temperature of a DNA double helix depends on its base composition. The change in fluorescence is measured after temperature is raised. Hence, determined by the melting point of the product at the end of the amplification reactions, it is ensured that the desired product is detected which makes it an important means of quality control. All PCR products for a particular primer pair should have the same melting temperature. For SYBR Green based PCR, it is important to run a melting curve following the real-time PCR as SYBR green does not distinguish between one
DNA and another. SYBR Green will detect any double stranded DNA including primer dimers (the primers can sometimes anneal to themselves and create small templates for PCR amplification) contaminating DNA and PCR product from misannealed primers. At the melting point, the two strands of DNA will separate and the fluorescence rapidly decreases which is plotted as the rate of change of the relative fluorescence units (RFU) with time (T) (-d(RFU)/dT) on the Y-axis versus the temperature on the X-axis, and this will peak at the Tm.
Chapter 4                                    Identification of NCX in arteriolar wall

Fig 4-1a.

Fig 4-1b.
Fig 4-7. Detection of NCX protein in rat cremaster arterioles by immunoblotting.

25 µg/lane was loaded in each lane; lanes 2 and 3 were loaded with protein from cremaster arteriole and lane 1 with protein from aorta (Fig 4-1a).

The protein from cremaster arterioles and aorta was electrophorised on 8% SDS-PAGE and blotted to a nitrocellulose membrane. Immunoblots were probed with anti canine cardiac NCX monoclonal antibodies (R3F1) (top panel) or actin monoclonal antibody (bottom panel) and were developed using horseradish peroxidase-conjugated mouse anti-rabbit IgG and enhanced chemiluminescence (ECL). NCX was detected in a strong 120 kDa band at is consistent with the previously described NCX1 form of the exchanger. There is another band at 70 kDa; 120 kDa represents the intact NCX1 and 70 kDa an N terminal fragment. Protein sizes are expressed in kDa are marked on left.

Western blots analysis of NCX1 in rat arterial smooth muscle (n = 6) and rat aorta (n = 12). The results are expressed as a ratio of the actin band to account for variations in sample loading (Fig 4-1b).
Fig 4-2a.
Fig 4-2b.
Chapter 4 Identification of NCX in arteriolar wall

Fig 4-2c.

NCX fold change relative to 18S

Liver Heart Aorta Arteriole Sk muscle
Fig 4-8. Real-time RT PCR showing detection of NCX1 (Fig 4-2a), 2 (Fig 4-2b) and 3 (Fig 4-2c).

RNA from heart, aorta, rat cremaster arteriole and skeletal muscle was isolated.

After RNA purity was established 1 µg of each RNA sample was reverse transcribed. Real-time Reactions were performed in 25 µl volumes that containing forward or reverse primer, 1 µl sample cDNA, and Sybr Green. Relative differences in mRNA were standardized by normalizing the Ct values of sample gene to the internal standard (18S) and the results were calculated and reported as fold changes.
**Fig 4-9. NCX amplification graph of actual data using Sybr Green.**

This graph displays the increase in fluorescence (i.e. increase in cDNA) as cycle number increases. Cycle number is shown along the X-axis and arbitrary fluorescence units (fold increase over background fluorescence) are shown on the Y-axis. These experiments were performed with SYBR Green, which has very low fluorescence in the absence of double stranded DNA and very high fluorescence in the presence of double stranded DNA.
Temperature, Celsius

**Fig 4-10. Melting curve analysis for NCX to determine the specificity of amplified PCR product.**

This graph displays the fluorescence data showing the typical decrease in fluorescence with temperature increase. Y-axis is the background subtracted relative fluorescence units (RFU) and on the X-axis is temperature. After the final amplification step, samples were gradually heated to reach the maximum temperature of 95° C. The degree of denaturation was measured automatically by the iCycler.
Fig 4-11. Derivative melt curve for NCX to determine the specificity of amplified PCR product.

This graph provides data normalized from Fig 4-4. On the Y-axis, the rate of change of the relative fluorescence units (RFU) with time (T) \((-\frac{d(RFU)}{dT})\) is displayed against temperature on the X-axis, showing the melting peak at the melting temperature (Tm). In all experiments, samples were run with the same primer pairs. There are no signs of primer dimer artifacts in the melt curve shown in Fig 4-5.
Fig 4-12. Autoradiogram of NCX cDNA products of PCR.

A portion of the PCR mixture (5 µl) was added to loading dye mix and electrophorosed on 2% (wt/vol) agarose gel electrophoresis and stained in ethidium bromide. The PCR products were visualized by UV illumination on an ethidium bromide-stained agarose gel to confirm successful amplification and crude variability in length of the final product. The lanes contain: lane 1 contains skeletal muscle; lane 2 liver, lane 3 aorta; lane 4 cremaster arteriole; lane 5 heart and lane M denotes DNA size marker.
DISCUSSION

There is evidence now that NCX is present in isolated VSM cells (Bova, Goldman et al. 1990; Slodzinski, Juhaszova et al. 1995; Slodzinski and Blaustein 1998). Western blotting was utilized to determine the presence of NCX in intact arterioles from rat cremaster muscle. Further, real-time PCR was employed to systemically analyze the tissue specific expression and the relative abundance of the NCX isoform in VSM of cremaster arteriole and in various other tissues.

We have used a monoclonal antibody against the canine cardiac form of NCX. This antibody cross-reacted with the protein homogenized from cremaster arterioles as shown by the Western blots indicating inter-tissue homology is present among NCX molecule. The presence of two bands was consistent with literature; a strong 70 kDa band which is attributed as a proteolytic fragment was found in addition to the 120 kDa of the integral exchanger (Philipson, Longoni et al. 1988). These are comparable to the bands from cardiac sarcolemma (Nicoll, Longoni et al. 1990; Durkin, Ahrens et al. 1991), human pulmonary smooth muscle cells (Sakai, Kinoshita et al. 2005; Zhang, Yuan et al. 2005) and smooth muscle of guinea pig stomach (Sakai, Kinoshita et al. 2005). The presence of NCX in vessels was not unexpected, as there are reports indicating the presence of NCX in arterial myocytes by functional studies and by immunoblotting and immunofluorescence (Juhaszova, Ambesi et al. 1994; Slodzinski, Juhaszova et al. 1995; Slodzinski and Blaustein 1998). But there is little data available identifying the presence of NCX in arterioles and also identifying the type of isoform present.
One of the important issues during this project was the difficulty in obtaining bands of comparable density from arterioles and aorta. A greater amount of tissue from the arterioles was required to give an equally strong signal, which was difficult due to the smaller size of the vessels. Therefore a weaker signal was typically obtained with cremaster vessels as compared to aorta although equal amounts of proteins were loaded in each lane. It could be argued that it can be due to the fact that we have employed an antibody directed against cardiac NCX. Therefore it may be possible that the vascular wall NCX does not share as great a sequence homology with the cardiac NCX, as compared to aorta, which may lead to the weaker signal obtained from arterioles. However, it can also be due to the fact that NCX is less abundant in the VSM cells of microvessels as compared to larger arteries such as aorta. There is evidence in the literature supporting such heterogeneity. There are reports that the velocity of NCX mediated transport ($V_{\text{max}}$) is greater in cardiac sarcolemmal vesicles (7.5 nM/mg protein/second) (Cheon and Reeves 1988) and rat brain pre-synaptic nerve terminals/synaptosomes (0.11 nM/mg protein/second) (Sanchez-Armass and Blaustein 1987) as compared to VSM sarcolemmal vesicles (0.071 nM/mg protein/second) (Matlib, Kihara et al. 1988).

There are three mammalian isoforms cloned to date. NCX1, NCX2 and NCX3 (Nicoll, Longoni et al. 1990; Furman, Cook et al. 1993; Li, Matsuoka et al. 1994; Nicoll, Quednau et al. 1996a). The primers designed in this study were isoform specific and the primers were chosen from the 5’ end. In the present study we have demonstrated that all the three isoforms of NCX are present in rat cremaster arterioles, with NCX1 being the abundant isoform of the three, and NCX3 being the least abundant. NCX1 was also found in abundance in other tissues like aorta and heart, and in lesser quantities in
skeletal muscle and liver. We also confirmed that NCX2 and NCX3 are abundantly found in the skeletal muscle of rat, consistent with the northern blot analysis showing that the transcripts of both isoforms are restricted to brain and skeletal muscle (Li, Matsuoka et al. 1994; Nicoll, Quednau et al. 1996a).

Our data demonstrate that the NCX is present in the wall of arterioles and presumably in the VSM. The most abundant form present appears to be similar to the cardiac form of NCX and is likely NCX1. The studies do however suggest vascular heterogeneity as there is lower density of NCX in cremaster arterioles (relative to actin expression) as compared to aorta. Cremaster vessels also appear to express lesser amounts of NCX2 and 3 as demonstrated by real-time PCR. The data support the studies described in the previous chapter, collectively suggesting a role for the NCX in arteriolar function.
CHAPTER 5

EFFECT OF ANTISENSE OLIGONUCLEOTIDES
DIRECTED AT THE SODIUM CALCIUM EXCHANGER
ON MYOGENIC REACTIVITY
INTRODUCTION

In VSM, depolarization-induced Ca$^{2+}$ entry via VGCC causes an increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ which is necessary for vasoconstriction (Somlyo and Somlyo 1994). Ca$^{2+}$ extrusion is by PMCA pump (Missiaen, De Smedt et al. 1992) and NCX (Bova, Goldman et al. 1990; Somlyo and Somlyo 1994). The plasma membrane of arterial smooth muscle cells contains NCX (Juhaszova, Ambesi et al. 1994) that can modulate the $[\text{Ca}^{2+}]_{\text{cyt}}$ and the intracellular Ca$^{2+}$ stores (Bova, Goldman et al. 1990; Borin, Tribe et al. 1994). As stated earlier the precise physiological role of NCX in VSM remains controversial with some investigators suggesting that NCX in VSM is latent in the unstimulated cell at basal intracellular Na$^+$ and Ca$^{2+}$ concentrations (Smith, Zheng et al. 1989a). In contrast some investigators propose that the NCX plays a major role in sarcoplasmic reticulum Ca$^{2+}$ homeostasis and thus vascular reactivity (Reuter, Blaustein et al. 1973; Bova, Goldman et al. 1990; Blaustein 1993).

To measure the effect of a particular ion transporter, the physiology of the cell or tissue is often investigated under normal conditions and compared to the response after selectively inhibiting the transporter. Cellular functions and the steady state kinetics of NCX have been characterized to an extent but relatively little is known of its molecular functions. These questions remain because there is a relative lack of specific and sensitive inhibitors of NCX, and removal of extracellular Na$^+$ also affects the intracellular [Na$^+$] and other Na$^+$ dependent-exchangers like Na$^+$/H$^+$ exchanger (Smith, Lyu et al. 1991) or VGCC (Kaczorowski, Slaughter et al. 1989) as explained earlier in Chapter 3. Thus, amiloride, and its analogues (for e.g. 3, 4-dichlorobenzamil), and the isothiourea
derivative KB-R7943 are not sufficiently selective inhibitors of NCX and also block other ion channels (Garcia, King et al. 1990; Smith, Lyu et al. 1991; Watano, Kimura et al. 1996; Iwamoto and Kita 2004b). As an alternate approach the exchanger inhibitory peptide (XIP) (Li, Nicoll et al. 1991) was developed to target CAM binding site domains of the NCX. However, the selectivity of this approach is limited as XIP would be expected to affect other CAM-dependent transport systems such as the PMCA (Enyedi and Penniston 1993). Thus a specific inhibitor of NCX would be of immense importance in understanding NCX function in VSM under physiological conditions.

In the absence of specific pharmacological inhibitors, an alternate approach to inhibiting the function of NCX is to interfere with the expression of the exchanger protein. To achieve this goal antisense ODNs have been utilized against the NCX mRNA by several groups (Van Eylen, Gourlet et al. 1994; Lipp, Schwaller et al. 1995; Slodzinski, Juhaszova et al. 1995; Takahashi, Bland et al. 1995; Bland, Takahashi et al. 1996; Niggli, Schwaller et al. 1996; Slodzinski and Blaustein 1998; Slodzinski and Blaustein 1998a; Eigel and Hadley 2001). Although antisense ODNs have mainly been used in cardiac myocytes (Lipp, Schwaller et al. 1995; Takahashi, Bland et al. 1995; Bland, Takahashi et al. 1996; Niggli, Schwaller et al. 1996; Slodzinski and Blaustein 1998a), this “knock down” approach has also been used in other cell types including arterial myocytes (Slodzinski and Blaustein 1998), rat pancreatic beta-cells (Van Eylen, Gourlet et al. 1994; Van Eylen, Lebeau et al. 1998), mouse distal convoluted tubule cells (White, Gesek et al. 1998) cerebellar Purkinje neurons (Kim, Park et al. 2005) and primary neurons from rat brain (Ranciat-McComb, Bland et al. 2000).
Takahashi and colleagues reported that Ca\(^{2+}\) influx from Na\(^+\) free medium was reduced by 20-30\% when they introduced a 20-mer antisense ODNs (10 µl) to the region near the un-translated 3' end of the NCX1 exchanger mRNA (Takahashi, Bland et al. 1995; Bland, Takahashi et al. 1996). Lipp et al. reported an 80\% reduction in exchanger function (reduction of \([\text{Ca}^{2+}]_{\text{cyt}}\)) in cardiac myocytes after 24 hours treatment with 3 µM of antisense ODNs (19-mer) (Lipp, Schwaller et al. 1995).

In contrast Slodzinski and colleagues used lower concentration (\(5\times10^{-7}\)M) and a tandem pair of short (15 and 17-mer) antisense ODNs to the region around the start codon of the NCX1 gene mRNA in cultured arterial smooth muscle cells (Slodzinski, Juhaszova et al. 1995; Slodzinski and Blaustein 1998) and cardiac myocytes (Slodzinski and Blaustein 1998a). The NCX was reversibly knocked down as determined by the effect of extracellular Na\(^+\) removal on \([\text{Ca}^{2+}]_{\text{cyt}}\). There was an increase in the \([\text{Ca}^{2+}]_{\text{cyt}}\) when control cells were exposed to [Na\(^+\)] free media; in contrast \([\text{Ca}^{2+}]_{\text{cyt}}\) did not rise in many antisense-ODN treated cells. In rat cardiac myocytes, NCX expression (based on Western blotting) and function was knocked down by 60\% after 4 hours of antisense ODN treatment.

Using an antisense strategy Matsuda and colleagues studied the role of the NCX in reperfusion injury in cultured rat astrocytes. There was an increase in the \([\text{Ca}^{2+}]_{\text{cyt}}\) immediately after reperfusion with \(\text{Ca}^{2+}\)-containing medium when astrocytes were perfused in \(\text{Ca}^{2+}\)-free medium for 15-60 minutes. The increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) was enhanced by low extracellular [Na\(^+\)] during reperfusion and blocked by inhibitors of the NCX, but not by Ca\(^{2+}\) channel antagonists. When antisense ODN treatment was compared with
the sense ODN, it was shown that NCX protein level and exchange activity was decreased with treatment of astrocytes with antisense (Matsuda, Takuma et al. 1996a).

A newer approach has been utilized to down regulate the expression of NCX and to demonstrate the effects of NCX depletion. RNA interference (RNAi) is a process in which double-stranded RNA triggers the degradation of a homologous messenger RNA (sharing sequence-specific homology to particular "target" mRNAs). This was first observed in *Caenorhabditis elegans* by Fire et al. (Fire, Xu et al. 1998). RNAi was utilized in conjunction with adenoviral transfection to down regulate NCX expression in postnatal rat myocytes. NCX expression was inhibited by ~ 94%, but spontaneous beating of the cardiac muscle cells was still maintained (Hurtado, Ander et al. 2005). It was observed to be more efficient than ODNs mediated down-regulation and thus is a powerful tool to study gene function. Recently it has been reported that cardio-specific NCX1 knock out was accomplished using Cre/loxP technology with viable cardiac functions and the mice surviving to adulthood (Henderson, Goldhaber et al. 2004).

The present study was designed to explore the possibility of using antisense ODN against NCX to inhibit NCX gene expression. Thus in the present chapter results are presented by directing antisense ODNs against NCX mRNA of rat isolated cremaster muscle arterioles. This approach was taken to alter expression/function in the vessel without the possible complications of whole animal effects. Antisense ODN may block translation and/or processing of certain mRNA by forming RNA-DNA duplexes. These duplexes have steric effects by blocking translation or translocation of the mRNA, or they may activate the degradation of the mRNA (Leonetti, Degols et al. 1993; Wagner 1994). The ODNs were introduced into functional arteriolar smooth muscle by the
method of reverse permeabilization (RP). It has been already shown that transfection
with polycationic lipids were limited due to the barrier provided by adventitia and also
that the conditions between isolated arterioles and cultured cells were different
(Richards, Davis et al. 2003). The relative levels of NCX expressed in antisense-versus
sense-treated arterioles were estimated by immunoblot analysis. This gave an additional
insight into the role of NCX in the development of myogenic response in a VSM and to
help evaluate the functional consequences of NCX loss in isolated rat cremaster
arterioles.
MATERIALS AND METHODS

First order arterioles were dissected, isolated, cannulated and monitored according to the protocol described in the Chapter 2 (General Methods) for studies.

Experimental protocols

As the approach taken in this chapter required considerable manipulation of the isolated arterioles (for example, permeabilization, ODN introduction, extended incubations and varying temperature conditions) there were a substantial number of control experiments to demonstrate maintained viability of the preparations.

Effects of time and temperature on myogenic responsiveness of isolated arterioles:

To see the effect of time and temperature on the viability of arterioles, three sets of experiments were performed. In the first set, arterioles were dissected and kept cannulated for 24 hours while continuously superfused with Krebs-bicarbonate buffer solution at 34º C. In the second and third groups vessels were kept at 34º C and at 4º C respectively, in Krebs-bicarbonate buffer solution for 24 hours. At the end of 24 hours arterioles from group two and three were cannulated, pressurized and superfused with Krebs-bicarbonate buffer solution as described in Chapter 2 (General Methods). After 24 hours the vessels were subjected to the following functional studies:

i. Pressure-diameter response curve
After recording the baseline diameter at 70 mmHg, a pressure-diameter response curve was obtained in each arteriole. Intra luminal pressure was reduced to 30 mmHg for 5 minutes, followed by sequential step increases in intra luminal pressure to 150 mmHg. Vessels were maintained at each pressure for 5 minutes, and the minimum intra luminal diameters were recorded. The intra luminal pressures of the vessels were returned to 70 mmHg at the end of the protocols. Diameters were represented relative to passive diameters obtained after 15 minute superfusion of the arterioles with Ca^{2+} free Krebs-bicarbonate buffer solution containing 2mM EGTA.

**ii. Pressure step**

After the arterioles were equilibrated at 70 mmHg they were subjected to a pressure step from 50 to 120 mmHg. Intra luminal pressure was reduced to 50 mmHg which was followed by a step increase in pressure to 120 mmHg. Vessels were maintained at each pressure for 5 minutes, and the minimum intra luminal diameters were recorded. The intra luminal pressures of the vessels were returned to 70 mmHg at the end of the protocol and the diameters reported relative to passive diameter at 70 mmHg.

**iii. Dose-response curve**

After the vessels stabilized at 70 mmHg, the inflow of Krebs-bicarbonate buffer solution a protocol followed in our lab. The intra luminal diameters were recorded in response to different concentrations of phenylphrine (10^{-9}-10^{-5}M). Vessels were maintained at each concentration for 5 minutes, before recording the diameters. At the end of the protocol the inflow of Krebs-bicarbonate buffer solution was resumed.
iv. Passive pressure response curve

After removal of phenylephrine by washing with Krebs-bicarbonate buffer solution, baseline intra luminal diameter at 70 mmHg was recorded. A passive pressure-diameter response curve was obtained after 15 minutes superfusion of the arterioles with Ca\textsuperscript{2+} free Krebs-bicarbonate buffer solution containing 2 mM EGTA.

Effects of RP on the myogenic response of the vessel:

In a separate study the effects of RP on the arterioles were examined. The vessels were exposed to a series of four solutions (without ODNs) as described in Chapter 2 (General Methods), to subject the arterioles to the RP procedure. The study was again divided into three groups. In the first group, arterioles were cannulated and reverse permeabilized as described in Chapter 2 (General Methods). At the end of RP, vessels were superfused continuously for 24 hours with Krebs-bicarbonate buffer solution. For the second and third groups RP was performed on vessels isolated, but not cannulated, and positioned in slotted slides. At the end of the protocol for RP, arterioles were kept at either 34\degree C or 4\degree C and incubated for 24 hours in fresh Krebs-bicarbonate buffer solution. At the end of the 24 hour period arterioles were cannulated, pressurized and superfused with Krebs-bicarbonate buffer solution as described in Chapter 2 (General Methods). The arterioles of the three sets were then subjected to the functional studies as described in materials and methods of this chapter (section 1).

Efficacy of RP as demonstrated by PKI peptide loading into arteriolar smooth muscle:

The arterioles were dissected, cannulated, and after developing tone at 70 mmHg, the arterioles were reverse permeabilized with and without protein kinase A inhibitor (PKI)
(10−6 M), added solution 2. Cell permeable (Calbiochem) and cell impermeable (Sigma-Aldrich) PKIs were used. At completion of the RP procedure the arterioles were superfused with fresh Krebs-bicarbonate buffer solution. A concentration-response curve was then obtained for forskolin (10^{-3}-10^{-4}M) in both PKI-treated and control groups. The concentration-response curves were later analyzed, to examine for inhibition of cAMP-mediated vasodilatation caused by forskolin, as an indicator of successful RP.

**Effects of NCX knockdown on the myogenic response of the vessel:**

After developing tone at 70 mmHg, the arterioles were reverse permeabilized with ODNs. The vessels were incubated with 10^{-6}M antisense or sense ODNs (see Chapter 2 for procedures and sequences) in solution 2 during RP procedure. Sense ODN treated vessels were used as a control group. At the end of this treatment the arterioles were incubated for 24 hours with Krebs-bicarbonate buffer solution at 34° C. Arterioles were cannulated, pressurized and superfused with Krebs-bicarbonate buffer solution as described in Chapter 2 (General Methods), at the end of the incubation period. The arterioles were later subjected to functional studies described earlier in materials and methods of this chapter. MI, a measure of myogenic responsiveness of an arteriole, was calculated as explained in Chapter 3 (Halpern, Mongeon et al. 1984; Jackson and Duling 1989).

**Effects of NCX knockdown on protein expression:**

At the end of experimental protocol 4, while the vessels were still cannulated and pressurized, arterioles were snap frozen, using dry ice/acetone cooled forceps. Keeping the arterioles under dry ice, vessel segments between the surgical ties were removed
using micro-fine scissors, and stored under -80º C until further use. After pooling 3-4 long (20-30 mm) vessel segments for each group the vessel were homogenized and protein resolved using 8% acrylamide gel as described in Chapter 2 (General Methods). The membrane was probed with NCX and α actin antibodies and band intensity was determined as described in Chapter 2 (General Methods). The NCX signal was expressed relative to the actin signal to account for variations in sample loading. Comparisons were made between the antisense and sense experimental groups. An internal standard of rat aorta homogenate was included in each electrophoresis.
RESULTS

Effects of time and temperature on the myogenic responsiveness of rat cremaster arterioles.

Vessels were kept overnight in Krebs-bicarbonate buffer solution at 4° C (in fridge), 34° C (in incubator) and 34° C (cannulated and superfused with Krebs-bicarbonate buffer solution). Fig 5-1 shows the pressure-diameter curves for isolated cremaster arterioles, after 24 hours (stored/superfused) in Krebs-bicarbonate buffer solution. With the increase in intra luminal pressure there was a corresponding decrease in the internal diameter of isolated arterioles, in all groups (63.2 ± 5.1% to 44.3 ± 4.1%; 63.7 ± 3.4% to 47.6 ± 6.6%; 59.0 ± 5.8% to 43.9 ± .7% in 4°, 34° and cannulated 34° C overnight respectively), while there were no significant differences between the groups (two-way ANOVA with a post hoc Bonferroni’s multiple comparison test, n = 4).

With an acute increase in the pressure from 50 to 120 mmHg (Fig 5-2) there was an associated decrease in the internal diameter (57.5 ± 5.2% to 43.3 ± 4%; 60.3 ± 4.9% to 46.4 ± 7.5%; 56.5 ± 6.8% to 47.5 ± .4% in 4°, 34° and cannulated 34° C overnight respectively; n = 4), which was not significantly different in the three groups of experiments (two-way ANOVA with a post hoc Bonferroni’s multiple comparison test, n = 4).

Further, dose-response curves for phenylephrine (Fig 5-3) were performed to study the effect of time on agonist responsiveness of rat cremaster arterioles. There was a significant concentration-dependent vasoconstriction with phenylephrine (10⁻⁹-10⁻⁵M;
P<0.0001, one-way ANOVA with a post hoc Bonferroni’s multiple comparison test, n = 4). There were no significant differences in the responses between vessels at 4°, 34° and cannulated 34° C after 24 hours, as shown by the IC₅₀ values of 7.1 ± 0.07; 7.3 ± 0.14; 7.1± 0.10 respectively (one-way ANOVA, n = 4). There was a pressure-dependent increase (74.8 ± 2.6% to 105.9 ± 3.4%; 74.1 ± 2.4% to 104.6 ± 1.3%; 73.3 ± 2.9% to 102.7 ± .2%) in the passive diameters of the arterioles which was not significantly different in the three treatment groups (Fig 5-4).

Effects of RP on the myogenic response of rat cremaster arterioles.

Vessels were reverse permeabilized as described in materials and methods section of this chapter, later kept overnight in Krebs-bicarbonate buffer solution at 4° C (in fridge), 34° C (in incubator) and 34° C (cannulated and superfused with Krebs-bicarbonate buffer solution) as explained earlier. Fig 5-5 shows the pressure-diameter curves for isolated cremaster arterioles, after 24 hours, in three different conditions described above. Vessels were kept overnight in Krebs-bicarbonate buffer solution at 4° C (in fridge), 34° C (in incubator) and 34° C (continuous superfusion with Krebs-bicarbonate buffer solution).

With the increase in intra luminal pressure there was a corresponding decrease in the internal diameter of an isolated arterioles, in all the groups (59.6 ± 2.0% to 51.3 ± 5.6%; 74.2 ± .3% to 51.7 ± 3.1%; 69.3 ± 2.9% to 47.9 ± 6.6% in 4°, 34° and cannulated 34° C overnight respectively), which was not significantly different between the three groups (two-way ANOVA with a post hoc Bonferroni’s multiple comparison test, n = 4).
With an increase in the intra luminal pressure from 50 to 120 mmHg we observed an associated decrease in the internal diameter (57.9 ± 4.8% to 45.7 ± 4.0%; 66.5 ± 2.6% to 55.6 ± 1.7%; 60.3 ± .9% to 45.6 ± 2.9% in 4°, 34° and cannulated 34° C overnight respectively; n = 4) (Fig 5-6). No significant difference was observed in the three groups of experiments (two-way ANOVA with a post hoc Bonferroni’s multiple comparison test, n = 4).

There was a significant concentration-dependent vasoconstriction with phenylephrine (10^{-9}-10^{-5}M; P<0.0001, one-way ANOVA with a post hoc Bonferroni’s multiple comparison test, n = 4) (Fig 5-7). However, no significant difference in the responses between vessels at 4°, 34° and cannulated 34° C after 24 hours was observed, as shown by the IC_{50} values of 7.1 ± 0.04; 7.2 ± 0.04; 7.3 ± 0.9 respectively (one-way ANOVA, n = 4). There was a pressure dependent increase (75.4 ± 1.7% to 105.1 ± 1.2%; 83.6 ± 5.7% to 110.5 ± 4.0%; 75.8 ± 1.8% to 106.6± 1.5%) in the passive diameters of the arterioles which was not significantly different in the three treatment groups (Fig 5-8).

**Use of a different experimental model to check the efficacy of RP by loading peptides into in-situ arteriolar smooth muscle.**

The efficacy of RP procedure was tested by delivery of peptides in arteriolar smooth muscle cells utilizing a cell impermeable inhibitor of protein kinase A. Arterioles were reverse permeabilized with (experimental) and without (control) 10^{-6}M PKI. Cell impermeable (after RP) or permeable (without RP) PKI was used, and a dose-response curve for forskolin (10^{-9}-10^{-5}M) was compared in both experimental and control groups. Although there was a concentration-dependent vasodilatation with forskolin in both the
experimental and control groups, there was a significant rightward shift in the presence of PKIs. There was a significant difference in the IC$_{50}$ values of 8.2 ± 0.4; 6.8 ± 0.04 in experimental and control groups respectively treated with cell permeable PKI (Fig 5-9) (P<0.05, unpaired t-test, n = 4). Cell impermeable PKI also caused a significant rightward shift of the forskolin dose-response curve (P<0.001, unpaired t-test, n = 4) with IC$_{50}$ values of 7.6 ± 0.1; 6.7 ± 0.1 in experimental and control groups respectively (Fig 5-10).

**Effects of oligonucleotide-induced NCX knockdown on myogenic responsiveness and protein expression in the rat cremaster arteriole.**

Vessels were reverse permeabilized with antisense or sense ODNs, as described in materials and methods section of this chapter, and stored overnight in Krebs-bicarbonate buffer solution at 34° C (in incubator). Fig 5-11 shows the pressure-diameter curves for isolated cremaster arterioles, after 24 hours, treated with ODNs. With the increase in intra luminal pressure there was a significant decrease in the internal diameter of an isolated arterioles, in both antisense (61.3 ± 2.03% to 43.1 ± 2.3%; n=9) and sense (68.5 ± 1.6% to 51.1 ± 3.8%; n = 5) treated arterioles. There was no significant difference between the two groups (two-way ANOVA with a post hoc Bonferroni’s multiple comparison test).

With an acute increase in the intra luminal pressure from 50 to 120 mmHg (Fig 5-12) there was a significant vasoconstriction with both antisense (58.8 ± 2.1% to 44.9 ± 2.07%; n = 9) and sense (68.2 ± 3.7% to 49.03 ± 2.6%; n = 5), ODNs however, it was not significantly different amongst the two groups (two-way ANOVA with a post hoc Bonferroni’s multiple comparison test).
There was a significant concentration-dependent vasoconstriction with phenylephrine ($10^{-9}$-$10^{-5}$M; $P<0.0001$, two-way ANOVA with a *post hoc* Bonferroni’s multiple comparison test) in antisense ($n = 9$) and sense ($n = 5$) treated vessels (Fig 5-13). IC$_{50}$ values of antisense and sense treated ODNs 7.4 ± 0.1 and 7.3 ± 0.1 respectively, were not significantly different (unpaired t-test). There was a pressure dependent increase (79.9 ± 1.9% to 106.1 ± .6%; 75.7 ± 1.5% to 106.4 ± 1.6%) in the passive diameters of the arterioles which was not significantly different in these two treatment groups (Fig 5-14).

Myogenic index was calculated to assess the myogenic reactivity for both antisense and sense ODN treated arterioles (Fig 5-15). There was a significant decrease (sense to antisense: -0.40 ± 0.02 to -0.24 ± 0.04) in the myogenic reactivity of antisense treated vessels ($P<0.05$, unpaired t-test with a *post hoc* Bonferroni’s multiple comparison test, $n = 5$).

**Fig 5-16** shows immunoblots of aorta (lane 1), sense (lane 2) and antisense (lane 3) treated rat cremaster arterioles. Anti NCX monoclonal (R3F1) antibody was used which specifically reacted with protein bands of 120 (molecular mass of mature glycosylated NCX) and 70 kDa (believed to be an N terminal proteolytic fragment) (Nicoll, Longoni et al. 1990; Juhaszova, Ambesi et al. 1994). The bottom panel shows actin band, at 42 kDa.

There was a significant decrease (0.1 ± 0.01 to 0.1 ± 0.009; sense to antisense) in NCX expression of rat cremaster arterioles, 24 hours after NCX knockdown in rat cremaster.
arterioles treated with antisense ODNs (P<0.05, unpaired t-test, n.=.5) NCX protein was expressed relative to α actin (Fig 5-17) to account for variations in sample loading.
Chapter 5                  Knockdown of NCX by ODNs

Fig 5-1.

Fig 5-2.
Fig 5-18. Effects of intra luminal pressure on arteriolar diameter of rat cremaster arterioles, after 24 hours.

The intra luminal pressure was increased sequentially from 30 mmHg up to 150 mmHg. Vessels were maintained at each pressure for 5 minutes, and the minimum intra luminal diameters were recorded. Vessel diameters are shown as a fraction of diameters under passive conditions (without Ca$^{2+}$ with 2 mM EGTA). Data are shown as mean ± SEM. Analysis shows no significant difference in responses between the three treatment groups (n = 4).

Fig 5-19. Effects of a pressure step on arteriolar diameter of rat cremaster arterioles, after 24 hours.

The intra luminal pressure was reduced to 50 mmHg, followed by a step increase in intra luminal pressure to 120 mmHg. Vessels were maintained at each pressure for 5 minutes, and the minimum intra luminal diameters were recorded. Vessel diameters are shown as a fraction of diameters under passive conditions (without Ca$^{2+}$ with 2 mM EGTA). Data are shown as mean ± SEM. Analysis shows no significant difference in responses between the three treatment groups (n = 4).
Fig 5-3.

Fig 5-4.
Fig 5-20. Concentration-response curve for phenylephrine ($10^{-9}$-$10^{-5}$M) in rat cremaster arterioles, after 24 hours.

Vessels were maintained at each concentration for 5 minutes, and the minimum intra luminal diameters were recorded. Vessel diameters are shown as a fraction of diameters under passive conditions (without Ca$^{2+}$ with 2 mM EGTA). Data are shown as mean ± SEM. Analysis shows no significant difference in responses between the three treatment groups (n = 4).

Fig 5-21. Effects of intra luminal pressure on passive arteriolar diameter of rat cremaster arterioles, after 24 hours.

The intra luminal pressure was sequentially increased from 30 mmHg up to 150 mmHg, and the minimum intra luminal diameters were recorded. Vessel diameters are shown as a fraction of diameters under passive conditions (without Ca$^{2+}$ with 2 mM EGTA) at 70 mmHg. Data are shown as mean ± SEM. Analysis shows no significant difference in responses between the three treatment groups (n = 4).
Fig 5-5.

Fig 5-6.
Fig 5-22. Effects of intra luminal pressure on arteriolar diameter of rat cremaster arterioles, 24 hours after RP.

The intra luminal pressure was increased sequentially from 30 mmHg up to 150 mmHg. Vessels were maintained at each pressure for 5 minutes, and the minimum intra luminal diameters were recorded. Vessel diameters are shown as a fraction of diameters under passive conditions (without Ca\(^{2+}\) with 2 mM EGTA). Data are shown as mean ± SEM. Analysis shows no significant difference in responses between the three treatment groups (n = 4).

Fig 5-23. Effects of a pressure step on arteriolar diameter of rat cremaster arterioles, 24 hours after RP.

The intra luminal pressure was reduced to 50 mmHg, followed by a step increase in intra luminal pressure to 120 mmHg. Vessels were maintained at each pressure for 5 minutes, and the minimum intra luminal diameters were recorded. Vessel diameters are shown as a fraction of diameters under passive conditions (without Ca\(^{2+}\) with 2 mM EGTA). Data are shown as mean ± SEM. Analysis no significant difference in responses between the three treatment groups (n = 4).
Fig 5-7.

Fig 5-8.
Fig 5-24. Concentration-response curve of phenylephrine \((10^{-9}-10^{-5}\text{M})\) in rat cremaster arterioles, 24 hours after RP. 

Vessels were maintained at each concentration for 5 minutes, and the minimum intra luminal diameters were recorded. Vessel diameters are shown as a fraction of diameters under passive conditions (without \(\text{Ca}^{2+}\) with 2 mM EGTA). Data are shown as mean ± SEM. Analysis shows no significant difference in responses between the three treatment groups \((n = 4)\).

Fig 5-25. Effects of intra luminal pressure on passive arteriolar diameter of rat cremaster arterioles, after 24 hours. 

The intra luminal pressure was sequentially increased from 30 mmHg up to 150 mmHg, and the minimum intra luminal diameters were recorded. Vessel diameters are shown as a fraction of diameters under passive conditions (without \(\text{Ca}^{2+}\) with 2 mM EGTA) at 70 mmHg. Data are shown as mean ± SEM. Analysis shows no significant difference in responses between the three treatment groups \((n = 4)\).
Fig 5-9.

Fig 5-10.
Fig 5-26. Concentration-response curve for Forskolin (10^{-9}-10^{-5}M) of rat cremaster arterioles after RP with 10^{-4}M cell permeable PKI.

Vessel diameters are shown as a fraction of diameters under passive conditions (without Ca^{2+} with 2 mM EGTA). Data are shown as mean ± SEM. Analysis shows a significant (P<0.01, Non linear regression, n = 6) rightward shift of the cell permeable PKI treated arterioles as compared to controls.

Fig 5-27. Concentration-response curve for Forskolin (10^{-9}-10^{-5}M) of rat cremaster arterioles after RP with 10^{-4}M cell impermeable PKI.

Vessel diameters are shown as a fraction of diameters under passive conditions (without Ca^{2+} with 2 mM EGTA). Data are shown as mean ± SEM. Analysis shows a significant (P<0.01, Non linear regression, n = 6) rightward shift of the cell impermeable PKI treated arterioles as compared to controls.
Fig 5-11.

Fig 5-12.
Fig 5-28. Effects of intra luminal pressure on arteriolar diameter of rat cremaster arterioles, 24 hours after antisense and sense ODN treatment.

The intra luminal pressure was increased sequentially from 30 mmHg up to 150 mmHg. Vessels were maintained at each pressure for 5 minutes, and the minimum intra luminal diameters were recorded. Vessel diameters are shown as a fraction of diameters under passive conditions (without Ca$^{2+}$ with 2 mM EGTA). Data are shown as mean ± SEM. Analysis shows no significant difference in response between antisense and sense treated arterioles (n = 5).

Fig 5-29. Effects of an acute pressure step on arteriolar diameter of rat cremaster arterioles 24 hours after antisense and sense ODNs.

The intra luminal pressure was reduced to 50, followed by a step increase in intra luminal pressure to 120 mmHg. Vessels were maintained at each pressure for 5 minutes, and the minimum intra luminal diameters were recorded. Vessel diameters are shown as a fraction of diameters under passive conditions (without Ca$^{2+}$ with 2 mM EGTA). Data are shown as mean ± SEM. Analysis shows no significant difference in response between antisense and sense treated arterioles (n = 5).
Fig 5-13.

![Graph showing the relationship between Log [Php, M] and D/D_max for Antisense and Sense groups.](image1)

Fig 5-14.

![Graph showing the relationship between Pressure (mmHg) and D/D_max for Antisense and Sense groups.](image2)
Fig 5-30. Concentration-response curve of phenylephrine (10^-9-10^-5M) in rat cremaster arterioles, 24 hours after antisense and sense ODNs treatment.

Vessels were maintained at each dose for 5 minutes, and the minimum intra luminal diameters were recorded. Vessel diameters are shown as a fraction of diameters under passive conditions (without Ca^{2+} with 2 mM EGTA). Data are shown as mean ± SEM. Analysis shows no significant difference in response between antisense and sense treated arterioles (two-way ANOVA with a post hoc Bonferroni’s multiple comparison test, n = 5).

Fig 5-31. Effects of intra luminal pressure on passive arteriolar diameter of rat cremaster arterioles, 24 hours after antisense and sense ODNs treatment.

The arterioles were superfused with 0 mM Ca^{2+} with 2 mM EGTA (passive conditions). The intra luminal pressure was sequentially increased from 30 mmHg up to 150 mmHg, and the minimum intra luminal diameters were recorded. Vessel diameters are shown as a fraction of diameters under passive conditions (without Ca^{2+} with 2 mM EGTA). Data are shown as mean ± SEM. Analysis shows no significant difference in response between antisense and sense treated arterioles (two-way ANOVA with a post hoc Bonferroni’s multiple comparison test, n = 5).
**Fig 5-15.**

<table>
<thead>
<tr>
<th>Pressure (mmHg)</th>
<th>Sense ODNs ( (D/D_{\text{max}}) )</th>
<th>Antisense ODNs ( (D/D_{\text{max}}) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>0.68 ± 0.03</td>
<td>0.57 ± 0.01</td>
</tr>
<tr>
<td>120</td>
<td>0.49 ± 0.02</td>
<td>0.47 ± 0.01</td>
</tr>
</tbody>
</table>

**Table 5-1.**
Fig 5-32. Effect of pressure step on MI in arterioles treated with antisense and sense ODNs.

The intra luminal pressure was reduced to 50 mmHg, followed by a step increase in intra luminal pressure to 120 mmHg. Vessels were maintained at each pressure for 5 minutes, and the minimum intra luminal diameters were recorded. Vessel diameters used were a fraction of diameters under passive conditions (without Ca\(^{2+}\) with 2 mM EGTA). Myogenic Index was calculated and data are shown as mean ± SEM. Analysis shows a significant decrease in MI in vessels treated with antisense ODNs as compared to sense (P<0.05, unpaired t-test, n = 5).

Table 5-2. Effects of a pressure step on arteriolar diameter of rat cremaster arterioles, 24 hours after sense and antisense treatment.

The vessels were subjected to a pressure step from 50 to 120 mmHg in cremaster arterioles 24 hours after antisense and sense ODN treatment. D/D\(_{max}\) is the vessel diameter expressed as a fraction of passive diameter (without Ca\(^{2+}\) with 2 mM EGTA). Data are shown as mean ± SEM.
Fig 5-16.

Fig 5-17.
Fig 5-33. Western blot comparing antisense treatment on NCX protein expression in rat cremaster arterioles to sense ODN treatment.

Arterioles show a band at 120 kDa consistent with the previously described NCX1 form of the exchanger. 120 kDa represents the intact NCX1 and 70 kDa a N terminal fragment.

Fig 5-34. Effects of NCX knockdown on protein expression in rat cremaster arterioles, 24 hours after ODN treatment.

Antisense suppressed protein expression in rat cremaster arteriole, as compared to sense ODNs. NCX protein expression is expressed relative to actin expression. Group data are presented as mean ± SEM. Analysis shows that at there was a significant (P<0.05, unpaired t-test, n=5) decrease in NCX expression in antisense-treated arterioles as compared to sense-treated arterioles.
Ca\(^{2+}\) is important physiologically as an intracellular signalling molecule hence it is essential to be able to control its intracellular levels in the VSM cells. NCX is an important regulator for Ca\(^{2+}\) homeostasis although the exact physiological role remains uncertain. By regulating intracellular Ca\(^{2+}\) levels NCX may thus play a role in myogenic responsiveness of the smaller vessels. It has been difficult to assign a physiological role to NCX due to lack of potent and selective pharmacological inhibitors, more so due to the complex signalling mechanisms and with various different pathways for Ca\(^{2+}\) entry and exit (Niggli, Schwaller et al. 1996). Thus the present studies were designed to examine the efficacy of delivery of oligonucleotides directed at the NCX so as to directly suppress protein expression.

The NCX protein was identified, sequenced and cloned in 1990 (Nicoll, Longoni et al. 1990), and its isoforms also identified in various tissues (Kofuji, Lederer et al. 1994; Li, Matsuoka et al. 1994; Niggli, Schwaller et al. 1996). This molecular information was an important milestone in NCX research and was utilized to design sense and antisense ODNs against NCX. Antisense ODNs selectively decrease the expression of NCX activity by blocking translation and by forming RNA-DNA duplexes (Leonetti, Degols et al. 1993; Wagner 1994). This helped in better understanding of physiological role of NCX in various tissues including cardiac and arterial myocytes (Lipp, Schwaller et al. 1995; Slodzinski, Juhaszova et al. 1995; Takahashi, Bland et al. 1995; Slodzinski and Blaustein 1998; Slodzinski and Blaustein 1998a; Takahashi, Azuma et al. 1999). While in Chapter 3 pharmacological inhibitors were utilized to elucidate a functional role for
Prior to performing the ODN administration experiments we performed extensive control experiments, to observe the effect of temperature and the RP procedure, per se, on the myogenic responsiveness of the arterioles. After keeping the vessels in Krebs-bicarbonate buffer solution for 24 hours, we observed that there was no change in the myogenic responsiveness of the vessels if kept cannulated for the entire period or if they were stored at 4 or 34 °C, as shown by the functional study (Fig 5-1 to Fig 5-4). We also observed that there was no effect of the RP procedure on the myogenic responsiveness of the arterioles, as shown by the functional study after the 24 hour incubation period (Fig 5-5 to Fig 5-8). However vessels were less reactive after 24 hr at 34 °C especially at higher intra luminal pressures (Fig 3-1 versus Fig 5-5). This procedure used similar methods as were to be used in ODN experiments with the exception that no ODNs were used.

To further confirm the efficacy of the RP procedure in our experimental setting we tested the technique on the delivery of an unrelated molecule. We confirmed the efficacy of RP by loading cell permeable and impermeable PKI into in-situ arteriolar smooth muscle. Forskolin is a vasodilator which acts by activating the enzyme adenylyl cyclase and therefore raising the intracellular levels of cAMP (Haynes, Robinson et al. 1992). Thus we postulated that successful delivery of PKI inside the cell would inhibit the vasodilator effects of Forskolin. We used two different PKIs; cell permeable and cell impermeable to show that the effects observed were not due to non-specific effects of PKI, but due to its
entry inside the cell by RP procedure. Data from this set of experiments showed that there was a significant rightward shift of the concentration-response curve with forskolin in PKI treated arterioles as compared to controls. The results were similar in both the cell permeable and cell impermeable PKIs treated experiments (Fig 5-9 and Fig 5-10). This rightward shift of the dose-response curve of forskolin was due to the fact that PKI was efficiently delivered inside the smooth muscle cells of the arterioles. Having established the efficacy of the RP technique subsequent experiments examined the delivery of ODNs with ODN experiments, which are discussed in the following section.

The antisense ODNs aimed to reduce or inhibit new protein formation, for which a lag period presumably depends on the half-life ($t_{1/2}$) of the protein and the kinetics of the ODN uptake. This implies that proteins with long $t_{1/2}$ will require a longer time before there is any evidence of decrease in protein expression by antisense ODNs. After antisense ODN treatment NCX should be efficiently eliminated from the plasma membrane of the VSM. Therefore any biological signals associated with NCX will be diminished or absent. Although such an approach had not been used in isolated arterioles, considerable research had previously been performed utilizing cultured myocytes, especially heart cells.

Lipp et al. have used 3 µM antisense ODNs in cultured rat cardiac myocytes and reported significant knockdown of NCX function within 24 hours (Lipp, Schwaller et al. 1995). Bland et al. used a higher concentration (10 µM) and obtained a reduction of $^{45}\text{Ca}^{2+}$ influx evoked by reduction of extracellular $[\text{Na}^+]$ within 24 hours (Bland, Takahashi et al. 1996). Kim et al. reported suppression of NCX by antisense ODNs in dissociated Purkinje neurons of rat cerebellum. A decrease in the fluorescence intensity
was observed 24 hours after the cells were treated with 1 µM of fluorescein-tagged NCX antisense ODNs as compared to control cells (Kim, Park et al. 2005). On the basis of this literature we incubated our arterioles with antisense ODNs for 24 hours.

In the present study we assessed viability of the vessel by its ability to develop spontaneous tone when cannulated after 24 hours of incubation with ODNs. The knockdown was confirmed by observing the expression of the target protein, NCX, and its effect on the myogenic response of the arterioles. We also made sure that the antisense and sense oligonucleotides were screened with the Gen bank so that there was no homology to any other described DNA sequence of NCX. We utilized a low concentration of antisense oligonucleotide of $10^{-6}$M, which was not toxic to the arterioles as most of the arterioles developed tone. Also in our experiments we used sense oligonucleotides as a control for non-specific effects relating to ODN administration per se.

There was a significant reduction (~ 25%) in the NCX protein expression shown in the antisense treated vessels as compared to the sense-treated vessels (Fig 5-16 and Fig 5-17). Our results are consistent with the results from Bland et al., who observed a 30% reduction in NCX expression in embryonic heart cells after 24 hours (Bland, Takahashi et al. 1996). Slodzinski et al. on the other hand observed a 50-60% reduction, in NCX protein in antisense oligonucleotide-treated heart cells after 7 days in the culture (Slodzinski and Blaustein 1998a). This could be due to the fact that Lipp et al. and Bland et al. used a much higher concentration (2 µM) of antisense oligonucleotides as compared to Slodzinski et al. However, the concentration of oligonucleotides and the exposure time in the present study were not sufficient to alter myogenic reactivity of the
isolated arterioles as assessed through comparisons of the pressure-diameter relationships for antisense and sense treated vessels.

While the calculated difference between the two pressure-diameter relationships (i.e. antisense compared to sense) did not reach the accepted level for significance (P=0.15), calculations of myogenic index did reveal a significant difference (P<0.05). The latter observation importantly points towards a functional significance for NCX in the myogenic response. That being said the reason for the apparent discrepancy may relate to normalization of between-vessel differences or that the knockdown of 25% was of borderline functional significance. To test for the latter, additional experiments will need to be performed so as to obtain a greater level of knockdown of the NCX protein (for example by increasing oligonucleotide exposure time).

A further limitation of these oligonucleotide studies was that vessels had to be pooled to allow Western blots to be performed. This could have obscured heterogeneity in the effectiveness of the knockdown procedure and prevented correlative analysis being performed on individual vessels. Recent advances in detection systems for Western blotting now enable much smaller amounts of protein to be detected (Li, Li et al. 2004; Bakalova, Zhelev et al. 2005). As such the sensitivity of our approach could be improved in future studies.

Thus taken together, the results indicate that antisense ODN knockout was successful in intact cremaster arterioles using the RP procedure. Although the actual reduction in NCX expression may not have been sufficient to show a clear effect on steady state pressure-diameter relationship, myogenic index calculations indicate a role for NCX in
arteriolar myogenic vasoconstriction. Additional studies are required to determine whether the conditions can be altered (increased ODN concentration or exposure time) for a higher and more effective level of NCX knockdown in isolated arterioles, or whether approaches such as the use of small interfering RNAs are more effective.
CHAPTER 6

GENERAL DISCUSSION AND

FUTURE DIRECTIONS
The myogenic response is the ability of an arteriole to contract, with an increase in the intra luminal pressure and dilation with a decrease in the intra luminal pressure (Johnson 1981; Davis and Hill 1999). Although the exact mechanisms linking changes in the intra luminal pressure to contraction are still unclear, it is known that increase in intra luminal pressure produces increase in $Ca^{2+}$ influx leading to tonic contraction (Harder 1984; Jaggar, Wellman et al. 1998). It is also known that intra luminal pressure leads to membrane depolarization (Harder 1984; Harder, Gilbert et al. 1987; Knot and Nelson 1995; Knot and Nelson 1998) which is responsible for $Ca^{2+}$ entry via VGCC (Davis, Donovitz et al. 1992; Meininger and Davis 1992; Wesselman, VanBavel et al. 1996; McCarron, Crichton et al. 1997; Schubert and Mulvany 1999). There are many reports suggesting that VGCC may not be the only route for $Ca^{2+}$ entry in VSM (Hwa and Bevan 1986; Hwa and Bevan 1986; Davis and Hill 1999). It is therefore important to know the contribution of mechanisms other than VGCC in regulating $[Ca^{2+}]_{cyt}$ in VSM cells.

NCX is thought to play an important role in the regulation of $Ca^{2+}$ homeostasis in VSM cells (Slodzinski, Juhaszova et al. 1995; Shimizu, Borin et al. 1997; Blaustein and Lederer 1999; Arnon, Hamlyn et al. 2000a). In order to understand the physiological significance of NCX in modulating $[Ca^{2+}]_{cyt}$ levels and hence, myogenic reactivity, tools which selectively inhibited the exchanger were utilized. This was followed by cellular approaches and NCX knockdown by antisense oligonucleotides. In the coming sections, I will discuss the overall results and physiological significance of the experiments included in the thesis.

**Functional significance of NCX in myogenic response**
Na\(^+\) gradient across the plasma membrane energizes NCX to move Na\(^+\) and Ca\(^{2+}\) ions in opposite directions. Therefore, manipulation of extracellular Na\(^+\) (to activate the exchanger in reverse mode and raise [Ca\(^{2+}\)\(_{\text{cyt}}\)] and pharmacological inhibitors were utilized to demonstrate the functional significance of the NCX in the setting of arteriole myogenic tone.

A decrease in the extracellular [Na\(^+\)] solution (to inhibit forward mode NCX) caused vasoconstriction and a simultaneous increase in [Ca\(^{2+}\)\(_{\text{cyt}}\)], consistent with reports from other investigators that reducing extracellular Na\(^+\) promotes Ca\(^{2+}\) entry (Takai, Yamada et al. 2004). NCX inhibitors KB-R7943 and SEA0400 caused a dose-dependent vasodilatation; SEA0400 being the more potent inhibitor of the two. This could be due to their inhibitory effects on Ca\(^{2+}\) uptake (Iwamoto, Kita et al. 2004c) in the VSM cells.

We observed the effect of NCX inhibitors on the myogenic response of de-endothelialized arterioles in reduced extracellular [Na\(^+\)]. Both the NCX inhibitors KB-R7943 and SEA0400 significantly attenuated the vasoconstriction induced by decreasing extracellular [Na\(^+\)] as compared to control vessels (where there was no change in extracellular [Na\(^+\)]). This was in support of our hypothesis; suggesting a role for NCX in regulating myogenic tone in rat cremaster arterioles. A possible candidate for vasoconstriction associated with decrease in extracellular [Na\(^+\)] could be Ca\(^{2+}\) entry via VGCC, as decrease in extracellular [Na\(^+\)] levels causes opening of VGCC (Blaustein and Lederer 1999). However, Nifedipine (L-type VGCC blocker), did not have any significant effects on contraction associated with the reverse mode of the NCX; ruling out VGCC as an ion channel involved in the response.
The present study examined the relationship between \( E_m \) and myogenic tone, and the role played by NCX, by utilizing NCX and VGCC inhibitors. It is interesting that NCX itself contributes to the action potential (Weidmann 1993; Benardeau, Hatem et al. 1996; Janvier and Boyett 1996; Janvier, Harrison et al. 1997) and on the other hand is itself regulated by the \( E_m \) (Blaustein and Lederer 1999). KB-R7943 caused vasodilatation associated with hyperpolarization, where as nifedipine was not associated with hyperpolarization, implicating NCX as these effects could not be attributed to non specific effects of KB-R7943 on VGCC.

We confirmed, that NCX was involved in the acute myogenic responsiveness of arterioles as shown by the decreasing myogenic reactivity (decreasing slope of the pressure step graph) with a reduction in extracellular \([\text{Na}^+]\). This decrease in slope was not seen in the control experiments (vessels not subjected to a decrease in extracellular \([\text{Na}^+]\)). Further, a significant decrease in myogenic index associated with a decrease in extracellular \([\text{Na}^+]\), was again in support of our hypothesis that NCX has a role to play in myogenic response of skeletal muscle arterioles.

Interpretation of these data must, however, be considered in the light of the fact that experimentally decreasing the transmembrane \( \text{Na}^+ \) gradient could modify the activity of \( \text{Na}^+ \) dependent exchange mechanisms such as \( \text{Na}^+/\text{H}^+ \) exchanger and the \( \text{Na}^+/\text{K}^+ \) pump (Bova, Goldman et al. 1990). Further, there is limitation of the use of NCX inhibitor KB-R7943, as non-specific effects have been suggested. It has been reported that KB-R7943 at relatively low concentrations blocks some other ion transporters, such as, \( \text{Na}^+/\text{H}^+ \) ATPase, \( \text{Na}^+/\text{K}^+ \) ATPase and \( \text{Ca}^{2+} \) ATPase (Iwamoto, Watano et al. 1996a; Matsuda, Arakawa et al. 2001). Thus the dilator effect of these inhibitors may not
necessarily indicate a specific action on the underlying myogenic contractile mechanism. But combining them with other approaches for modulating NCX activity such as reduction in extracellular [Na⁺] can be used as a strong tool to study NCX mediated responses in VSM.

There are reports suggesting that cardiotonic steroids, such as endogenous ouabain may contribute to etiology and patho-physiology of salt-sensitive hypertension and that NCX1 plays a major role (Iwamoto, Kita et al. 2005). Thus there is potential for vascular NCX1 to be explored, and exploited, as a new therapeutic target (Iwamoto, Kita et al. 2005).

**Identification of NCX in arteriolar wall**

As outlined above, treatment with NCX inhibitors provided evidence that NCX may exert an influence on the level of myogenic tone in arterioles, as vasodilatation was associated with the use of inhibitors. In support of the functional and pharmacological evidence of NCX presence in VSM, the results of this section confirmed the expression of NCX protein in intact arterioles from rat cremaster muscle. Further, real-time PCR was employed to analyze the tissue specific expression and the relative abundance of the various NCX isoforms in cremaster arteriole and in various other tissues.

A monoclonal antibody, raised against the canine cardiac form of NCX, cross-reacted with the protein homogenized from cremaster arterioles. A strong 70 kDa band was attributed to a proteolytic fragment of NCX in addition to the 120 kDa band of the integral exchanger (Philipson, Longoni et al. 1988).
Out of the three NCX isoforms cloned to date (NCX1, 2 and 3), message for NCX1 was the most abundant in VSM, whereas message for NCX3 was the least abundant. As a control we also confirmed that message for NCX2 and NCX3 are found in the skeletal muscle of rat. Data from this study confirms the presence of NCX in the VSM of cremaster arterioles, with NCX1 being the most abundant isoform. Thus these data support the functional studies discussed in the first section and collectively suggest a role for the NCX in arteriolar function.

**NCX knockdown utilizing oligonucleotides**

After confirming the presence of NCX and the type of isoform present in the VSM, we next altered the expression of NCX. By regulating levels of NCX, $[\text{Ca}^{2+}]_{\text{cyt}}$, and hence myogenic tone, may be regulated. This was an important technique for assessing the role played by NCX in myogenic responsiveness of the smaller vessels, especially due to lack of potent and selective pharmacological inhibitors. Hence we examined the efficacy of delivery of oligonucleotides directed at the NCX so as to directly suppress protein expression.

Control studies confirmed that there were no temperature-and RP procedure-dependent changes in the myogenic responsiveness of the arterioles (after 24 hours), as shown by the functional studies. As a control to demonstrate efficacy of the loading procedure, PKI peptides were loaded into arteriolar smooth muscle in-situ by utilizing the RP procedure. Efficacy was confirmed by successful delivery of PKI resulting in inhibition of the vasodilator effects of Forskolin. Following establishment and verification of the methods, antisense oligonucleotides were delivered by RP.
We assessed viability of the vessel by its ability to develop spontaneous tone 24 hours after incubation with oligonucleotides. The knockdown was confirmed by observing the expression of the target protein, NCX, and its effect on the myogenic response of the arterioles. There was a significant reduction (~ 25%) in the NCX protein expression when the vessels were treated with antisense oligonucleotides. This was consistent with results of other studies (Bland, Takahashi et al. 1996; Slodzinski and Blaustein 1998). Further, our calculations of MI showed that antisense treatment was able to reduce myogenic responsiveness of the arterioles pointing towards functional significance of NCX in the myogenic response.

Thus taken together, the results of this study indicate that antisense oligonucleotide knockout was successful in intact cremaster arterioles; however, it was not sufficient to alter sustained or acute myogenic reactivity of the arterioles. Thus, future studies are required to adapt the oligonucleotide knockdown procedure so as to determine whether a greater inhibition of NCX expression is required to demonstrate an effect on the myogenic response or whether the exchanger only plays a complementary role in arteriolar smooth muscle Ca\(^{2+}\) handling.

**Future directions**

An array of ion channels participates in regulation of vascular tone. However, due to factors such as differences in channel regulation and expression there remain gaps in our knowledge. Clearly there is a complex relationship between E\(_m\) and arteriolar tone such that modulation of E\(_m\) can be a used as an important mechanism to manipulate vascular tone. Therefore, hyperpolarizing vasodilators which act via inhibition of NCX
may be useful in understanding pathophysiological states like hypertension, and hence should be explored further.

Additional experiments should be conducted using different sets of antisense and control ODNs (differing/overlapping sequences) which will further confirm that the ODN knockdown of the protein was not due to non-specific antisense effect. Importantly, different concentrations of antisense ODN and with longer incubations than 24 hours may be required. Such approaches can further help to evaluate the efficacy of antisense ODNs and help confirm whether any effect seen is specific to the antisense sequence utilized.

Apart from antisense ODNs other gene silencing methods, including siRNA, can be utilized in conjunction with conventional pharmacology to provide a powerful set of tools to examine the function of NCX in the microcirculation. These approaches, along with the simultaneous measurement of membrane potential, [Ca$^{2+}$]$_{cyt}$ levels, and diameter should also be studied under in vivo conditions so as to establish the role played by NCX in the regulation of microcirculation in both health and disease states. Further, exchanger distribution and function should be studied by utilizing new ion-selective fluorescent dyes and antibody probes combined with digital imaging methods.

The maintenance of Ca$^{2+}$ homeostasis is important for all cells, especially smooth muscle cells. Mechanisms involved in unloading the cells of Ca$^{2+}$ include, PMCA, SERCA, Na$^+$/K$^+$ ATPase and NCX. The relative contributions from each are still an issue of debate, and may be achieved with the recent advances in the field of gene targeting and transgenic mice. By removal, or addition, of individual components and isoforms a
better understanding of the functional role(s) of NCX and other Ca\(^{2+}\) removing channels/exchangers (and individual isoforms) can be achieved. Consistent with this there are recent reports that there are ties between various components of Ca\(^{2+}\) homeostasis (e.g. SERCA and NCX via the 2\(\alpha\) isoform of Na\(^+\)/K\(^+\)-ATPase) (Lencesova, O’Neill et al. 2004). Hence, the use of genetically altered mice the potential interactions between NCX and other Ca\(^{2+}\) clearance components could be explored further. The significance of altered expression of NCX relative to its activity must also be investigated further (Ishida and Paul 2005).

A change in Intra luminal pressure leads to activation of multiple signalling pathways including NCX and TRP channels. Future research could study if these operate in parallel or is there some interaction between these two signalling pathways in relation to myogenic reactivity. Some work has already been done in this area showing that Ca\(^{2+}\) entry via the reverse mode of Na\(^+\)/Ca\(^{2+}\) exchange contributes to store depletion-mediated increase in [Ca\(^{2+}\)]\(_{\text{cyt}}\). TRP channels that are permeable to Na\(^+\) and Ca\(^{2+}\) are believed to form functional SOCC (Zhang, Yuan et al. 2005). It is also important to mention here that it is difficult to functionally distinguish between NCX and SOCC hence SOCC might be operating in parallel with NCX in regulation of [Ca\(^{2+}\)]\(_{\text{cyt}}\) and hence myogenic tone.

Recent studies by Dong et al. have shown a role of NCKX in regulation of [Ca\(^{2+}\)]\(_{\text{cyt}}\) and contractility in arterial smooth muscle (Dong, Jiang et al. 2006). Further studies are needed to show if NCX and NCKX both have physiological relevance in the regulation of myogenic tone.
Functional studies, of myogenically active arterioles, should also be extended to
determine the role of the $\text{Na}^+\text{K}^+$ ATPase (in particular isoforms exhibiting high ouabain
sensitivity) in cation handling. The results of this study confirmed the presence of NCX
isoforms in VSM; the contribution of isoforms of the NCX 1, 2 and 3 should be examined
further. Such studies will allow testing of the hypothesis that the differential expression
of isoforms confers specific cellular functions in arteriolar smooth muscle.

In conclusion, based on the results from this thesis, a conceptual diagram is presented
in Fig. 6-1 to illustrate the possible signaling pathways in the regulation of arteriolar
myogenic response and the setting of myogenic tone. This figure also provides a
framework for further investigation.
Fig 6-2. Myogenic signalling pathway for NCX.

The mechanical stimulus provided by an increase in intra luminal pressure initiates the opening of non-selective cation channels and non-selective cation entry, predominately Na⁺. This leads to depolarization of the membrane with subsequent opening of L-type VGCCs. NCX functions in the reverse mode to remove Na⁺ from the space between the plasma membrane and sarcoplasmic reticulum and supply Ca²⁺ from the extracellular space, leading to muscle contraction.
BIBLIOGRAPHY


Appendix of suggested corrections


Jones, T. W. (1852). "Discovery that the veins of the bat's wing (which are furnished with valves) are endowed with rhythmical contractility and the onward flow of blood is accelerated by each contraction." Phil. Trans. 1: 131-136.


Ringer, S. (1883). "A further contribution regarding the influence of the different constituents of the blood on the contraction of the heart." J. Physiol. 4: 29-42.


Satoh, H., K. S. Ginsburg, et al. (2000). "KB-R7943 block of Ca(2+) influx via Na(+)/Ca(2+) exchange does not alter twitches or glycoside inotropy but prevents Ca(2+) overload in rat ventricular myocytes." Circulation 101(12): 1441-6.


Zhuge, R., K. E. Fogarty, et al. (2002). "Spontaneous transient outward currents arise from microdomains where BK channels are exposed to a mean Ca(2+) concentration on the order of 10 microM during a Ca(2+) spark." J Gen Physiol 120(1): 15-27.
