RHYTHMICITY IN VASCULAR SMOOTH MUSCLE

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The data contained in this thesis, are original and obtained by the author under the supervision of Professor Caryl Hill.

All calcium imaging, electrophysiology, immunohistochemistry and contraction studies were carried out by myself with the exception of electrophysiological studies in chapter 3, which were performed by Professor Caryl Hill and Professor David Hirst and selected experiments in chapter 4 which were performed by Professor Caryl Hill. The electron microscopy in Chapter 5 was performed by Dr Therese Brackenbury and Dr Shaun Sandow, and the quantitative PCR studies carried out in the Appendix were performed by Dr Hilton Grayson.

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ABSTRACT

Spontaneous, rhythmical contractions or vasomotion can be observed in both small vessels of the microcirculation and in large vessels both *in vivo* and *in vitro*. Although vasomotion is considered to have both physiological and pathophysiological relevance, relatively little is known about the cellular events that underlie this phenomenon. Thus, using electrophysiology to study changes in membrane potential, the ratiometric calcium (Ca\(^{2+}\)) indicator Fura-2 AM to study changes in intracellular calcium ([Ca\(^{2+}\)]\(i\)) in both the arterial wall and in individual smooth muscle cells (SMCs), and video microscopy to study changes in vessel diameter, this thesis has sought to provide a greater understanding of the mechanisms underlying vasomotion in the iris arteriole and basilar artery of the juvenile rat.

In the iris arteriole, vasomotion occurred independently of the endothelium. Spontaneous contractions were preceded by spontaneous depolarizations and [Ca\(^{2+}\)]\(i\) oscillations. Arteriolar vasomotion was shown to be dependent on the cyclical release of Ca\(^{2+}\) from intracellular stores, but not those operated by ryanodine receptors (RyR). Oscillations were little changed when the membrane potential of short segments of arteriole was either depolarized or hyperpolarized. When the segments were voltage clamped, oscillating inward currents were recorded, indicating that the changes in membrane potential were voltage independent. Vasomotion was abolished by inhibitors of phospholipase C (PLC), phospholipase A\(_2\) (PLA\(_2\)) and protein kinase C (PKC). Further inhibition of the lipoxygenase, cyclo-oxygenase and cytochrome P450 arms of the PLA\(_2\) pathway suggested the involvement of metabolic products of the lipoxygenase pathway in the generation of rhythmical activity. Thus observations suggest that irideal arteriolar vasomotion is voltage independent, resulting from the cyclical release of Ca\(^{2+}\) from IP\(_3\)-sensitive stores which are activated by cross talk between the PLC and PLA\(_2\) pathways in vascular smooth muscle.

In the rat basilar artery, rhythmical oscillations in both membrane potential and [Ca\(^{2+}\)]\(i\) were also found to precede rhythmical contractions. Vasomotion was shown to be dependent on Ca\(^{2+}\) influx through L-type voltage dependent calcium channels (VDCCs) since inhibition of these channels depolarized SMCs and abolished rhythmical contractions and depolarizations. Activity of VDCCs was critical for synchronizing [Ca\(^{2+}\)]\(i\) oscillations in adjacent SMCs. Abolition of vasomotion, reduction in basal [Ca\(^{2+}\)]\(i\) levels, SMC hyperpolarization and relaxation were observed following the addition of inhibitors of...
[Ca\(^{2+}\)]_i handling or the PLC signaling pathway. Calcium-dependent chloride channel (Cl\(_{Ca}\)) antagonists produced similar effects, suggesting the involvement of Cl\(_{Ca}\) channels. Rhythmical activity was also abolished following chloride substitution. Antagonists of Ca\(^{2+}\) release from the Ry-sensitive intracellular store and of intermediate conductance Ca\(^{2+}\)-activated potassium channels (IK\(_{Ca}\)) depolarized SMCs, constricted the vessel and increased the frequency of rhythmical depolarizations and [Ca\(^{2+}\)]_i oscillations. Together, these findings suggest that vasomotion in the basilar artery depends on the release of intracellular calcium from IP\(_3\)-sensitive stores which activates Cl\(_{Ca}\) channels to depolarize SMCs. Depolarization in turn activates VDCCs, synchronizing contractions of adjacent cells through influx of extracellular calcium. Subsequent calcium-induced calcium release from Ry-sensitive stores activates an IK\(_{Ca}\) channel, hyperpolarizing the SMCs and providing a negative feedback pathway for regeneration of the contractile cycle.

In contrast to the iris arterioles, removal of the vascular endothelium from the basilar artery resulted in asynchronous and irregular contractions. [Ca\(^{2+}\)]_i oscillations in adjacent SMCs became unsynchronized, but spontaneous depolarizations persisted. Using immunohistochemistry, connexins (Cx) 37, 40 and 43 were found in the endothelium, while Cx45 and to a lesser extent Cx37 were expressed in SMCs. Serial section electron microscopy demonstrated that the endothelial and smooth muscle layers were well coupled by myoendothelial gap junctions (MEGJs). Studies of the role of the endothelial-derived relaxing factors nitric oxide (NO) and endothelium derived hyperpolarizing factor (EDHF) confirmed that these vasoactive substances play a modulatory but not essential role in vasomotion. Thus, inhibition of NO in intact preparations augmented rhythmical activity and further synchronized [Ca\(^{2+}\)]_i oscillations in adjacent cells, while inhibition of EDHF failed to completely abolish vasomotion. Data suggests that [Ca\(^{2+}\)]_i oscillations are generated within the smooth muscle, but that the SMCs are poorly coupled. The vascular endothelium is thus essential for facilitating the spread of voltage along the arterial wall, in order to coordinate calcium fluxes through VDCCs. Transfer of electrical current passes from the SMCs to the endothelium via functional MEGJs and vasomotion takes place.

Short synthetic peptides designed to target amino acid sequences in the extracellular loops of the vascular connexins were used to further investigate the role of cellular coupling in vasomotion in the rat basilar artery.\(^{37,43}\) Gap 27 or the combined peptides Gap 37 and Gap 43, possessing homology with sequences in the second extracellular loop of Cx37 and 43 rapidly and reversibly inhibited vasomotion, decreased basal [Ca\(^{2+}\)]_i levels,
hyperpolarized and relaxed SMCs. Incubation in $^{43}$Gap 26 and Gap 43, targeting amino acid sequences important for docking of the first and second extracellular loops of Cx43 respectively and the mimetic peptides $^{45}$Gap27 or Gap 45, corresponding to different amino acids in the second extracellular loop of Cx45, produced similar results. In the absence of the endothelium, $^{37}$,$^{43}$Gap 27 was shown to have no effect. On the other hand, $^{40}$Gap 27 and Gap 40, targeting the second extracellular loop of Cx40, significantly reduced the amplitude of the rhythmical depolarizations and caused the appearance of irregular, uncoordinated contractions and [$Ca^{2+}$]$_i$ oscillations in the arterial wall. [$Ca^{2+}$]$_i$ oscillations in adjacent SMCs were unsynchronized. In the absence of the endothelium, $^{40}$Gap 27 caused the irregular activity to regain rhythmicity and the small uncoordinated [$Ca^{2+}$]$_i$ oscillations in individual SMCs to become synchronized. Inhibition of Cx37 with Gap 37, targeting the second extracellular loop of Cx37 resulted in small rhythmical depolarizations and reduced [$Ca^{2+}$]$_i$ oscillations in adjacent SMCs, similar to that observed in the presence of $^{40}$Gap 27 or in the absence of the endothelium. Thus while data would indicate an important role for gap junctional communication during vasomotion, additional effects on [$Ca^{2+}$]$_i$ oscillations in SMCs, membrane potential and vessel tone suggest that the peptides may have non-specific actions at intracellular Ca$^{2+}$ stores. Alternatively, since results obtained with peptides against different extracellular sequences of the same connexin were remarkably consistent, a functional interaction between the conductance of gap junctions and the activity of intracellular Ca$^{2+}$ stores cannot be ruled out.

In summary, this thesis has investigated the basic ionic mechanisms and intracellular signaling pathways that are active during spontaneous vasomotion in the iris arteriole and basilar artery of juvenile rats. It is concluded that during rhythmical activity, different vascular beds possess both common and unique control mechanisms, most likely reflecting the differential distribution of ion channels and receptors, which may ultimately allow for specific targeting of therapeutic agents. The role of cellular coupling via gap junctions has been examined specifically in the cerebral vascular bed using Cx mimetic peptides.
PUBLICATIONS ARISING FROM DATA PRESENTED 
IN THIS THESIS

The following papers resulting in work presented in this thesis have been published:


A number of presentations of this work were made at scientific meetings. The following abstracts were published in conjunction with these presentations:


Hill C.E and Haddock R.E. (2002). Regulation of cerebral vasomotion by calcium stores and voltage dependent ion channels. 22nd Meeting of the European Society of Microcirculation, Exeter, UK.


The following is a list of abbreviations used in the text of this thesis:

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>BK&lt;sub&gt;Ca&lt;/sub&gt;</td>
<td>large conductance K&lt;sub&gt;Ca&lt;/sub&gt; channel</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>calcium</td>
</tr>
<tr>
<td>[Ca&lt;sup&gt;2+&lt;/sup&gt;]&lt;sub&gt;i&lt;/sub&gt;</td>
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<tr>
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<td>calcium induced calcium release</td>
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<td>calmodulin</td>
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<tr>
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<tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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<td>prostaglandin</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>-------------</td>
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<td>phospholipase A&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
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<td>protein kinase C</td>
</tr>
<tr>
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<td>plasma membrane Ca&lt;sup&gt;2+&lt;/sup&gt;-ATPase</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PLB</td>
<td>phospholamban</td>
</tr>
<tr>
<td>ROC</td>
<td>receptor operated channel</td>
</tr>
<tr>
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<td>resting vessel diameter</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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<tr>
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<td>smooth muscle cell</td>
</tr>
<tr>
<td>SRCA</td>
<td>sarcoplasmic reticulum Ca&lt;sup&gt;2+&lt;/sup&gt;-ATPase</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
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<td>small conductance K&lt;sub&gt;Ca&lt;/sub&gt; channel</td>
</tr>
<tr>
<td>TRP</td>
<td>transient receptor potential</td>
</tr>
<tr>
<td>VDCC</td>
<td>voltage dependent calcium channel</td>
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</table>
CHAPTER 1: INTRODUCTION.................................................................1

1.1 CALCIUM HOMEOSTASIS IN SMOOTH MUSCLE CELLS.........................1

1.2 SOURCES OF CALCIUM FOR CONTRACTION.........................................4
  1.2.1 Extracellular calcium.................................................................4
  1.2.2 Intracellular calcium.................................................................6

1.3 MECHANISMS UNDERLYING SMOOTH MUSCLE CONTRACTION..............8
  1.3.1 Calcium dependent contraction of smooth muscle.........................8
  1.3.2 Calcium independent contraction of smooth muscle.......................9

1.4 MECHANISMS UNDERLYING SMOOTH MUSCLE RELAXATION...................9
  1.4.1 Relaxation and the role of the endothelium.................................10
  1.4.2 NO/cGMP/PKG.................................................................10
  1.4.3 Prostacyclin.............................................................................11
  1.4.4 Endothelium derived hyperpolarizing factor (EDHF).......................12

1.5 CELLULAR COUPLING: GAP JUNCTIONS..............................................13

1.6 ELEMENTARY CALCIUM RELEASE EVENTS..........................................14
  1.6.1 Calcium sparks...........................................................................15
  1.6.2 Calcium waves............................................................................16
    1.6.2.1 Smooth muscle.................................................................16
    1.6.2.2 Endothelium.................................................................19

1.7 CALCIUM DYNAMICS AND MYOENDOTHELIAL GAP JUNCTIONS..............20

1.8 VASOMOTION.....................................................................................21
  1.8.1 Vasomotion and calcium oscillations.........................................22
  1.8.2 Role of cellular coupling..........................................................25
  1.8.3 Endothelium.............................................................................26
  1.8.4 Ionic mechanisms.....................................................................28
  1.8.5 Modelling.................................................................................33
3.2.4 Effect of nifedipine on rhythmical contractions, membrane potential and calcium ........................................57
3.2.5 Current clamp of short segments ............................................................................................................57
3.2.6 Voltage clamp of short segments ............................................................................................................58
3.2.7 Effect of BAPTA-AM on rhythmical contractions and membrane potential ...........................................58
3.2.8 Effect of ryanodine and caffeine on rhythmical contractions ....................................................................59
3.2.9 Effect of DIDS on rhythmical contractions, membrane potential and calcium ...........................................59
3.2.10 Effect of phospholipase C pathway inhibition on rhythmical contractions and calcium .......................59
3.2.11 Effect of 2-APB on rhythmical contractions ..........................................................................................60
3.2.12 Effect of phospholipase A2 pathway inhibition on rhythmical contractions and calcium ......................60
3.2.13 Effect of phospholipase A2 pathway agonists on rhythmical contractions ............................................61
3.2.14 Role of the vascular endothelium in rhythmical contractions ...................................................................62
3.2.15 Confirmation of endothelial removal .....................................................................................................62

3.3 DISCUSSION ............................................................................................................................................63
3.3.1 Spontaneous vasomotion in rat iris arterioles ..........................................................................................63
3.3.2 Spontaneous contractions are voltage independent ..................................................................................65
3.3.3 Absence of a role for the ryanodine sensitive intracellular calcium store .............................................66
3.3.4 A role for phospholipase C and the IP3 sensitive intracellular calcium store ............................................67
3.3.5 Chloride channel inhibition abolishes rhythmical activity .......................................................................68
3.3.6 A role for the phospholipase A2 pathway in spontaneous vasomotion ..................................................70
3.3.7 Rhythmical contractions occur independently of the endothelium ....................................................71
3.3.8 Proposed mechanism underlying spontaneous contractions of iris arterioles .......................................72

CHAPTER 4: VOLTAGE DEPENDENCE OF VASOMOTION IN THE RAT BASILAR ARTERY ..............................................74
4.1 INTRODUCTION .....................................................................................................................................74
4.2 RESULTS ...................................................................................................................................................75
4.2.1 General observations .................................................................................................................................75
4.2.1.1 Contractions ..................................................................................................................................75
4.2.1.2 Measurement of smooth muscle cell membrane potential ................................................................76
4.2.1.3 Measurements of smooth muscle cell $[Ca^{2+}]_{i}$ ............................................................................76
4.2.1.4 Rhythmical activity over time .............................................................................................................76
4.2.1.5 Variations in vasomotion and synchronicity of $Ca^{2+}$ oscillations ....................................................77
4.2.2 Role of extracellular calcium in rhythmical contractions and $[Ca^{2+}]_{i}$ ..................................................78
4.2.3 Role of intracellular calcium stores .......................................................................................................79
4.2.3.1 Effect of phospholipase C pathway inhibition on spontaneous rhythmical activity ..........................79
4.2.3.2 Effect of ryanodine on spontaneous rhythmical activity .....................................................................80
4.2.3.3 Spatial distribution of intracellular calcium stores ...........................................................................81
4.2.4 Effect of phospholipase A2 pathway .......................................................................................................81
4.2.5 Role of ion channels .................................................................................................................................82
4.2.5.1 Involvement of calcium dependent chloride ($Cl_{Ca}$) channels .........................................................82

xiii
4.3 DISCUSSION ......................................................................................................................... 84

4.3.1 Spontaneous vasomotion in the juvenile rat basilar artery .................................................. 85
4.3.2 Variation in cellular coupling during spontaneous vasomotion ............................................ 85
4.3.3 Essential role for phospholipase C and the IP₃ sensitive intracellular calcium store ........... 86
4.3.4 Protein kinase C inhibition modulates rhythmic activity ......................................................... 87
4.3.5 Rhythmic contraction require extracellular calcium ................................................................. 88
4.3.6 Voltage dependent calcium channels synchronize rhythmic activity ..................................... 88
4.3.7 Organization of intracellular calcium stores ............................................................................. 89
4.3.8 Chloride channel inhibition abolishes rhythmic activity .......................................................... 90
4.3.9 A role for intermediate conductance calcium activated potassium (IKCa) channels ............ 90
4.3.10 Absence of a role of phospholipase A₂ signaling pathway ....................................................... 91
4.3.11 Proposed mechanisms underlying spontaneous vasomotion in the rat basilar artery ............ 92

CHAPTER 5: ROLE FOR THE ENDOTHELium IN RAT BASILAR ARTERY VASOMOTION .93

5.1 INTRODUCTION ....................................................................................................................... 93
5.2 RESULTS .................................................................................................................................... 94

5.2.1 Anatomical characteristics and morphological identification of myoendothelial gap junctions .... 94
5.2.2 Connexin specific immunohistochemistry in the juvenile rat basilar artery ............................... 95
5.2.3 Rhythmic activity in dye identified cells .................................................................................. 95
5.2.4 Role of the vascular endothelium in spontaneous rhythmic vasomotion ................................. 96
5.2.5 Confirmation of successful endothelial removal ....................................................................... 97
5.2.6 Effect of nitric oxide inhibition on spontaneous rhythmic activity .......................................... 98
5.2.7 Effect of cGMP on spontaneous rhythmic activity in endothelium intact and endothelium denuded preparations ........................................................................................................ 98
5.2.8 Effect of EDHF inhibition on spontaneous rhythmic activity ..................................................... 99

5.3 DISCUSSION ............................................................................................................................ 100

5.3.1 Cell coupling in the arterial wall ............................................................................................... 100
5.3.2 Connexin distribution in the endothelial and smooth muscle cell layers .................................... 101
5.3.3 Role of the endothelium in spontaneous vasomotion ............................................................... 102
5.3.4 Role of NO and cGMP in spontaneous vasomotion .................................................................. 104
5.3.5 Role of EDHF in spontaneous vasomotion .............................................................................. 107
5.3.6 Proposed model of cellular coupling in rat basilar artery vasomotion ........................................ 108

CHAPTER 6: EFFECT OF GAP MIMETIC PEPTIDES ON RAT BASILAR ARTERY VASOMOTION ................................................................................................................................. 110

6.1 INTRODUCTION ....................................................................................................................... 110
6.2 RESULTS ................................................................................................................................... 111

6.2.1 Cx mimetic peptides homologous to the extracellular Gap 27 sequence ............................... 111
6.2.1.1 Effect of $^{37,43}$Gap 27 ...............................................................111
6.2.1.2 Effect of $^{40}$Gap 27 .................................................................112
6.2.1.3 Effect of $^{45}$Gap 27 .................................................................113
6.2.2 Cx mimetic peptides homologous to the extracellular Gap 26 sequence .................................................................113
6.2.2.1 Effect of $^{37,40}$Gap 26 ...............................................................113
6.2.2.2 Effect of $^{43}$Gap 26 .................................................................114
6.2.3 Cx mimetic peptides homologous to additional extracellular sequences .................................................................114
6.2.3.1 Effect of Gap 37 .................................................................114
6.2.3.2 Effect of Gap 43 .................................................................115
6.2.3.3 Effect of Gap 37 and 43 in combination .................................................................115
6.2.3.4 Effect of Gap 40 .................................................................115
6.2.3.5 Effect of Gap 45 .................................................................116
6.3 DISCUSSION .................................................................................................116
6.3.1 $^{37,43}$Gap 27 and Gap 37 and Gap 43 in combination abolish vasomotion .................................................................117
6.3.2 Role of Cx43 in spontaneous vasomotion .................................................................118
6.3.3 Cx40 in the endothelium synchronizes spontaneous rhythmical activity .................................................................119
6.3.4 Role of Cx37 in spontaneous vasomotion .................................................................120
6.3.5 Role of Cx45 in spontaneous vasomotion .................................................................120
6.3.6 Conclusions .................................................................................................120

CHAPTER 7: GENERAL DISCUSSION .........................................................................................122
7.1 A comparative study of vasomotion .................................................................................................123
7.2 Vasomotion in the basilar artery: a working model .................................................................................125
7.3 Methodological limitations .................................................................................................................................129
7.4 Implications for future research .................................................................................................................................130

APPENDIX: DIFFERENTIAL EXPRESSION OF INOSITOL 1,4,5-TRISPHOSPHATE RECEPTOR
SUBTYPES BETWEEN THE SMOOTH MUSCLE AND ENDOTHELIAL CELL LAYERS OF RAT
ARTERIES .................................................................................................................132
A.1 INTRODUCTION .................................................................................................................................132
A.2 METHODS .................................................................................................................................................133
A.2.1 Messenger RNA extraction and reverse transcription .................................................................................133
A.2.2 Quantitative PCR .............................................................................................................................................134
A.2.3 Immunohistochemistry .............................................................................................................................................135
A.3 RESULTS .................................................................................................................................................136
A.3.1 Differential mRNA expression for each IP$_3$R subtype .................................................................................136
A.3.2 Differential protein expression for each IP$_3$R subtype .................................................................................137
A.3.2.1 Distribution of IP$_3$R1 .............................................................................................................................................137
A.3.2.2 Distribution of IP$_3$R2 .............................................................................................................................................137
CHAPTER 1
INTRODUCTION

Changes in intracellular calcium concentration ([Ca\(^{2+}\)]_i) control many fundamental cellular responses including transcriptional activation, cell differentiation and proliferation, hormone secretion, apoptosis and muscle contraction (Berridge et al., 2000). In this regard, calcium (Ca\(^{2+}\)) is considered to be the universal intracellular messenger. However, the ability to control such a multitude of physiological outcomes by a single ion relies on the capacity of individual cells to generate and shape calcium signals with different spatial, temporal and kinetic patterns (Iino, 1999; Bootman et al., 2001; Berridge et al., 2003). The mechanisms by which such intracellular heterogeneity can occur are thought to arise from variations in the distribution and interaction of membrane receptors, ion channels and the production/ and or diffusion of second messengers (Pabelick et al., 2001). Moreover, in blood vessels, it is becoming critically evident that functional cellular communication, via gap junctions, between the individual cells that comprise the vascular wall is necessary for the integration, modulation and coordination of these different Ca\(^{2+}\) signals in order to ensure the appropriate control of vascular tone and proper tissue function (Christ et al., 1996).

1.1 CALCIUM HOMEOSTASIS IN SMOOTH MUSCLE CELLS

In vascular smooth muscle cells (SMCs) a sufficient increase in intracellular calcium concentration ([Ca\(^{2+}\)]_i), leads to activation of the contractile apparatus. At rest, however, the rate of calcium (Ca\(^{2+}\)) entry is considered low. The [Ca\(^{2+}\)]_i of a resting cell varies between cell types, tissues and species (Kuriyama et al., 1998), however, in SMCs it is generally accepted to be ~100 nM compared to the concentration of extracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]_e) at ~ 1 mM (Alvarez et al., 1999; Marin et al., 1999; Baylor & Hollingworth, 2000; Rottingen & Iversen, 2000; Bootman et al., 2001). Stimulation of SMCs is thought to increase [Ca\(^{2+}\)]_i as high as 1 M (Becker et al., 1989; Orallo, 1996; Bootman et al., 2001; Sanders, 2001). Sustained, elevated levels of [Ca\(^{2+}\)]_i are thought to be toxic to SMCs (Hamm & Opie, 1983; Savineau & Marthan, 2000; Bootman et al., 2001; Lee et al., 2002a),
therefore, it is not surprising that a number of mechanisms exist in order to decrease [Ca$^{2+}$]$_i$ and restore Ca$^{2+}$ homeostasis. In SMCs, Ca$^{2+}$ homeostasis is achieved by transporting [Ca$^{2+}$]$_i$ either from the cytosol to the cell exterior, or conversely, into intracellular organelles, such as the sarcoplasmic reticulum (SR) (Gustafsson, 1993; Shuttleworth, 1999) thus restoring the large imbalance between [Ca$^{2+}$]$_i$ and [Ca$^{2+}$]$_e$.

The two major mechanisms by which Ca$^{2+}$ efflux from SMCs occurs is via the plasma membrane Ca$^{2+}$-ATPase (PMCA), which has a high affinity for Ca$^{2+}$ and the sodium/calcium (Na$^+$/Ca$^{2+}$) exchanger, which has a low affinity for Ca$^{2+}$ but high capacity for transporting Ca$^{2+}$ (Guerini, 1998; Blaustein & Lederer, 1999). The PMCA uses energy from ATP to actively pump Ca$^{2+}$ against its electrochemical gradient from the cytosol to the extracellular space and is thought to be electroneutral, pumping 1 Ca$^{2+}$ out for every 2 H$^+$ in (Niggli, 1982; Furukawa et al., 1989; Salvador et al., 1998). The PMCA is activated by the binding of calmodulin to the C terminal calmodulin-binding domain of the pump, causing a conformational change and increasing its affinity for calcium. Conversely, in the absence of calmodulin, the PMCA undergoes autoinhibition and has been shown to be further regulated by various protein kinases, most notably PKC (Missiaen et al., 1992; Kuriyama et al., 1995; Guerini, 1998; Zylinska & Soszynski, 2000; Sanders, 2001). Differences in the expression of PMCAs in vascular SMC tissues have been observed between species and tissue beds (Eggermont et al., 1988). Interestingly, the PMCA appears to be less abundant in SMCs from small blood vessels than in SMCs of larger arteries (Kwan et al., 1986).

In contrast to the PMCA, the Na$^+$/Ca$^{2+}$ exchanger is electrogenic and transports 1 Ca$^{2+}$ out for every 3 Na$^+$ (Ashida & Blaustein, 1987; Slaughter et al., 1989). On the other hand, like the PMCA, the relative contribution of the Na$^+$/Ca$^{2+}$ exchanger to Ca$^{2+}$ efflux is thought to vary between different vascular beds and in vessels of different size (Furukawa et al., 1988; Karaki et al., 1997; Nazer & van Breemen, 1998). Although the precise function of this exchanger in vascular SMC tissues remains somewhat controversial (Blaustein & Lederer, 1999), it is generally accepted the Na$^+$/Ca$^{2+}$ exchanger assists in the bulk ejection of excess [Ca$^{2+}$]$_i$ following contraction. Hence extrusion of Ca$^{2+}$ via the Na$^+$/Ca$^{2+}$ exchanger would appear to dominate over extrusion via the PMCA pump in many smooth muscle tissues (Orallo, 1996; Kuriyama et al., 1998). The Na$^+$/Ca$^{2+}$ exchanger can also perform in 'reverse mode' whereby extracellular Ca$^{2+}$ is transported into the cell in exchange for Na$^+$, depending on the electrochemical gradient for Na$^+$ and Ca$^{2+}$ across the cell membrane (Vennekens, 2002).
[Ca\(^{2+}\)], is also maintained at low levels by uptake and storage of Ca\(^{2+}\) into a number of cellular organelles. The endoplasmic reticulum (ER) or sarcoplasmic reticulum (SR) in smooth muscle is an interconnected, tubular network and is considered to be the most important intracellular organelle in terms of Ca\(^{2+}\) homeostasis due to its ability to store high concentrations of Ca\(^{2+}\) (5-10mM) (van Breemen & Saida, 1989). The membrane of the SR is impermeable to Ca\(^{2+}\) and uptake is mediated by SR Ca\(^{2+}\)-ATPases (SRCA) which actively pump Ca\(^{2+}\) from the cytosol to the SR lumen (1 Ca\(^{2+}\) in for 1 K\(^{+}\) and H\(^{+}\) out) (Grover & Khan, 1992). Structurally, the SRCA is similar to the PMCA except that it appears to lack the calmodulin binding domain. Instead it is regulated by the small protein, phospholamban (PLB) which physically interacts with the SRCA to inhibit its activity (Frank et al., 2003; MacLennan & Kranias, 2003). Phosphorylation of PLB by protein kinases leads to dissociation of PLB and disinhibition of the SRCA, thus increasing SRCA activity (Simmerman & Jones, 1998). Pharmacologically, the SRCA is inhibited by thapsigargin, and cyclopiazonic acid (Darby et al., 1993). These agents appear to differentially inhibit the SRCA depending on the vascular bed or species investigated and it has been suggested that these different sensitivities may reflect functionally, or spatially separate calcium stores within the SR (Golovina & Blaustein, 1997). After Ca\(^{2+}\) is pumped into the SR, it is buffered by calcium binding proteins, such as calsequestrin or calreticulin. Binding of Ca\(^{2+}\) to these proteins decreases [Ca\(^{2+}\)] in the SR lumen, thereby decreasing the energy required to pump Ca\(^{2+}\) in from the cytosol against its concentration gradient (Milner et al., 1992; Sanders, 2001).

Until recently, it was thought that mitochondria played a significant role in Ca\(^{2+}\) homeostasis only in those cells with pathologically high [Ca\(^{2+}\)]\(_i\) levels (Somlyo & Himpens, 1989), however it is now generally accepted that [Ca\(^{2+}\)]\(_i\), removal from the cytoplasm to mitochondria may also be important at low [Ca\(^{2+}\)]\(_i\) levels (Kamishima & Quayle, 2002; Sward et al., 2002; Szado et al., 2003). In vascular SMCs, increases in [Ca\(^{2+}\)]\(_i\) have been shown to elevate mitochondrial [Ca\(^{2+}\)]\(_m\), suggesting that mitochondria can directly modulate cellular calcium dynamics by buffering calcium diffusion (Karaki & Weiss, 1981; Drummond & Tuft, 1999; Monteith & Blaustein, 1999; Gurney et al., 2000; Szado et al., 2003; Poburko et al., 2004). Transport of Ca\(^{2+}\) from the cytosol into the mitochondria has been shown to occur via a low affinity, high capacity uniporter which is coupled to oxidative phosphorylation. The electrochemical gradient present across the inner
mitochondrial membrane is considered to drive $\mathrm{Ca}^{2+}$ into the mitochondrial lumen (Gunter & Pfeiffer, 1990).

The role of the nucleus as a possible store of calcium remains unclear. Because the SR has been reported to be contiguous with the nuclear membrane (Somlyo, 1985) and has been shown regulate its $[\mathrm{Ca}^{2+}]$ independently of that in the cytoplasm (Himpens et al., 1994), it has been speculated that the nucleus may also play a role in calcium homeostasis in SMCs.

1.2 SOURCES OF CALCIUM FOR CONTRACTION

Under *in vivo* conditions, vascular SMCs are rarely at rest (Dora, 2001b). In other words, $[\mathrm{Ca}^{2+}]_i$ exceeds the 100 nM threshold that defines the resting state and the muscle is in a partially contracted state. This is referred to as vascular tone and is due to the interaction of various vasoactive factors, including neural, hormonal and autoregulatory mechanisms, which act to increase basal $[\mathrm{Ca}^{2+}]_i$ levels (Johnson, 1986). SMC contraction occurs following elevation of $[\mathrm{Ca}^{2+}]_i$, which can attributed to at least three different mechanisms; one based entirely on voltage dependent events, such as through the influx of extracellular $\mathrm{Ca}^{2+}$ through voltage dependent $\mathrm{Ca}^{2+}$ permeable ion channels in the plasma membrane, one involving entirely voltage independent events, such as following the release of $\mathrm{Ca}^{2+}$ from intracellular stores, and the other relying on both voltage dependent and independent events (McDonald et al., 1994; Kuriyama et al., 1995; Karaki et al., 1997; Kuriyama et al., 1998).

1.2.1 Extracellular calcium

Most commonly, extracellular $\mathrm{Ca}^{2+}$ enters SMCs through voltage dependent calcium channels (VDCCs) which are activated by depolarization of the membrane. In blood vessels, the VDCCs are predominantly dihydropyridine sensitive L-type channels (Nelson et al., 1990), although the presence of T-type and dihydropyridine-insensitive rapidly inactivating voltage dependent channels has also been recently described (Triggle, 1997; Hansen et al., 2001; Morita et al., 2002). Moreover, modulation of the membrane potential and thus control of $\mathrm{Ca}^{2+}$ influx through VDCCs and vascular tone has been shown to be tightly coupled to the activation and inactivation of a number of different ion channels in the SMC membrane, including chloride ($\mathrm{Cl}^-$) and potassium ($\mathrm{K}^+$) channels. Agonist
induced Cl\(^{-}\) conductances have been described in a number of vascular smooth muscle tissues including the rabbit aorta, ear artery, portal vein and renal arteriole, the rat mesenteric, cerebral, pulmonary, coronary arteries, anococcygeus muscle, aorta and portal vein and guinea-pig mesenteric artery, the activation of which leads to depolarization of the membrane and opening of VDCCs, ultimately increasing [Ca\(^{2+}\)], and initiating contraction (Byrne & Large, 1984, 1985; Van Helden, 1988; Pacaud et al., 1989; Amedee et al., 1990; Hogg et al., 1994a, b; Wang et al., 1997; Hansen et al., 1998; Lamb & Barna, 1998; Hirakawa et al., 1999; Graves et al., 2000; Lamb et al., 2000; Doughty & Langton, 2001; Kitamura & Yamazaki, 2001). Conversely the activation of K\(^{+}\) channels has been shown to hyperpolarize SMCs and decrease the opening probability of the VDCCs, thus reducing Ca\(^{2+}\) influx into the cell and inducing relaxation. Modulation of VDCCs through alterations in K\(^{+}\) conductances have been described (Byrne & Large, 1984, 1985; Nelson et al., 1990; Brayden & Nelson, 1992; Bolotina et al., 1994; Faraci & Heistad, 1998; Standen & Quayle, 1998; Chrissobolis et al., 2000; Gurney et al., 2002; Cole & Clement-Chomienne, 2003).

Extracellular Ca\(^{2+}\) can also cross the plasma membrane by passing through non-voltage gated, Ca\(^{2+}\) permeable channels including the receptor operated channels (ROCs) which open as a result of an agonist binding to a G-protein coupled receptor, and store-operated channels (SOCs), which open in response to depletion of the SR Ca\(^{2+}\) stores (Hurst, 1998; Putney & McKay, 1999; Ma et al., 2000; McFadzean & Gibson, 2002). Both of these channels have been found to be present in SMCs from a number of vascular beds and mounting evidence would suggest an important role for the TRP (Transient Receptor Potential) gene family which encodes the proteins that form these channels (Fellner & Arendshorst, 1999; Xu & Beech, 2001; Albert & Large, 2002; Flemming et al., 2002; Jung et al., 2002; Large, 2002; Lee et al., 2002b; Wilson et al., 2002). At least 20 mammalian TRP proteins have been identified in this family, which have been divided into three main subfamilies on the basis of sequence homology, TRPC (TRPC1-7), TRPV (TRP1-5) and TRPM (TRPM1-8) (Clapham et al., 2001; Minke, 2002; Montell et al., 2002). In brief, the activation of the mammalian TRPC channels is varied and thought to occur following the activation of a G-protein coupled receptor or tyrosine kinase coupled receptor and the subsequent activity of phospholipase C (PLC) and/or production of DAG (Montell, 2001). Activity of PLC also leads to the formation of IP\(_{3}\) causing depletion of Ca\(^{2+}\) from the IP\(_{3}\) sensitive intracellular stores. Some TRPC channels in the plasma membrane are thought to be directly activated by IP\(_{3}\) receptors, due to conformational coupling, in response to IP\(_{3}\).
binding or subsequent to store depletion (Boulay et al., 1999; Ma et al., 2000), while depletion of the SR activates the TRPs through unknown mechanisms (Vennekens, 2002). PLC dependent but store independent mechanisms have also been proposed, suggesting that DAG can also activate TRPC channels either directly or through its breakdown products such as arachidonic or linoleic acid (Hoffman, 1999; Lintschinger, 2000; Inoue, 2001). TRPV channels are activated through mechanical and physical stimuli such as low pH, heat and osmotic stress, or through the binding of ligands such as capsaicin and anadamide (Caterina & Julius, 1999; Vennekens, 2002). On the other hand, little information is available regarding the regulation of TRPM channels. Methanol and changes in temperature have been shown to activate TRPM8, while others are gated by ADP-ribose (TRPM2) and phosphorylation (TRPM7) (Clapham et al., 2001; Runnels et al., 2001; Minke, 2002; Vennekens, 2002). Thus, while the investigation of the role of these channels remains outside the scope of the present study, it is recognized that TRP channels most likely play an important role in the normal regulation of blood vessel function (Friechel, 2001; Inoue, 2001; Schilling, 2001; Xu & Beech, 2001; Sweeney, 2002; Welsh, 2002; Nilius, 2003).

Ca\(^{2+}\) may also enter the cell through stretch activated channels (SACs) which open in response to a mechanical stimulus, such as shear stress during turbulent blood flow (Kirber et al., 2000; Cornelissen et al., 2001; Wu & Davis, 2001; Ji et al., 2002; Mohanty & Li, 2002)

### 1.2.2 Intracellular calcium

Release of Ca\(^{2+}\) from the SR is mediated through both ryanodine (RyR) and inositol 1,4,5-trisphosphate (IP\(_3\)) receptor channels, via mechanisms termed Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) and IP\(_3\)-induced Ca\(^{2+}\) release (IICR), respectively.

Calcium induced calcium release involves the process by which a rise in [Ca\(^{2+}\)], triggers further Ca\(^{2+}\) release from the SR. Therefore, in vascular smooth muscle cells, it is not surprising that RyRs are primarily activated by [Ca\(^{2+}\)], (1-10 \(\text{M}\)), typically following entry of extracellular calcium through L-type voltage dependent calcium channels (Sutko & Airey, 1996; Collier, 2000; Fill & Copello, 2002).

Molecular cloning studies have identified at least three mammalian isoforms of the RyR (RyR1-3), which have subsequently been shown to be widely distributed both across species and from different tissues within the same species (Sutko & Airey, 1996; Franzini-
Armstrong & Protasi, 1997). The RyR1 isoform is predominantly expressed in skeletal muscle (Takeshima et al., 1989; Shoshan-Barmatz & Ashley, 1998; Ogawa et al., 1999), although it has also been identified in some parts of the brain (Furuichi et al., 1994; Giannini et al., 1995) and in smooth muscle (Neylon et al., 1995; Coussin et al., 2002). RyR2 is most widely distributed in cardiac muscle and is the major isoform present in brain (McPherson & Campbell, 1993; Giannini et al., 1995; Tunwell et al., 1996), however some expression in smooth muscle has also been reported (Neylon et al., 1995; Coussin et al., 2000). RyR3 is thought to be the least widely distributed of the three isoforms, having been detected in minor amounts in skeletal muscle, brain and smooth muscle tissues (Neylon et al., 1995; Sutko & Airey, 1996; Franzini-Armstrong & Protasi, 1997; Flucher et al., 1999; Sorrentino & Reggiani, 1999; Jiang et al., 2003). RyR channels have also been shown to be activated by caffeine and are locked into an open subconductance state by low concentrations of ryanodine (1-10 μM) (Rousseau et al., 1987; Smith et al., 1988; Karaki et al., 1997; Marin et al., 1999). Conversely, RyRs are inhibited by high concentrations of [Ca²⁺]ᵢ (>10 μM) and ryanodine (~100 μM) (Bootman et al., 2001).

The IP₃ receptor operated SR Ca²⁺ store is present in all SMCs and therefore appears to be the most important in physiological terms (Orallo, 1996). IP₃ induced calcium release occurs following the activation of phospholipase C (PLC) and the generation of IP₃, which binds to the IP₃R channels, releasing Ca²⁺ from the SR. Although Ca²⁺ release through this channel absolutely requires IP₃, activation of the receptor has also been shown to be dependent on Ca²⁺ (Kuriyama et al., 1995). Furthermore, this relationship appears to be biphasic, whereby small increases in [Ca²⁺]ᵢ (0.5-1 μM) open the channel, while high [Ca²⁺]ᵢ have been shown to inhibit the channel (Bootman et al., 2001).

Like the RyR, there are three different isoforms of the IP₃R (IP₃R1-3), all of which show subtle differences in their gating kinetics for IP₃ and Ca²⁺ (Thrower et al., 2001; Bultynck et al., 2003). In the low nM range of [Ca²⁺]ᵢ, the IP₃Rs bind IP₃ with different affinities (IP₃R2> IP₃R1> IP₃R3) (Newton et al., 1994; Wojcikiewicz & Luo, 1998). In addition, IP₃R1 has been shown to be inactivated by [Ca²⁺]ᵢ of >0.3 μM, whereas IP₃R2 and IP₃R3 stay open at higher [Ca²⁺]ᵢ (Haberichter et al., 2002; Swatton & Taylor, 2002). Compounds such as heparin, xestospongin and 2-aminoethoxydiphenyl borate (2-APB) are commonly used to inhibit the IP₃R and thus IP₃ induced calcium release, however, the specificity of these inhibitors appears to vary between species and tissues (Ehrlich et al., 1994; Bootman et al., 2002). Changes in membrane potential and [Ca²⁺]ᵢ may even affect
IP$_3$ production itself (Itoh et al., 1992; Van Helden et al., 2000). A recent study in rat cerebral arterial myocytes has raised the intriguing possibility that calcium release from IP$_3$ sensitive intracellular stores may be directly regulated by changes in membrane potential, since membrane depolarization has been shown to evoke Ca$^{2+}$ release from these stores in a graded manner that can produce cell contraction in the absence of agonists or extracellular Ca$^{2+}$ influx. Coupling of myocyte depolarization to Ca$^{2+}$ release from the SR depends on VDCC that, after sensing the change in membrane potential, activate G-proteins and subsequently the PLC/IP$_3$ signalling pathway (del Valle-Rodriguez et al., 2003).

A number of studies have suggested that, in smooth muscle tissues, RyR and IP$_3$Rs actually define two functionally and anatomically distinct compartments within the SR (Tribe, 1994; Elmoselhi et al., 1996; Golovina & Blaustein, 1997; Janiak et al., 2001). Others have reported that SMCs contain one type of calcium store, either ryanodine sensitive or IP$_3$ sensitive, only (Burdyga et al., 1998; Hill et al., 1999; Boittin et al., 2000). On the other hand, significant evidence also exists to suggest that RyR and IP$_3$R sensitive calcium release may involve partially or completely overlapping calcium stores (Komori & Bolton, 1991; Zholos et al., 1994; Komori et al., 1995; Pacaud & Loirand, 1995; Flynn et al., 2001; Janiak et al., 2001). Nevertheless, it is clear that in some smooth muscle tissues, calcium release from IP$_3$ sensitive calcium stores activates RyRs in order to amplify the calcium signal in response to agonist stimulation (Boittin et al., 1999; Bayguinov et al., 2000; Gordienko & Bolton, 2002). The scope for such interactions will vary depending on receptor distribution and isoform interaction in different cells.

1.3 MECHANISMS UNDERLYING SMOOTH MUSCLE CONTRACTION

1.3.1 Calcium dependent contraction of smooth muscle

Contraction of vascular smooth muscle occurs via a complex cascade of activated protein kinases, typically following a global rise in [Ca$^{2+}$]$_i$. It is generally accepted that contraction is initiated by the binding of Ca$^{2+}$ to calmodulin (CaM) (Kamm, 1985). The Ca$^{2+}$-CaM complex binds to the catalytic subunit of myosin light chain (MLC) kinase which then phosphorylates the MLC, causing a conformational change. The phosphorylated myosin interacts with actin to produce contraction (Kamm, 1985; Somlyo & Somlyo, 2000). Phosphorylated MLC is dephosphorylated by MLC phosphatase. The amount of phosphorylated MLC is therefore dependent on the balance between the MLC kinase and
MLC phosphatase (Lee et al., 1997; Woodrum & Brophy, 2001), both of which are highly regulated through phosphorylation by a number of signalling molecules, such as PKC and Rho kinase in a process known as calcium sensitization (Chrissobolis, 2001).

1.3.2 Calcium independent contraction of smooth muscle

Calcium sensitization is the process by which the contractile force of smooth muscle persists in the presence of constant cytoplasmic levels of Ca²⁺ (Somlyo & Somlyo, 1994) or at low levels of [Ca²⁺]ᵢ, such as under basal conditions, in vivo (Chrissobolis, 2001). This mechanism is important for sustained contraction, particularly as elevation of the [Ca²⁺]ᵢ within SMCs is often transient, being maintained at low levels by a system of homeostatic regulatory mechanisms as discussed previously (Orallo, 1996; Laher & Zhang, 2001). In smooth muscle cells, calcium sensitization is typically brought about by the inhibition or down regulation of myosin light chain phosphotase activity or phosphorylation of filament associated proteins such as caldesmon and calponin (Kamm, 1985; Masuo, 1994; Somlyo & Somlyo, 1994; Wesselman et al., 2001; Shaw et al., 2003). The activity of multiple second messengers/signalling pathways, including Rho A / Rho-kinase (Kureishi et al., 1997; Sauzeau et al., 2000; Chrissobolis, 2001; Bolz et al., 2003), PKC (Masuo, 1994; Gokina et al., 1999; Wesselman et al., 2001) and arachidonic acid (Gong, 1992) pathways have been linked to calcium sensitization mechanisms. Agonists and second messengers have also been reported to directly modify the MLC kinase/MLC phosphatase activity ratio independently of [Ca²⁺]ᵢ (Yagi et al., 1988; Lee et al., 1999; Schlossmann et al., 2003; Somlyo & Somlyo, 2003) and several recent studies have suggested a mechanism involving interactions between the extracellular matrix, cytoskeleton and important downstream signalling molecules in the calcium sensitization process (Deng et al., 2001; Taggart, 2001). The implication of these latter pathways in vasomotor responses is not clear at this stage.

1.4 MECHANISMS UNDERLYING SMOOTH MUSCLE RELAXATION

Typically, vascular smooth muscle relaxation is induced following restoration of [Ca²⁺]ᵢ levels to below the threshold for [Ca²⁺]ᵢ to trigger contraction (Orallo, 1996). Under these circumstances, calmodulin dissociates from the MLC kinase, which is thereby inactivated and myosin is dephosphorylated by MLC phosphatase and remains detached from actin
SMC relaxation can also occur independently of changes in \([\text{Ca}^{2+}]\), following a reduction in the sensitivity of the contractile apparatus to \(\text{Ca}^{2+}\) (Somlyo & Somlyo, 2000, 2003). Since \(\text{Ca}^{2+}\) can be raised by both voltage dependent and independent mechanisms, it seems logical that the mechanisms underlying relaxation should have a similar basis.

1.4.1 Relaxation and the role of the endothelium

The endothelium plays an important role in the regulation of vascular tone through the production and release of a number of potent vasoactive factors, including nitric oxide (NO), prostacyclins (PGI\(_2\)) and endothelium derived hyperpolarizing factor (Furchgott & Zawadzki, 1980; Taylor & Weston, 1988; Mombouli & Vanhoutte, 1997). These mediators, which act on underlying SMCs to cause relaxation, are generated in response to a wide variety of chemical factors, including acetylcholine (ACh), ATP and bradykinin, or in response to haemodynamic forces such as shear stress (Moncada \textit{et al.}, 1991). The importance of each vasodilatory factor as a mediator of SMC relaxation has been reported to vary depending on the vascular bed and animal species studied (Hill \textit{et al.}, 2001). Thus, while both NO and EDHF have been shown to play major roles in endothelium-dependent vasodilation, the contribution of each of these factors has been reported to vary inversely proportional to vessel diameter (Feletou & Vanhoutte, 1996; Shimokawa \textit{et al.}, 1996). On the other hand, prostaglandins are generally regarded to play a more minor role (Bauersachs \textit{et al.}, 1997; Mombouli & Vanhoutte, 1999).

1.4.2 NO/cGMP/PKG

NO is formed by the conversion of L-arginine to L-citrulline in a reaction catalyzed by NO synthase (NOS) (Palmer & Moncada, 1989). Several isoforms of NOS have been identified, including endothelial NOS (eNOS), neuronal NOS and inducible NOS (Moncada \textit{et al.}, 1991; Andrew & Mayer, 1999). eNOS can be stimulated to produce NO in response to agonist or in response to increased vasoconstriction and shear stress (Vargas \textit{et al.}, 1990; Fleming & Busse, 1995). Release of eNOS has also been observed under basal conditions (Moncada \textit{et al.}, 1991). NO produced in the endothelium diffuses to the smooth muscle where it activates guanylate cyclase, which subsequently converts GTP to cGMP (Rapoport & Murad, 1983). In vascular smooth muscle cells, the effects of cGMP are mediated through protein kinase G (PKG), the activity of which typically leads to a
reduction in [Ca\(^{2+}\)]_i (McDaniel et al., 1992), or a reduction in the sensitivity of the contractile system to Ca\(^{2+}\), and vasodilatation. (Carvajal et al., 2000).

Thus the mechanisms proposed to account for cGMP dependent relaxation include the direct phosphorylation and inhibition of VDCCs (Tewari & Simard, 1997), activation of K\(_{Ca}\) channels and hyperpolarization of the membrane potential (Chen & Rembold, 1992; Robertson et al., 1993; White et al., 1993; Hampl et al., 1995; Carrier et al., 1997), activation of SRCA due to phosphorylation of phospholamban and uptake of [Ca\(^{2+}\)]_i into the SR (Cornwell et al., 1991; Karczewski et al., 1992), stimulation of the PMCA or the Na\(^+\)/Ca\(^{2+}\) exchanger and extrusion of [Ca\(^{2+}\)]_i from the cell (Furukawa et al., 1991; Yoshida et al., 1999), a reduction in IP\(_3\) formation following the direct inhibition of PLC or inhibition of the G-protein coupled receptor that stimulates PLC (Lincoln & Cornwell, 1993) and direct phosphorylation of the IP\(_3\)R, reducing channel activity in response to IP\(_3\) (Komalavilas & Lincoln, 1994). Evidence would also suggest that in certain arteries, NO can directly activate K\(_{Ca}\) channels independently of cGMP (Bolotina et al., 1994; Yuan et al., 1996). In SMCs, cGMP/PKG pathway activity has also been shown to increase MLC phosphatase activity, independently of MLCK, resulting in an overall decrease in MLC phosphorylation and a reduction in the sensitivity of the contractile apparatus to calcium (Wu et al., 1996; Lee et al., 1997). Others have demonstrated that cGMP/PKG can inhibit PKC activation and decrease Ca\(^{2+}\) sensitivity in those SMCs in which PKC activity contributes to contraction (Kumar et al., 1997).

### 1.4.3 Prostacyclin

Metabolism of arachidonic acid by the cyclooxygenase enzymes leads to the formation of prostaglandins, including prostaglandin H\(_2\). Cell specific synthesis of individual prostanoid products including the vasodilator, prostacyclin and the vasoconstriclor thromboxane A\(_2\), occurs via the action of specific synthases (Davis & Hill, 1999; Smith et al., 1991). Prostacyclin receptors are coupled to adenylate cyclase which elevates cAMP levels in vascular SMC (Kukovetz et al., 1979; Miwa et al., 1997; Vanhoutte, 1999). The mechanisms by which cAMP induces SMC relaxation are believed to occur through the activation of PKA which, like PKG, can phosphorylate key regulatory proteins involved in the control of muscle tone (Rybalkin et al., 2003). Thus, endothelial derived prostaglandins, via cAMP/PKA activity in the vascular smooth muscle, are thought to stimulate K\(_{ATP}\) channels causing hyperpolarization of membrane potential and closure of
VDCCs (Parkington et al., 1995). Increased levels of cAMP have also been reported to inhibit Ca\(^{2+}\) release from intracellular stores, cause a reduction in Ca\(^{2+}\) influx across the cell membrane and increase the sequestration of \([Ca^{2+}]_i\) into intracellular stores. Prostacyclin dependent SMC relaxation via Ca\(^{2+}\) desensitization of the contractile apparatus can occur either by inhibiting MLCK or activating MLC phosphatase (Karaki et al., 1997; Bukoski et al., 1989; Abe & Karaki, 1992; Pelligrino & Wang, 1998). Interestingly, prostacyclin has been shown to facilitate the release of eNOS (Shimokawa et al., 1988). Conversely, the action of prostacyclin in vascular SMCs can be potentiated by NO (Delpy et al., 1996). Indeed the increase in cGMP in some vascular SMCs can inhibit the break down of cAMP (Delpy et al., 1996). Therefore, NO can directly increase the cellular activity of prostacyclin within the smooth muscle.

1.4.4 Endothelium derived hyperpolarizing factor (EDHF)

By definition, EDHF is a non-NO, non-prostanoid factor that is synthesized and released from the endothelium to cause hyperpolarization and relaxation of the underlying smooth muscle (Sandow, 2004). While EDHF activity has been shown to vary within and between vascular beds, between species, strains or sex, during aging and disease, it is generally accepted that the actions of EDHF are mediated by the activation of \(K_{Ca}\) channels proposed to be located on the endothelium (McGuire et al., 2001; Campbell & Gauthier, 2002; Ding, 2003; Griffith et al., 2004; Sandow, 2004). Thus the activation of \(K_{Ca}\) channels results in hyperpolarization of the endothelium, followed by the hyperpolarization of the smooth muscle, closure of the VDCCs and inhibition of Ca\(^{2+}\) influx across the plasma membrane and relaxation of the smooth muscle. Evidence would also suggest that EDHF-mediated SMC hyperpolarization may reduce cell \([Ca^{2+}]_i\) via the inhibition of phospholipase C and Ca\(^{2+}\) release from intracellular stores (Itoh et al., 1992; Yamagishi et al., 1992; Drummond & Cocks, 1996). The inhibition of \(K_{Ca}\) channels with a combination of apamin, selective for small conductance \(K_{Ca}\) (SK\(_{Ca}\) channels and charybdotoxin, which blocks large conductance \(K_{Ca}\) (BK\(_{Ca}\) channels, intermediate conductance \(K_{Ca}\) (IK\(_{Ca}\) channels and some voltage dependent \(K^+\) channels, in the absence of NO and protaglandins, is considered characteristic of the EDHF mediated response (Adeagbo & Triggle, 1993; Cowan et al., 1993; Busse et al., 2002). To date, considerable controversy remains surrounding the precise nature of EDHF and many hypotheses have been proposed concerning the precise mechanisms that mediate this vascular response. One suggestion is
that EDHF mediated relaxation is dependent on the release of a chemical factor that diffuses from the endothelium, to the smooth muscle across the internal elastic lamina. Indeed several putative candidates for a diffusible factor have been proposed, including epoxyeicosatrienoic acids (EETs), anandamide, K⁺ ions, hydrogen peroxide, C-type natriuretic peptide (CNP) and L-NAME insensitive NO (non-NOS) (Sandow, 2004). Others have suggested that there is no chemical factor but that direct electrical coupling via myoendothelial gap junctions (MEGJs) and the passive electrotonic spread of hyperpolarization from the endothelium to the smooth muscle may provide a simple mechanism to explain ‘EDHF’ activity in some vascular beds (Sandow et al., 2002).

1.5 CELLULAR COUPLING: GAP JUNCTIONS

In the vasculature, coordinated tissue responses initiated by the release of neurotransmitters or other stimuli, such as those of humoral or myogenic origin are critical to normal physiological function and rely on the electrical and chemical coupling of the cells that comprise the vascular wall (Christ et al., 1996). Thus in the vasculature, cell coupling via gap junctions occurs between adjacent SMCs, between endothelial cells (ECs) and in some cases, between smooth muscle and ECs (Sandow & Hill, 2000; Hill et al., 2002).

Gap junctions are specialized intercellular protein channels formed in the plasma membranes of adjacent cells that, as previously mentioned, permit the transfer of electrical current and small molecules of less than one kilo Dalton (1 kD) in size between the cytoplasmic domains of coupled cells (Kumar & Gilula, 1996). The protein channels that make up gap junctions consist of two opposing hemichannels or connexons. Each connexon is constructed from six radially arranged connexin protein subunits, consisting of four α-helical transmembrane segments, (three cytoplasmic domains and two extracellular loops) which transverse the plasma membrane of a single cell to create one half of the central aqueus pore. Thus a complete gap junction channel results from the docking of two connexons, one from each of two opposing cells (Musil & Goodenough, 1993; Falk et al., 1997). Moreover, connexons can be formed from a single connexin isotype (homomeric), while others are comprised of multiple connexin isotypes (heteromeric). Consequently, gap junction channels may be homotypic, consisting of two identical homomeric or heteromeric connexons, or heterotypic, containing different homomeric or heteromeric connexin...
subtypes. Channels are known to aggregate within the plane of the membrane to form gap junction plaques (Kumar & Gilula, 1996; Yeh et al., 1998).

Connexins are encoded by a large gene family predicted to comprise at least 20 isoforms in humans (Evans & Martin, 2002; Willecke et al., 2002). Connexin proteins are commonly distinguished by their molecular weight in kDa, and are encoded by a single gene consisting of one exon and one or two introns within the 5' untranslated region (White & Paul, 1999; Saez et al., 2003). In blood vessels, four connexins have been identified, namely Cx37, Cx40, Cx43 and Cx45 (Rummery & Hill, 2004). Thus although only four different connexins are expressed in the vascular wall, the number of structurally different and thus physiologically distinct channels that may be formed is large (Brink et al., 1997). Since homocellular coupling between adjacent ECs and between adjacent smooth muscle cells in addition to heterocellular coupling between endothelial and smooth muscle cells have been observed, significant heterogeneity can occur in connexin expression within and between the cell layers of different vascular beds. Cx37, 40 and 43 have been described in the endothelium of a number of different vessels (Hill et al., 2001). In large conduit arteries, such as the aorta, Cx43 is the most abundant gap junctional protein within the vascular smooth muscle (Rummery & Hill, 2004). The distinct functional properties of each Cx protein arise from amino acid sequences specific for each Cx, located in the intracellular loop and carboxy terminus (Kumar & Gilula, 1996; Saez et al., 2003; Sohl & Willecke, 2004). The extracellular loops and the four transmembrane regions contain many highly conserved residues which are thought to be involved in mediating the docking of the two connexons during the formation of gap junction channels, as well as voltage gating of the channel and formation of the aqueous pore (Unwin, 1989; Kumar & Gilula, 1996; Dhein, 1998; Sosinsky, 1996; Yeager & Nicholson, 1996; Evans & Martin, 2002; Sohl & Willecke, 2004).

1.6 ELEMENTARY CALCIUM RELEASE EVENTS

In vascular smooth muscle, global cytosolic [Ca\textsuperscript{2+}]\textsubscript{i} represents the average Ca\textsuperscript{2+} level throughout the entire cytoplasm of the cell, and as such, has been considered to be the key regulator of smooth muscle contraction. However, Ca\textsuperscript{2+} sensitive, Ca\textsuperscript{2+} released from the SR does not necessarily lead to an obligatory all or none response within smooth muscle cells (Wang et al., 2004). Indeed, over the last decade in particular, advances in
fluorescence imaging have lead to the identification of several localized Ca\textsuperscript{2+} signalling events within the SMCs that challenge the traditional ideology that homogenous changes in cytoplasmic [Ca\textsuperscript{2+}]\textsubscript{i} control vessel diameter (Jaggar et al., 2000; Wang et al., 2004). Elementary calcium signals appear to have a limited spatial range (typically 1-6\,\mu m) and since the [Ca\textsuperscript{2+}]\textsubscript{i} declines sharply with distance from the site of origin, it has been argued that regulation of cellular activities must rely on the proximity of calcium channels to their targets in order for Ca\textsuperscript{2+} to have a specific effect (Jaggar et al., 2000). The two most common elementary Ca\textsuperscript{2+} signalling events that have been described in vascular smooth muscle cells are Ca\textsuperscript{2+} sparks and Ca\textsuperscript{2+} waves.

16.1 Calcium sparks

Release of Ca\textsuperscript{2+} from RyR can result in highly localized, transient increases in [Ca\textsuperscript{2+}]\textsubscript{i}, caused by the coordinated opening of a cluster of at least 10 channels in the SR (Nelson et al., 1995; Mejia-Alvarez, 1999). These events have been termed Ca\textsuperscript{2+} sparks (Nelson et al., 1995) and a single spark is known to be capable of producing a very high (10-100\,\mu M) local (~1\% of the cell volume) increase in [Ca\textsuperscript{2+}]\textsubscript{i}, while increasing the global [Ca\textsuperscript{2+}]\textsubscript{i} by <2nM (Jaggar et al., 1998b). Ca\textsuperscript{2+} sparks in SMCs were first described in myocytes from rat cerebral arteries (Nelson et al., 1995), but have subsequently been shown to occur in smooth muscle cells from a wide variety of tissues, including the rat mesenteric, pulmonary and coronary arteries, human cerebral arteries, rat portal vein, porcine trachea and guinea pig vas deferens, urinary bladder and ileum (Arnaudeau, 1996; Sieck, 1997; Gordienko, 1998; Jaggar et al., 1998a; Porter et al., 1998; Zhuge, 1998; Miriel et al., 1999; Remillard et al., 2002; Wellman et al., 2002; Heppner et al., 2003). Perhaps, more importantly, Ca\textsuperscript{2+} sparks have been reported in intact, non-pressurized and pressurized cerebral and mesenteric arteries (Gollasch et al., 1998; Jaggar et al., 1998a; Miriel et al., 1999), suggesting that these elementary signalling events may be physiologically relevant to the control of vascular tone.

In SMCs, Ca\textsuperscript{2+} sparks have been shown to activate large conductance Ca\textsuperscript{2+}-sensitive (BK\textsubscript{Ca}) channels to elicit a transient outward K\textsuperscript{+} current (Jaggar et al., 2000; Perez et al., 2001; Zhuge et al., 2002), also termed a spontaneous transient outward current or STOC (Benham & Bolton, 1986). In arteries at physiological levels of pressure, inhibition of Ca\textsuperscript{2+} sparks or BK\textsubscript{Ca} channels leads to membrane depolarization, activation of voltage dependent \textit{Ca}\textsuperscript{2+} channels, an elevation in arterial wall [Ca\textsuperscript{2+}]\textsubscript{i} and contraction (Nelson et al., 1995;
Thus it is widely recognized that in arterial SMCs, Ca^{2+} sparks lead to membrane hyperpolarization, decreased global [Ca^{2+}], and relaxation, particularly in vessels of the cerebral circulation (Nelson et al., 1995; Jaggar et al., 1998a; Jaggar et al., 1998b; Jaggar et al., 2000). Recent evidence would suggest that the frequency of Ca^{2+} sparks can be regulated by NO through the activity of PKG and PKA in the smooth muscle, resulting in increased spark frequency, and subsequently increases in the activation of K_{Ca} channels and enhanced relaxation (Jaggar et al., 1998b). On the other hand, in some non-vascular SMC tissues, Ca^{2+} sparks have been shown to trigger Cl^- efflux to promote membrane depolarization and contraction (Kotlikoff & Wang, 1998; ZhuGe, 1998). In cerebral vascular SMCs, agonists of the PKC signalling pathway have been reported to reduce spark frequency, causing decreased K_{Ca} channel activation and reduction of membrane hyperpolarization (Bonev et al., 1997). In vascular smooth muscle, RyR1 and RyR2 subtypes, but not RyR3, appear to play a major role in the generation of calcium sparks (Coussin et al., 2000), while another study in RyR3 deficient mice would suggest that activity of RyR3 may be important in altering Ca^{2+} spark frequency and hence contributing to the maintenance of vascular tone (Lohn et al., 2001a).

### 1.6.2 Calcium waves

#### 1.6.2.1 Smooth muscle.

A calcium wave may be defined as a transient rise in [Ca^{2+}]_c, typically starting from a specific region of the cell, propagating along its length in a wave like manner (Neylon et al., 1990; Wier & Blatter, 1991; Mayer et al., 1992). In contrast to Ca^{2+} sparks, Ca^{2+} waves can travel considerable distances and thereby have the potential to contribute to global cellular events. The velocity of wave propagation has been described in the range of 10-50 μm.s^{-1}, although higher values have also been obtained (Amundson & Clapham, 1993) (Rottingen & Iversen, 2000). It has been suggested that the spread of the Ca^{2+} wave cannot be due to passive diffusion of Ca^{2+} because such a process will not propagate at a constant speed, as has been described to occur in numerous smooth muscle tissues (Iino et al., 1994; Jaggar & Nelson, 2000; Shaw et al., 2004). It has also been argued that, if Ca^{2+} waves were simply a process of Ca^{2+} release and diffusion along the cell, the peak [Ca^{2+}]_c amplitude attained at any one point within the cell would decrease with an increase in diffusion distance, contradictory to observations made in vascular smooth muscle tissues (Iino, 1999). Thus, the peak [Ca^{2+}]_c remains constant during the propagation of Ca^{2+} waves (McCarron,
and therefore, must be due to a mechanism by which Ca\(^{2+}\) release at the wave front is regeneratively enhanced. In this regard, Ca\(^{2+}\) waves are thought to occur as the result of Ca\(^{2+}\) release from intracellular stores due to the activation of IP\(_3\)R and/or RyR (McCarron, 2004).

Two main models have been proposed to explain the mechanism by which Ca\(^{2+}\) waves can be generated based on Ca\(^{2+}\) release from intracellular stores. Since in SMCs, the number of IP\(_3\)R has been shown to drastically exceed the number of RyR (Wibo, 1994), it is not surprising that the most common mechanism through which Ca\(^{2+}\) waves are evoked is thought to rely solely on the release of Ca\(^{2+}\) from the IP\(_3\) sensitive intracellular store (Iino, 1999). In this model, PLC activity and the subsequent generation of IP\(_3\) releases Ca\(^{2+}\) from the IP\(_3\) sensitive intracellular Ca\(^{2+}\) store. Elevations in [Ca\(^{2+}\)]\(_i\) are thought to provide a positive feedback mechanism to further stimulate PLC and increase IP\(_3\) production, thus increasing the activation of the IP\(_3\)R itself (Mahoney et al., 1993). In addition, Ca\(^{2+}\) released from the IP\(_3\) sensitive intracellular store can activate neighbouring IP\(_3\)R in a CICR manner, causing a regenerative Ca\(^{2+}\) cascade along the length of the cell and formation of the Ca\(^{2+}\) wave (Iino, 1999). Since IP\(_3\)Rs have been shown to be biphasically responsive to increases in [Ca\(^{2+}\)]\(_i\), (Newton et al., 1994; Hagar, 1998; Wojcikiewicz & Luo, 1998; Ramos-Franco et al., 2000), the subsequent increases in [Ca\(^{2+}\)]\(_i\), in turn inhibit the IP\(_3\)Rs, leading to the cessation of Ca\(^{2+}\) release and Ca\(^{2+}\) homeostatic mechanisms act to restore [Ca\(^{2+}\)]\(_i\) to resting levels (Parker, 1990). This cyclical feedback mechanism provides the basis for successive cycles of CICR from the IP\(_3\) sensitive intracellular store. Over time, regenerative [Ca\(^{2+}\)]\(_i\) waves can be observed as repetitive Ca\(^{2+}\) transients and in individual SMCs, are widely described throughout the literature as [Ca\(^{2+}\)]\(_i\) oscillations (Rottingen & Iversen, 2000). A second model relies on the additional recruitment of RyRs and CICR from the ryanodine sensitive store to amplify the Ca\(^{2+}\) signal initiated via Ca\(^{2+}\) release from the IP\(_3\) sensitive store (Berridge, 1990; Lee, 2002). Thus although the relative involvement of IP\(_3\)R and RyR is thought to vary between smooth muscle preparations (Ruehlmann et al., 2000; Poburko et al., 2004), it is generally agreed that the initiation of Ca\(^{2+}\) waves is critically dependent on the activation of IP\(_3\)Rs (Iino, 1993). Ca\(^{2+}\) influx across the plasma membrane, via VDCCs, ROCs/SOCs and the reverse mode Na\(^+\)/Ca\(^{2+}\) exchanger, refill the intracellular stores allowing repetitive Ca\(^{2+}\) waves, or oscillations to be maintained in the long term (Iino et al., 1994; Miriel et al., 1999; Ruehlmann et al., 2000; Lee et al., 2001).
Ca$^{2+}$ waves, or oscillations, have been recorded from a number of vascular smooth muscle tissues, including both isolated SMCs and intact preparations (Weissberg et al., 1989; Hamada et al., 1997; Mironneau et al., 1996; Hyvelin et al., 1998; Blatter & Wier, 1992; Kasai et al., 1997; Pauvert et al., 2000; Lee et al., 2002a). Ca$^{2+}$ waves have been described to occur spontaneously, in response to electrical stimulation or following the application of agonists (Iino et al., 1994; Miriel et al., 1999; Jaggar & Nelson, 2000; Heppner et al., 2002). In blood vessels, Ca$^{2+}$ waves can cause an increase in vessel tone or contraction (Kasai et al., 1997; Sward et al., 2002), although Ca$^{2+}$ waves that do not produce such effects have also been observed (Miriel et al., 1999). Ca$^{2+}$ waves, are thought to be important for reducing some of the more deleterious effects of sustained increases in [Ca$^{2+}$], thus allowing frequency dependent events, such as the activation of calmodulin, that occur within the SMCs to take place (Savineau & Marthan, 2000; Lee et al., 2002a).

Ca$^{2+}$ waves, were first recorded in an intact blood vessel by Iino et al. (1994), who observed that Ca$^{2+}$ oscillations in adjacent SMCs of the rat tail artery occurred in an asynchronous manner following stimulation of sympathetic nerves or application of agonist. In this preparation, Ca$^{2+}$ oscillations were abolished by caffeine and ryanodine, indicating the involvement of intracellular Ca$^{2+}$ stores. Oscillations were also inhibited in the absence of extracellular Ca$^{2+}$, highlighting the critical importance of extracellular Ca$^{2+}$ influx to the maintenance of this phenomenon (Iino et al., 1994). Asynchronous calcium waves have subsequently been reported in other vascular beds and under various experimental conditions. Thus agonist induced asynchronous Ca$^{2+}$ waves have been observed in SMCs of the intact rabbit inferior vena cava (Lee et al., 2001; Ruehlmann et al., 2000) and rat cerebral (Jaggar & Nelson, 2000) and mesenteric arteries (Miriel et al., 1999; Mauban et al., 2001; Peng et al., 2001; Sell et al., 2002; Lamboley et al., 2003; Shaw et al., 2004). Such transients appear to have little effect on global [Ca$^{2+}$], changes, as asynchronous waves cannot effectively sum to cause a significant increase in the average [Ca$^{2+}$], across the arterial wall (Mauban et al., 2001).

One important issue raised by these studies then, is the effect of such Ca$^{2+}$ transients on vascular tone. Several studies would suggest that increasing concentrations of agonist appear to be critical to the frequency and velocity at which these asynchronous events occur (Iino et al., 1994; Miriel et al., 1999; Jaggar & Nelson, 2000; Ruehlmann et al., 2000; Mauban et al., 2001). Thus, very high concentrations of agonist lead to an elevated mean [Ca$^{2+}$], within the arterial wall, due to the summation of Ca$^{2+}$ waves, and finally to tonic
contraction (Lamboley et al., 2003), while at low concentrations, changes in global \([\text{Ca}^{2+}]_i\) remain low and very little force appears to develop (Miriel et al., 1999). The number of responding cells has also been shown to increase with dose dependent changes in agonist concentration (Ruehlmann et al., 2000). Such responses to agonist application are thought to be a consequence of heterogeneous receptor/channel distribution. In addition, data from these studies is consistent with the suggestion that SMCs in intact preparations from which these events are recorded must be poorly coupled in terms of \(\text{Ca}^{2+}\) diffusion between adjacent cells (Christ et al., 1996). Paradoxically however, in some vascular smooth muscle preparations, the application of agonists above certain concentrations gives rise to synchronized \([\text{Ca}^{2+}]_i\) oscillations within all SMCs across the vascular wall (Mauban et al., 2001; Peng et al., 2001; Sell et al., 2002; Lamboley et al., 2003; Shaw et al., 2004). This simultaneous recruitment gives rise to global \([\text{Ca}^{2+}]_i\) oscillations and coordinated rhythmic contractions otherwise known as vasomotion.

1.6.2.2 Endothelium.

As discussed previously, the vascular endothelium plays a critical role in the regulation of vascular tone through the release of NO, PGs and EDHF. It is also widely recognized that these factors are \([\text{Ca}^{2+}]_i\) dependent (Himmel et al., 1993; Coleman et al., 2004) and several studies of cultured ECs have reported the presence of \(\text{Ca}^{2+}\) waves in individual cells in response to application of agonists, such as ACh, or mechanical stimulation (Jacob et al., 1988; Missiaen et al., 1996; Huser & Blatter, 1997; Moerenhout et al., 2001). Like SMCs, \(\text{Ca}^{2+}\) waves in cultured ECs are dependent on activation of PLC and increased IP\(_3\) production (Jacob et al., 1988; Tran et al., 2000). Similarly, CICR from the Ry-sensitive but not the IP\(_3\)-sensitive intracellular \(\text{Ca}^{2+}\) store is thought to play a more important role in development of \(\text{Ca}^{2+}\) waves in some cultured EC systems (Neylon & Irvine, 1990; Laskey et al., 1992). In this regard, Lesh et al., (1993) have directly identified RyRs in cultured porcine thoracic aorta ECs through immunohistochemical techniques. Several studies have also shown that cultured ECs possess voltage-independent calcium-dependent ion channels, which permit extracellular \(\text{Ca}^{2+}\) influx into the cell and contribute to the amplification of the \(\text{Ca}^{2+}\) signal (Sage et al., 1989; Neylon & Irvine, 1990; Laskey et al., 1992; Moerenhout et al., 2001).

\(\text{Ca}^{2+}\) waves have been observed to propagate from individual cells to neighbouring cells in cultured EC monolayers (Sage et al., 1989; Laskey et al., 1992; Domenighetti et al., 1998; Moerenhout et al., 2001) and studies in porcine coronary artery and bovine aortic and
umbilical vein cultured ECs have demonstrated both dye and electrical coupling between cells within cell monolayers (Larson et al., 1983; Domenighetti et al., 1998). Others have reported that the putative gap junction inhibitors, palmitoleic acid and octanol inhibit synchronized Ca\(^{2+}\) fluxes across cell populations (Laskey et al., 1992; Domenighetti et al., 1998). Together, these results would suggest Ca\(^{2+}\) waves are propagated within endothelial cell monolayers through functional gap junctions.

Ca\(^{2+}\) imaging studies of the intact rat tail artery and rat ureteric arterioles have reported Ca\(^{2+}\) waves which occur spontaneously in individual cells or following the application of vasodilators (Kasai et al., 1997; Burdyga et al., 2003). Spontaneous Ca\(^{2+}\) oscillations have also been observed in individual ECs from intact mouse skeletal muscle arterioles in vivo (Duza & Sarelius, 2004). However, unlike many of the studies in cultured systems, cells of the intact vascular endothelium appear to respond heterogeneously to stimulation, rather than developing intercellular Ca\(^{2+}\) waves that spread along the vessel length (Huang et al., 2000; Marie & Beny, 2002). Since studies in the hamster cheek pouch arteriole in vivo, have shown that ACh elevates EC [Ca\(^{2+}\)]\(_i\) at the site of stimulation, but not at distant sites, despite the spread of hyperpolarization along the vessel segment (Dora et al., 2003), data would suggest that Ca\(^{2+}\) does not readily pass between ECs through gap junctions. Thus under more physiologically relevant conditions, it may be that cellular coupling within the endothelium is critical for coordinating integrated vessel responses through the conduction of electrical responses, rather than the transfer of small signalling molecules such as IP\(_3\) or Ca\(^{2+}\)(Christ et al., 1996; Dora, 2001b). Indeed, several studies have demonstrated the ability of the endothelium to rapidly conduct electrical signals via gap junctions over large distances within a vascular bed (Segal & Duling, 1989; Xia & Duling, 1995; Segal, 2000; Dora, 2001a; Yamamoto et al., 2001; Sandow et al., 2003a).

1.7 CALCIUM DYNAMICS AND MYOENDOTHELIAL GAP JUNCTIONS

In the vasculature, cellular coupling via gap junctions is critical to the ability of blood vessels to act as a coordinated functional unit (Christ, 1996). Thus while changes in [Ca\(^{2+}\)]\(_i\) within the EC and SMC layers ultimately control contraction and dilatation, control of vascular tone must ultimately rely on the ability of the vessel to intergrate cellular responses between the two cell layers. In this regard, electrical and chemical coupling via myoendothelial gap junctions (MEGJs) is thought to provide a critcal pathway through
which intercellular communication is able to directly regulate vascular function. MEGJs form when projections arising from an EC or a smooth muscle cell abut the plasma membrane of a cell from the opposite layer (Sandow & Hill, 2000). MEGJs are smaller and less frequent than homocellular EC gap junctions and their incidence varies throughout the vasculature (Sandow & Hill, 2000; Sandow et al., 2002). On the other hand, like homocellular gap junctions, MEGJs allow the passage of small signalling molecules, and facilitate the propagation of electrical signals, between the two cell layers that comprise the vascular wall. Communication between ECs and SMCs in terms of Ca\(^{2+}\) dynamics has been reported by several investigators. Thus in the hamster microcirculation \textit{in vivo}, Ca\(^{2+}\) has been shown to diffuse relatively short distances between the smooth muscle and EC layers following application of SMC agonists (Yashiro & Duling, 2000; Dora et al., 2003). Heterocellular coupling has also been observed in the rat mesenteric and porcine coronary arteries in which changes in SMC [Ca\(^{2+}\)]\(_i\) directly alter [Ca\(^{2+}\)]\(_i\) responses in the underlying endothelium (Budel et al., 2001; Oishi et al., 2001). Ca\(^{2+}\) coupling between SMCs and ECs has also been suggested in the rat mesenteric artery during vasomotion, since an oscillatory pattern of [Ca\(^{2+}\)]\(_i\) has been shown to develop in the endothelium of this artery during rhythmic contractions (Schuster et al., 2001).

1.8 VASOMOTION

Many types of smooth muscle tissues, including the gastrointestinal tract, urinary tract and lymphatic vessels have been observed to undergo spontaneous, rhythmic contractions (Tomita, 1981; Van Helden, 1993; Hashitani et al., 1996). Rhythmic contractions also appear to be an inherent feature of vascular smooth muscle tissues, occurring independently of neural or hormonal activity (Hayashida et al., 1986; Bouskela & Grampp, 1992; Lee & Earm, 1994; Hill et al., 1999).

Rhythmic contractions of blood vessels, or vasomotion, has commonly been observed \textit{in vivo} in small resistance vessels of the microcirculation, as well as in larger arteries from a number of different species and vascular beds (Auer & Gallhofer, 1981; Hundley et al., 1988; Fujii et al., 1990b; Morita-Tsuzuki et al., 1992; Bertuglia et al., 1994; Hill et al., 1999). The frequency and amplitude of these contractions has been reported to vary between species, as well as within and between vascular beds (Colantuoni et al., 1984; Slaaf et al., 1987; Oude Vrielink et al., 1990; Bertuglia et al., 1994; Morita et al., 1995;
Hill et al., 1999). Under physiological conditions, it has been proposed that vasomotion may be important in maintaining blood flow and oxygenation to metabolically active organs, such as the brain (Fujii et al., 1990b), while in other vascular beds, such as in skeletal muscle, it has been suggested that vasomotion may only be induced as a protective mechanism under conditions of critical perfusion (Rucker et al., 2000).

Other studies have reported vasomotion in vitro in isolated pressurized arterioles (Duling et al., 1981), following treatment with agonists or increases in extracellular concentration of K+ or when arterial rings are set up under isometric conditions (Katusic et al., 1988; Gustafsson, 1993; Lee & Earm, 1994; Stork & Cocks, 1994). Rhythmical contractions have also been described to occur spontaneously in some elastic arteries in vivo, and after exposure to agonists in vitro (Hayashida et al., 1986; Chemtob et al., 1992; Porret et al., 1995; Eddinger & Ratz, 1997). So although changes in vasomotion have been described in response to alterations in blood pressure and metabolic conditions, the effect of vasomotion itself on peripheral resistance, remains controversial (Funk et al., 1983; Fujii et al., 1990b; Bouskela & Grampp, 1992; Gratton et al., 1998; Meyer et al., 2002). A striking feature of the literature then, is that considerable diversity exists in both the incidence and mode of generation of rhythmical contractions in blood vessels. It is possible that these variations may be due heterogeneity in the expression of receptors, ion channels and intracellular signaling pathways in different vascular beds.

1.8.1 Vasomotion and calcium oscillations

In intact blood vessels, vasomotion has been shown to be associated with underlying oscillations in SMC Ca^{2+} (Mauban et al., 2001; Peng et al., 2001; Schuster et al., 2001; Sell et al., 2002; Lamboley et al., 2003; Filosa et al., 2004; Lamont & Wier, 2004; Matchkov et al., 2004b; Mauban & Wier, 2004; Schuster et al., 2004; Shaw et al., 2004). In the majority of studies in which changes in tension have been directly correlated with changes in [Ca^{2+}], the peak of the Ca^{2+} oscillation has been shown to precede the peak of the contraction (Mauban et al., 2001; Schuster et al., 2001; Lamboley et al., 2003; Schuster et al., 2004). Since [Ca^{2+}] has been shown to be the major regulator of vascular tone, the phase shift between the two peaks is likely to reflect the time required for activation of the contractile apparatus and thus the two phenomena are considered to be causally associated (Shimamura et al., 1999; Schuster et al., 2001).
At a cellular level, $[\text{Ca}^{2+}]_j$, oscillations underlying vasomotion in the rat mesenteric artery, have been resolved as spatially uniform, synchronized non-wavelike Ca$^{2+}$ transients or repetitive Ca$^{2+}$ flashes (Peng et al., 2001; Sell et al., 2002; Lamboley et al., 2003; Mauban & Wier, 2004). Thus, in contrast to those vessels in which low agonist concentrations elicit asynchronous Ca$^{2+}$ waves, vasomotion appears to be characterized by the simultaneous recruitment of all SMCs within the arterial wall. As discussed previously, the initial release of Ca$^{2+}$ and the asynchronous Ca$^{2+}$ waves observed in intact blood vessels following the application of low concentrations of adrenergic agonists is dependent on the release of Ca$^{2+}$ from the IP$_3$ sensitive intracellular Ca$^{2+}$ store. In some preparations, this signal has been shown to be amplified following additional release of Ca$^{2+}$ from the ryanodine sensitive intracellular store. During vasomotion however, because the Ca$^{2+}$ signals occur simultaneously it has been suggested that the mechanisms leading to the generation of such events must involve changes in membrane potential that could rapidly coordinate events in all SMCs across the vascular wall (Mauban et al., 2001). In this regard, vasomotion has been shown to be associated with rhythmical depolarizations in the membrane potential of SMCs (Hill et al., 1999; Bartlett et al., 2000; Oishi et al., 2002).

While the precise mechanisms leading to the generation of vasomotion still remain somewhat controversial, Peng et al., (2001) have clearly shown that the synchronized Ca$^{2+}$ flashes recorded during rhythmical activity depend on both the release of Ca$^{2+}$ from intracellular stores and the influx of extracellular Ca$^{2+}$ for their generation. Thus in the presence of VDCC inhibitors or Ca$^{2+}$ free bathing solution, synchronized Ca$^{2+}$ events were abolished. Under these conditions, asynchronous Ca$^{2+}$ waves could be observed, the presence of which were shown to be dependent on the release of Ca$^{2+}$ from intracellular stores. These findings have led to the intriguing hypothesis that asynchronous Ca$^{2+}$ waves may provide a basic pacemaker mechanism underlying vasomotion, the synchronization of which is capable of initiating coordinated contraction (Peng et al., 2001).

In agreement with this proposal, additional studies in the rat mesenteric artery by Lamont and Wier (2004) have demonstrated that the application of agonist results in the appearance of asynchronous Ca$^{2+}$ waves, followed by repetitive synchronized Ca$^{2+}$ flashes, or oscillations and vasomotion. Schuster et al., (2004) have reported that inhibition of L-type VDCCs leads to the abolition of global $[\text{Ca}^{2+}]_j$, oscillations and inhibition of rhythmical contractions. Similarly, Miriel et al., (1999) have demonstrated the loss of vasomotion and the return of asynchronous Ca$^{2+}$ waves following inhibition of VDCCs. Contraction or
tension studies performed both in vivo and in vitro have also identified an essential role for extracellular Ca$^{2+}$ influx and activation of VDCCs during vasomotion in the rat mesenteric and basilar arteries, afferent arterioles, thoracic aorta and portal vein, as well as the rabbit basilar, ear, mesenteric, femoral arteries, cerebral arterioles and porcine retinal and hamster skinfold arterioles (Colantuoni et al., 1984; Hayashida et al., 1986; Hundley et al., 1988; Fujii et al., 1990b; Chemtob et al., 1992; Omote et al., 1992; Gustafsson, 1993; Omote & Mizusawa, 1993, 1996; Burt, 2003; Hesselund et al., 2003; Takenaka et al., 2003). One exception is the rat iris arteriole in which spontaneous rhythmical contractions have been shown to depend entirely on the release of Ca$^{2+}$ from intracellular stores (Hill et al., 1999). Since numerous studies have investigated rhythmical activity following the application of adrenergic agonists which are known to produce contraction dependent on the entry of Ca$^{2+}$ through VDCCs, the abolition of vasomotion in these vessels may simply be due to a generalized decrease in [Ca$^{2+}$], across the vessel wall (Hill et al., 2001). Such a supposition highlights the critical importance of correlating changes in membrane potential, [Ca$^{2+}$], and diameter during vasomotion, so that the contribution of each mechanism can be fully elucidated.

Concerning the role of intracellular Ca$^{2+}$ stores, Lamont and Wier, (2004) have demonstrated the presence of both vasomotion and synchronized Ca$^{2+}$ flashes in the rat mesenteric artery following the application of ryanodine, but not in the presence of the putative IP$_3$R channel inhibitor 2-APB, suggesting a critical role for the involvement of IP$_3$Rs and not RyRs in providing the Ca$^{2+}$ for the initiation of these events. This is in direct contrast to the findings of Peng et al., (2001), whereby Ca$^{2+}$ released from the ryanodine-sensitive store was shown to contribute to the underlying asynchronous Ca$^{2+}$ waves within this same vascular bed. It can be suggested that these discrepancies may be due to differences in experimental technique. Nevertheless, the dependence of rhythmical contractions on the release of Ca$^{2+}$ from intracellular stores has long been recognized and several studies have demonstrated that inhibitors of SR Ca$^{2+}$ release can rapidly abolish both agonist induced and spontaneous vasomotion in blood vessels from a number of different vascular beds (Gustafsson et al., 1994; Hill et al., 1999; Burt, 2003; Takenaka et al., 2003). Thus when taken together, these results would suggest that in the majority of vascular beds, rhythmical activity may result from interplay between extracellular Ca$^{2+}$ influx and release of Ca$^{2+}$ from intracellular stores, similar to that previously described by
Peng et al., (2001). The relative importance of these events may vary due to the differential expression of ion channels and intracellular receptor distribution.

1.8.2 Role of cellular coupling

It is apparent from the above discussion that vasomotion must be critically dependent on the coordination of Ca\(^{2+}\) signals within individual SMCs leading to the synchronized Ca\(^{2+}\) responses and development of simultaneous contraction along the vessel length. In this regard, it has long been recognized that cellular coupling via gap junctions is mandatory to the coordination of many cellular events (de Wit, 2004). Synchronization is thought to be mediated by the transfer of small intracellular signaling molecules between cells of the vascular wall, such as IP\(_3\) or Ca\(^{2+}\) or by the rapid conduction of current along the vessel length (Christ et al., 1996). A number of studies have investigated the role of gap junctions in rhythmically active blood vessels using a range of substances thought to interfere with cellular coupling. In the rat mesenteric artery, the gap junction inhibitors, heptanol, octanol and 18-glycyrrhetinic acid result in the loss of synchronized Ca\(^{2+}\) transients and the subsequent appearance of asynchronous Ca\(^{2+}\) waves, consistent with inhibition of gap junctions (Dhein, 1998; Sell et al., 2002; Matchkov et al., 2004b). A role for cellular coupling is further supported by contraction studies in the rat iris arteriole, rat pulmonary artery and hamster aorta, in which these putative inhibitors have also been shown to abolish rhythmical activity (Jackson et al., 1991; Hill et al., 1999; Bonnet et al., 2001). Thus on the basis of these findings, it is generally accepted that cellular coupling via gap junctions within the vascular wall must be critical to the development of rhythmical activity in these vascular beds. On the other hand, since a growing body of evidence would suggest that these compounds are most likely having additional nonspecific effects on targets other than gap junctions (Spray & Burt, 1990; Chaytor et al., 1997; Hashitani & Suzuki, 1997; Yamamoto et al., 1998; Coleman et al., 2001, 2002; Tare et al., 2002; Matchkov et al., 2004b), the results of these studies must be interpreted with caution. Nevertheless, since Chaytor et al., (1997) have demonstrated loss of vasomotion in the rabbit mesenteric artery using synthetic mimetic peptides designed specifically to disrupt the docking of connexins between adjacent cells, it reasonable to suggest that gap junctions within the vascular wall are essential for coordinating Ca\(^{2+}\) oscillations and the consequent contractions, by providing a pathway for chemical and electrical signals between neighbouring SMCs. However, the relative degree to which these cells are coupled has
recently been called into question. A study by Shaw et al., (2004) in pressurized rat mesenteric arteries, would suggest that during vasomotion, complete synchronization of the [Ca$^{2+}$]$_i$ transients is not a prerequisite for the genesis of rhythmical contractions in this artery. Others have found that under similar conditions, synchronization within the mesenteric vascular bed is a necessary but not sufficient condition for vasomotion to occur (Schuster et al., 2004). It may be that variations exist in the degree of cellular coupling between SMCs which, depending on the vascular bed, species or methodological approach, result in differential patterns of Ca$^{2+}$ release, presumably through differences in the regulation of these channels.

1.8.3 Endothelium

In the rat mesenteric artery, asynchronous Ca$^{2+}$ oscillations can be observed in the absence of a functional endothelium but under conditions in which synchronized Ca$^{2+}$ oscillations and vasomotion are recorded in intact vessels (Peng et al., 2001). Such a finding has led to the proposal that a functional endothelium is obligatory for synchronizing rhythmical activity and thus initiating vasomotion within this vascular bed. Although most existing knowledge regarding the influence of the endothelium on vasomotion comes from studies of diameter changes or tension oscillations, it is nevertheless apparent that different and even contradictory effects of the endothelium on rhythmical activity have been observed. Thus, an essential role for the endothelium has been described in the rat mesenteric artery (Omote & Mizusawa, 1993; Dora et al., 2000a; Okazaki et al., 2003; Mauban & Wier, 2004), hamster aorta and cheek pouch arteriole (Jackson, 1988; Jackson et al., 1991; Verbeuren et al., 1997), canine basilar artery and rabbit femoral artery (Katusic et al., 1988; Omote & Mizusawa, 1995), while a more modulatory influence has been reported in rat cerebral and irideal arterioles (Dirnagl et al., 1993; Hill et al., 1999), rat basilar (Hempelmann et al., 1998) and mesenteric arteries (Sell et al., 2002; Lambolely et al., 2003; Schuster et al., 2004), rabbit ear (Omote & Mizusawa, 1994), basilar (Omote & Mizusawa, 1996) and mesenteric arteries (Chaytor et al., 1997), canine basilar artery (Katusic et al., 1988), hamster skeletal muscle arterioles (Bertuglia et al., 1994) and porcine carotid artery (Stein, 1984). In this regard then, endothelium dependent and independent vasomotion has been described both within the same preparation, as well as between different vascular beds or different species. It has been suggested that such discrepancies may be due to poor experimental technique or alternatively, when both endothelium
dependent and independent activity are observed in the same study, to underlying differences in the mechanisms that regulate these activities (Katusic et al., 1988; Omote & Mizusawa, 1994; Sell et al., 2002; Schuster et al., 2004).

The mechanisms by which the endothelium regulates vasomotion remain obscure. Simultaneous oscillations in both SMC and EC \([\text{Ca}^{2+}]\), have been observed during adrenergic induced vasomotion in the rat mesenteric artery (Schuster et al., 2001). These findings suggest a role for functional myoendothelial coupling and heterocellular Ca\(^{2+}\) flux during this activity. They also suggest a role for endothelial derived substances such as NO and/or EDHF since increases in endothelial cell Ca\(^{2+}\) are known to catalyse the formation of these products. Thus, in the hamster aorta and rat mesenteric artery, vasomotion has been shown to be dependent on the release of NO and the subsequent activity of cGMP in the SMCs (Jackson et al., 1991; Gustafsson et al., 1993). In these studies, an essential role for NO/cGMP was confirmed when addition of cell permeable cGMP analogues were shown to restore vasomotion in endothelium denuded tissues (Jackson et al., 1991; Gustafsson et al., 1993). Subsequently it has been hypothesized that smooth muscle cGMP activity, as a consequence of NO released from the endothelium, is responsible for triggering a depolarizing current that can synchronize Ca\(^{2+}\) released from intracellular stores in neighbouring SMCs, resulting in the initiation of vasomotion (Peng et al., 2001). While the recent identification of a novel cGMP activated Ca\(^{2+}\) dependent chloride channel in isolated rat mesenteric SMCs would argue in favour of the importance of a NO/cGMP synchronizing current for the generation of such activity (Matchkov et al., 2004a; Piper & Large, 2004), others have reported that the synchronized Ca\(^{2+}\) transients underlying rhythmical contractions in the same vessels are resistant to inhibition of NOS (Sell et al., 2002). Indeed it has been further demonstrated that application of the vasodilator ACh to endothelium intact mesenteric arteries results in the loss of synchronized Ca\(^{2+}\) signals and the appearance of asynchronous SMC \([\text{Ca}^{2+}]\), oscillations, while incubation in the NO donor SNP was shown to mimic the effects of ACh (Sell et al., 2002). Since Peng et al., (2001) failed to examine a change in electrical coupling in the absence of functional endothelium, one possible interpretation of the results is that the absolute synchronization of SMC Ca\(^{2+}\) transients during vasomotion simply reflects electrochemical signaling via cellular coupling between the endothelium and SMC layers (Lamboley et al., 2003) and that factors generated by the endothelium provide a modulatory, but not essential role during rhythmical activity within this vascular bed. The importance of such a proposal is
highlighted by the fact that SMCs in the rat mesenteric artery have been shown to exhibit poor electrical coupling and Ca\(^{2+}\) transients do not appear to be propagated between adjacent cells during vasomotion (Weidelt, 1997; Sell et al., 2002). A modulatory influence of the NO/cGMP pathway in rat mesenteric arteries has also been described by others (Watts et al., 1994; Tsai et al., 1995; Huang & Cheung, 1997; Okazaki et al., 2003; Lamont & Wier, 2004). Indeed, recent studies examining the role of EDHF in agonist induced vasomotion in the rat mesenteric artery raise the possibility that an endothelial factor other than NO is responsible for the generation of vasomotion within this vascular bed (Dora et al., 2000b; Okazaki et al., 2003; Mauban & Wier, 2004). In support of such a proposal, Lamont and Weir (2004) have shown that while Ca\(^{2+}\) transients are synchronous between SMCs when the artery is undergoing vasomotion, only asynchronous Ca\(^{2+}\) signals can be detected following inhibition of EDHF after application of apamin and charybdotoxin. Thus a need for careful interpretation of results is critical in order to further our understanding of endothelium dependent vasomotion, particularly in blood vessels other than those found in the mesenteric vascular bed.

1.8.4 Ionic mechanisms

Despite the surrounding controversy, the initiation of a depolarizing current and the propagation of electrical signals along the vessel length are most likely to be critical to the synchronized activation of VDCCs, as well as other voltage dependent ion channels, and the development of coordinated contraction in the majority of vascular beds from which vasomotion is recorded. Regrettably, while rhythmical activity has been shown to associated with not only oscillations in SMC [Ca\(^{2+}\)], and fluctuations in diameter, but also with oscillations in membrane potential, the extent of our knowledge regarding the relationship between these components, or the sequence of ionic events leading to the initiation and maintenance of a repetitive cycle, remains unclear. Moreover, advances within the field are made difficult by the fact that few studies have directly investigated the ionic mechanisms underlying vasomotion using electrophysiology.

During spontaneous vasomotion, rhythmical depolarizations have been recorded in hamster cheek pouch arterioles in vivo (Segal & Beny, 1992; Bartlett et al., 2000) and rat iris arterioles (Hill et al., 1999), rat aorta (Hayashida et al., 1986) and human pial arteries in vitro (Gokina et al., 1996). Rhythmical depolarizations have also been observed during vasomotion in the rat mesenteric artery (Gustafsson, 1993; Peng et al., 2001; Oishi et al.,
202), following application of an adrenergic agonist or nerve stimulation and under in the
dg coronary artery and in human pial arteries following changes in K⁺ permeability
(‘oshinaka & Uchida, 1986; Gokina et al., 1996). Depolarizations typically range between
1-30 mV in amplitude and precede or coincide with the rhythmical contractions. Large
rhythmical depolarizations have been observed to precede spontaneous contractions in
other smooth muscle tissues, such as the guinea-pig lymphatics and the rabbit urethra (Van
Helden, 1993; Hashitani et al., 1996). In vascular tissues, it has been suggested that the lag
between the peak depolarization and peak contraction, likely reflects the time required for
underlying intracellular events, such as Ca²⁺ release from the SR or activation of the
contractile apparatus to occur (Bartlett et al., 2000).

In most blood vessels exhibiting spontaneous vasomotion, the most negative
membrane potential of the vascular SMCs is relatively depolarized, around -40 mV
(Iayashida et al., 1986; Gokina et al., 1996; Bartlett et al., 2000). In contrast, quiescent
vessels, such as the rat mesenteric artery, exhibit more hyperpolarized resting membrane
potentials of around -50 to -60 mV, only depolarizing to similar potentials (~ -40 mV)
following stimulation (Molvany et al., 1982; Yoshinaka & Uchida, 1986; Gokina et al.,
D96; Peng et al., 2001; Oishi et al., 2002). Thus the apparent threshold for initiation of the
rhythmical depolarizations and hence contractions, in the majority of preparations appears
to lie within the range of membrane potentials at which L-type voltage dependent calcium
cannels are active (Nelson et al., 1990). Such findings support experimental data obtained
from contraction and Ca²⁺ imaging studies in which voltage dependent events have been
shown to be necessary for the development of vasomotion. The exception is the rat iris
teriole which, during vasomotion, appears to maintain a more hyperpolarized membrane
potential around -60 mV (Hill et al., 1999). In addition, since inhibition of L-type VDCCs
with nifedipine, was shown to reduce or abolish the rhythmical depolarizations and
contractions, hyperpolarize the membrane potential, and relax hamster cheek pouch
terioles in vivo and in human pial arteries in vitro (Gokina et al., 1996; Bartlett et al.,
D00), it is likely that Ca²⁺ influx through these channels must also be substantially
contributing to the maintenance of vascular tone as well as being responsible for the
activation of a depolarizing current. Inhibition of the L-type VDCCs has been shown to
result in the abolition, or reduction in tension oscillations/ rhythmical contractions and loss
d of vessel tone in the rat mesenteric artery and dog coronary artery (Uchida, 1985;
ustafsson & Nilsson, 1993; Oishi et al., 2002).
Since activation of Cl\text{Ca} channels has previously been implicated in depolarization of the membrane potential and opening of voltage dependent calcium channels in response to agonist stimulation (Large & Wang, 1996) and in the depolarization associated with nyogenic contraction to pressurization (Doughty & Langton, 2001), it is reasonable to suggest that the generation of such a current is critical for synchronizing Ca\textsuperscript{2+} transients and thus coordinated contraction during vasomotion in many vascular beds. Cl\text{Ca} channel activation and the generation of a depolarizing current has been shown to be responsible for the pacemaker activity and coordinated contraction observed in rhythmically active gastric and intestinal smooth muscle tissues and lymphatic vessels (Van Helden, 1993; Crowe et al., 1997; Hirst et al., 2002; Hirst & Edwards, 2004; Sanders et al., 2004). Thus while the depolarizing current required to open the VDCCs in spontaneously active blood vessels remains unidentified, Cl\text{Ca} channels have been proposed as the likely candidate (Bartlett et al., 2000). In this regard, the putative Cl\text{Ca} channel inhibitor has been shown to abolish adrenergic induced vasomotion in the rat portal vein (Burt, 2003). Furthermore, since in hamster cheek pouch arterioles, irregular depolarizations can be recorded in the presence of the VDCC inhibitor nifedipine, Ca\textsuperscript{2+} released from intracellular stores appears to be essential to the activation of these channels (Bartlett et al., 2000). Incidentally, inhibition of the SR Ca\textsuperscript{2+}-ATPase inhibitor with cyclopiazonic acid, abolished rhythmical depolarizations and contractions and perhaps more importantly, hyperpolarized and relaxed SMCs, demonstrating that intracellular Ca\textsuperscript{2+} release is essential to the activation of a depolarizing current in these vessels (Bartlett et al., 2000). These findings would appear to correlate well with Ca\textsuperscript{2+} imaging studies in which Ca\textsuperscript{2+} released from intracellular stores is essential to the development of synchronized Ca\textsuperscript{2+} transients in arteries from the mesenteric vascular bed (Peng et al., 2001).

In the rat mesenteric artery under quiescent conditions, inhibition of CICR with ryanodine has been shown to abolish caffeine induced depolarization and increased vessel tension (Peng et al., 2001), suggesting that the ryanodine sensitive Ca\textsuperscript{2+} stores contribute significantly to the activation of the depolarizing current in this preparation. However, in this vascular bed, Ca\textsuperscript{2+} released from intracellular stores are thought to activate a novel cGMP-dependent Cl\text{Ca} current (Peng et al., 2001), whose properties have been shown to differ from the classical Cl\text{Ca} currents observed in other vascular smooth muscle tissues (Large & Wang, 1996; Matchkov et al., 2004a; Piper & Large, 2004). In contrast, Gustafsson and Nilsson et al., (1994) have reported that inhibition of the Na\textsuperscript{+}-K\textsuperscript{+} ATPase,
following incubation in ouabain, effectively abolishes adrenergic induced rhythmical contractions in endothelium denuded mesenteric arteries exposed to exogenous cGMP, indicating that membrane depolarization is not simply due to the release of NO from the endothelium. Since the Na\(^+\)-K\(^+\) ATPase is an electrogenic membrane bound enzyme which is thought to play an important role in maintaining the electrochemical Na\(^+\)/K\(^+\) gradient across the membrane, the activity of which typically contributes to the resting membrane potential by around 10mV (Gustafsson, 1993), it is not unreasonable to suggest that its activity may contribute to the synchronizing depolarizing current that appears to be essential for the initiation of vasomotion in many vascular beds. Thus, the Na\(^+\)-K\(^+\) ATPase has been suggested to play an important role, albeit to various degrees, in the rhythmical activity observed in a number of vascular beds including the human coronary artery, canine basilar artery, rabbit inferior vena cava in vitro and the rat basilar artery in vivo (Katusic et al., 1988; Sabouni & Mustafa, 1989; Fujii et al., 1990a; Lee et al., 2001; Oishi et al., 2002). Nevertheless, since at certain concentrations, ouabain has been shown to attenuate cellular coupling through inhibition of gap junctions (Harris et al., 2000), and cellular coupling is essential to the development of vasomotion, the results of these studies should be interpreted with caution. Interestingly, recent studies in the rat cerebral artery have demonstrated a critical role for the TRPC6 and TRPM4 channels in pressure-induced SMC depolarization and vasoconstriction (Welsh, 2002; Earley et al., 2004), and so it is possible that TRP channels may contribute to the depolarizing current in rhythmically active blood vessels.

The role of K\(^+\) channels in vasomotion is also controversial. In hamster cheek pouch arterioles in vivo, the K\(_{Ca}\) channel inhibitor TEA has been reported to depolarize SMCs, abolish spontaneous contractions and decrease the amplitude and frequency of rhythmical depolarizations (Bartlett et al., 2000), suggesting that these channels contribute to the hyperpolarizing phase of the membrane potential oscillations. Although few investigators have examined the role on K\(^+\) currents during vasomotion using electrophysiology, a number of tension or contraction studies have also demonstrated both mandatory and modulatory roles for K\(_{Ca}\) channels in a variety of vascular beds. In this regard, the dual IK\(_{Ca}\)/BK\(_{Ca}\) channel inhibitor, charybdotoxin has been reported to abolish spontaneous vasomotion in the rabbit basilar artery and CPA induced rhythmical contractions in the rat mesenteric artery exposed to phenylephrine (Omote & Mizusawa, 1996; Huang & Cheung, 1997). The SK\(_{Ca}\) channel inhibitor apamin, applied with charybdotoxin, both luminally and
abluminally, has been reported to abolish adrenergically induced vasomotion in the rat mesenteric artery (Dora et al., 2000b; Okazaki et al., 2003; Mauban & Wier, 2004), while incubation in apamin alone had no effect (Gustafsson & Nilsson, 1994). Charybdotoxin alone has been described to abolish agonist induced vasomotion in the rabbit ear artery and CPA evoked, adrenergic induced vasomotion in the intact rabbit femoral and endothelium denuded ear arteries (Omote & Mizusawa, 1993, 1994, 1995). Rhythmic contractions have also been shown to be inhibited following K\textsuperscript{+} channel inhibition in endothelium denuded rabbit mesenteric arteries (Omote & Mizusawa, 1993). However K\textsubscript{Ca} channel inhibition has no significant effects on adrenergic induced vasomotion in the hamster cheek pouch arteriole and aorta or in CPA evoked rhythmic contractions in the rabbit mesenteric artery (Jackson, 1988; Bouskela & Grampp, 1992). On the other hand, K\textsuperscript{+} channels have been described to play a modulatory, but not obligatory, role in rhythmic activity observed the hamster aorta and rat mesenteric and cerebral vascular beds (Jackson, 1988; Gustafsson & Nilsson, 1994; Hempelmann et al., 1998), but not in the generation of rhythmic activity in the canine basilar artery (Katusic et al., 1988). Nevertheless, activation of a K\textsuperscript{+} current, most likely mediated via K\textsubscript{Ca} or K\textsubscript{V} channels is thought to be important for rhythmic activity in the human and canine coronary arteries and hamster cheek pouch arteriole (Sabouni & Mustafa, 1989; Yoshinaka & Uchida, 1986; Bouskela & Grampp, 1992). However, since the K\textsubscript{ATP} channel inhibitor glibenclamide has been described to have no effect on rhythmic contractions in the hamster cheek pouch arteriole, rat mesenteric artery, rabbit basilar, femoral and ear artery, it is likely that K\textsubscript{ATP} channels do not contribute to the ionic mechanisms underlying vasomotion in a majority of vascular beds (Bouskela & Grampp, 1992; Gustafsson & Nilsson, 1994; Omote & Mizusawa, 1994, 1995, 1996; Huang & Cheung, 1997). Despite the apparent heterogeneous involvement of K\textsuperscript{+} channels during vasomotion, the importance of these channels during rhythmic activity cannot be underestimated. Since K\textsuperscript{+} channels have been reported to form a functional unit with RyR and VDCCs (Jaggar et al., 2000), it is reasonable to suggest that activation of a hyperpolarizing current may provide an important negative feedback mechanism for regeneration of the contractile cycle. The diversity of responses to K\textsuperscript{+} channel inhibition may also reflect the relative contribution of the ryanodine and IP\textsubscript{3} sensitive intracellular stores to the initiation or maintenance of the repetitive cycle.
1.8.5 Modelling

Several investigators have created theoretical models in an attempt to assist the understanding of how the ionic mechanisms underlying the generation and maintenance of rhythmical oscillations may influence the regulation of vascular tone in those blood vessels displaying vasomotion (Funk et al., 1983; Bertuglia et al., 1996; Griffith, 1996; Ursino et al., 1998; Parthimos et al., 1999; Goldman & Popel, 2001; Loewenstein et al., 2001; Gratton, 2002; Bakker et al., 2004; Koenigsberger et al., 2004). These studies have theoretically confirmed that feedback between oscillating systems is not only essential for the generation of vasomotion in many vascular systems, but also for the large variety of vasomotion patterns observed throughout the vasculature. For example, Parthimos et al., (1999) have demonstrated that interactions between the membrane oscillator (voltage dependent), which controls the activation of channels such as the VDCCs and K_{Ca} channels and the cytosolic oscillator (voltage independent), relying on the intermittent release of Ca^{2+} from intracellular stores, are able to explain the diverse patterns observed experimentally during vasomotion in the rabbit ear artery. On the other hand, Ursino et al., (1998) have shown that interactions between mechanical stimuli, for example blood flow, and vessel regulatory mechanisms, such as the myogenic response, can influence vasomotion patterns in hamster skeletal muscle arterioles. More recently, however, advances in our understanding of the cellular Ca^{2+} dynamics that occur during vasomotion, have led to theoretical models examining the importance of cellular coupling in the recruitment and synchronization of Ca^{2+} signals that underlie rhythmical contractile events (Loewenstein et al., 2001; Koenigsberger et al., 2004). Thus, Koenigsberger et al., (2004) have theoretically demonstrated that vasomotion in the rat mesenteric artery is dependent on the propagation of electrical signals through weak cellular coupling via gap junctions, and not through the diffusion of the signaling molecule, IP_{3}. Perhaps more importantly, theoretical models have been used in an attempt to explain vasomotion in terms of an adaptive haemodynamic mechanism, particularly under pathophysiological conditions such as hypoxia or haemorrhagic shock (Intaglietta, 1991; Gratton et al., 1998; Ursino et al., 1998; Goldman & Popel, 2001).

1.8.6 Pathophysiology

It has been suggested that vasomotion may play an important role under pathophysiological conditions in which blood flow to tissues has become compromised.
Indeed, the incidence of vasomotion has been shown to be altered during disease states. This may occur due to alteration in the underlying mechanism or alteration in the expression or activation of component ion channels.

Changes in both the frequency and amplitude of vasomotion have previously been described in response to changes in blood pressure (Fujii et al., 1990b; Oude Vrielink et al., 1990; Gustafsson et al., 1994; Achakri et al., 1995). Furthermore, vasomotion has been shown to be upregulated in hypertensive animals and under these conditions, rhythmical activity generally occurs independently of the endothelium (Shimamura et al., 1999), suggesting that the endothelium derived vasodilators are not essential for vasomotion. Spontaneous vasomotion has been observed in posterior cerebral arteries from both spontaneously hypertensive (SHR) and spontaneously hypertensive, stroke prone (SHRSP) rats (Osol & Halpern, 1988). In the basilar artery of SHR, but not normotensive WKY rats, rhythmical tension oscillations are recorded in vitro, following inhibition of BKCa channels (Kamouchi et al., 2002). Since, increases in vascular tone and blood pressure were reported in vivo in mice with a targeted deletion of the 1 subunit of the BKCa channel (Brenner et al., 2000; Pluger et al., 2000), it was suggested that alterations in K+ channel activity play an important role in upregulating vasomotion in cerebral vessels in SHR. As such, decreased 1 subunit expression in hypertensive animals has been shown to result in activity of BKCa channels with reduced sensitivities for Ca2+ and an inability to respond normally to Ca2+ sparks (Brenner et al., 2000; Lohn et al., 2001b; Amberg & Santana, 2003). Thus changes in the 1 subunit of BKCa channels are thought to lead to a rise in global [Ca2+]i and increased vascular tone. Paradoxically, others have reported increased expression of the BKCa channel 1 subunit and an increase in overall BKCa function during hypertension (Sobey, 2001; Cox, 2002). These findings can be reconciled if changes in the BKCa subunit composition results in channels that show an overall decreased expression of the 1 subunit which compensate for increased subunit expression and are thus less likely to be activated by physiological levels of [Ca2+]i (Sobey, 2001). Decreases in voltage gated (Kv) and ATP-sensitive potassium (KATP) channel activity and impaired Kir channel function are also thought to contribute to the alterations in vascular tone associated with hypertension (McCarron & Halpern, 1990; Cox, 2002; Sadanaga et al., 2002). Thus the decrease in K+ channel activity described during hypertension may facilitate SMC depolarization. Since SMC depolarization is critical for the initiation of vasomotion, such alterations may explain the prevalence of rhythmical activity under such altered conditions.
Norepinephrine has been shown to induce rhythmical tension oscillations in the tail artery of SHRSP, but not in the SHR or WKY rat. Activity was blocked by inhibitors of VDCCs and K+ channels, while disruption of the endothelium appeared to have no effect on tension oscillations (Myers, 1984; Myers et al., 1985). Since under normotensive conditions, VDCCs, KCa channels and ryanodine receptor channels are thought to form a functional unit to control levels of [Ca2+]i and promote relaxation (Jaggar et al., 2000) and in the SHRSP tail artery, neither the ryanodine receptor inhibitor, ryanodine, nor the KCa channel inhibitors apamin, charybdotoxin or TEA, had an effect on rhythmical activity in the SHRSP tail artery (Myers et al., 1985; Lamb & Webb, 1989), alterations in K+ channels other than KCa may be involved in the tension oscillations observed in this artery in these animals. Conversely, calcium influx through VDCCs, calcium release from the ryanodine sensitive intracellular calcium store and both BKCa and SKCa channel activity have been shown to contribute to the development of spontaneous vasomotion in endothelium denuded pulmonary arteries isolated from monocrotaline (MCT)-induced pulmonary hypertensive rats (Kiyoshi et al., 2003). Non-endothelial cell derived prostaglandin H2 has also been shown to promote the rhythmical activity observed in MCT-induced hypertension.

Vasomotion has also been reported in Dahl-salt (DS) and deoxycorticosterone acetate (DOCA)-salt induced hypertensive models (Holloway & Bohr, 1973; Boegehold, 1993). In skeletal muscle arterioles of the DS hypertensive rat, spontaneous vasomotion showed an increase in contraction amplitude compared to control normotensive animals. It was suggested that this difference could be accounted for by suppression of basal NO during hypertension (Boegehold, 1993) and would appear to correlate with a diminished role of the endothelium under pathologically hypertensive conditions. On the other hand, tension oscillations in the thoracic aorta from DOCA-salt rats were abolished by gap junctional inhibitors, suggesting that cell coupling via gap junctions is essential for the initiation and/or maintenance of vasomotion during hypertension (Watts & Webb, 1996).

Since cell coupling via gap junctions has been suggested to play an important role in rhythmically active blood vessels under normotensive conditions (Chaytor et al., 1997; Hill et al., 1999), it is not surprising that a role for gap junctional communication has been suggested in vasomotion recorded under hypertensive conditions (Tsai et al., 1995). In skeletal muscle arterioles of Cx40 deficient mice, loss of Cx40 has been shown to be associated with hypertension and altered vasomotion patterns which could not be accounted for by alterations in endothelium derived relaxing substances (de Wit et al., 2000; de Wit et
al., 2003). On the other hand, tension oscillations were observed more frequently in the carotid artery and thoracic aorta from wild type compared to Cx43 deficient mice (Slovut et al., 2004). In addition increased Cx43 expression has been reported to underlie the rhythmical oscillations observed in the rat thoracic aorta and rat tail artery during hypertension (Watts & Webb, 1996; Haefliger et al., 1997; Slovut et al., 2004).

Taken together, these findings suggest that the increased incidence of rhythmical activity in vessels from hypertensive animals may directly contribute to the high blood pressure indicative of this disease state. Alternatively, vasomotion may be an adaptive process that may counteract the reduction in perfusion observed under these conditions. Regardless, it would seem that in animal models of hypertension, the increased incidence of vasomotion appears to be associated with alterations in the activity of K+ channels and a decrease in endothelial derived relaxing factors. Increasing evidence also suggests that alterations in cell communication via gap junctions may play a critical role in the increased incidence of vasomotion during hypertension. Since rhythmical contractions of blood vessels have also been recorded in human hypertension (Hollenberg & Sandor, 1984; Aalkjaer et al., 1985; Webb et al., 1992) it is important to gain a better understanding of the mechanisms and pathophysiological significance of this activity during the hypertensive state.

Although upregulation of vasomotion has long been considered a feature of the vasculature under high blood pressures, such as during hypertension, it has also been observed under conditions of low blood pressure. For example, rhythmical oscillations in vessel diameter have been reported in vivo during hemorrhagic hypotension in the rat mesenteric artery and in arterioles of the rat pancreas and skeletal muscle (Gustafsson et al., 1991; Vollmar et al., 1994; Torres Filho et al., 2001). Blood pressure has also been reported to be significantly lower in rabbit skeletal muscle during vasomotion, compared to those vessels exhibiting stationary diameters (Schmidt-Lucke et al., 2002). Under conditions of low blood pressure, it has been suggested that vasomotion may provide an important mechanism by which vascular perfusion can be maintained so that tissue function and integrity remains unaffected (Intaglietta, 1991). A similar role has been proposed for the appearance of spontaneous tension oscillations in vitro in the rat pulmonary artery under hypoxic conditions (Bonnet et al., 2001).

The characteristics of vasomotion may be altered in blood vessels during pregnancy. Thus, vasomotion in arteries obtained from pregnant rats exhibited reduced contraction
amplitudes and more hyperpolarized smooth muscle cell membrane potentials, compared to non-pregnant rats (Meyer et al., 1993; Gratton et al., 1998). It has been suggested that under these conditions, the reduction in vasomotion is due to decreased Ca\(^{2+}\) influx across the cell membrane, as a direct result of the more negative membrane potentials (Meyer et al., 1993; Gratton et al., 1998). In contrast, vasomotion has been reported to be upregulated in epigastric and omental arteries under pre-eclamptic, or pregnancy-induced hypertensive conditions in humans (Ebeigbe & Ezimokhai, 1988; Pascoal, 1995). Thus during pre-eclampsia, alterations in vasomotion are associated with more depolarized membrane potentials and increased Ca\(^{2+}\) influx across the smooth muscle cell membrane (Ebeigbe & Ezimokhai, 1988; Pascoal, 1995). Others have suggested that impaired Ca\(^{2+}\) extrusion/sequestration may increase global [Ca\(^{2+}\)]\(_i\) levels and account for the increased vascular responsiveness observed in these vessels (Aalkjaer et al., 1985).

Alterations in the prevalence of vasomotion have also been studied under other pathophysiological conditions including diabetes. Studies of flow motion using laser Doppler flow techniques have demonstrated a reduction in both the incidence and amplitude of vasomotion in human microvessels of the skin in diabetic compared to non-diabetic subjects (Stansberry et al., 1996). Others have reported impaired flow motion with no apparent change in vasomotion amplitude (Benbow et al., 1995). These observations are apparently consistent with an overall decrease in the incidence of vasomotion during diabetes (Nilsson & Aalkjaer, 2003). Additional evidence obtained from rat skeletal muscle arterioles, suggesting that the prevalence of vasomotion is reduced after infusion of insulin and increased following the infusion of glucose is consistent with this hypothesis (Renaudin et al., 1998; Wiernsperger, 2000). Impaired vasomotion has also been reported in diabetic humans with peripheral neuropathy and with diabetic maculopathy (Bek, 1999; Lefrandt et al., 2003; Meyer et al., 2003).

1.9 RHYTHMICAL ACTIVITY IN GASTRIC SMOOTH MUSCLE

Many types of non-vascular smooth muscle tissues undergo rhythmic contractions which are preceded by underlying rhythmic depolarizations (Tomita, 1981). In the large smooth muscle tissues, such as the stomach, these depolarizations are recorded as slow waves, which appear to be driven by specific pacemaker cells (Hashitani et al., 1996; Dickens et al., 1999; Van Helden et al., 2000; Dickens et al., 2001). In the gastrointestinal
tract, these cells are termed interstitial cells of Cajal (ICC) and are connected by numerous gap junctions to the smooth muscle cell layers, producing synchronized membrane potential changes in all cells (Thuneberg, 1982; Liu & Huizinga, 1993; Sanders, 1996; Dickens et al., 1999). In general, ICCs can be divided into two groups. Firstly, a thin layer of ICCs have been identified in the myenteric region between the circular and longitudinal smooth muscle cell layers in most regions of the gastrointestinal tract. These cells form a network and are termed ICC\textsubscript{MY} (Burns et al., 1997). The second group termed ICC\textsubscript{IM}, are distributed intramuscularly, mostly throughout the circular muscle layer, with individual ICC\textsubscript{IM} scattered amongst the SMCs. The distribution of ICC appears to show considerable regional variation (Hirst & Edwards, 2004).

The ionic mechanisms underlying the pacemaker activity in the gastrointestinal tract have yet to be fully elucidated. This is made more difficult by the fact that the electrical properties of the gastric smooth muscle appear to differ depending on the species or region studied (Suzuki, 2000; Sanders et al., 2004). In the gastric antrum however, slow waves consist of two components, an initial rapid component, followed by a prolonged plateau component (Hirst & Ward, 2003). Much of our knowledge of slow wave activity has arisen from studies in mutant mice with impaired ICC development, where it was found that those animals lacking ICC\textsubscript{MY} fail to generate slow waves (Ward et al., 1994; Ward et al., 1997), while in tissues lacking ICC\textsubscript{IM}, the plateau phase of the slow wave is absent (Dickens et al., 2001). In the gastrointestinal tract then, slow waves appear to depend on pacemaker activity initiated by the ICC\textsubscript{MY}, and subsequently augmented by a regenerative potential arising from the ICC\textsubscript{IM}. Each slow wave therefore reflects the sum of contributions made by ICC\textsubscript{IM} and ICC\textsubscript{MY}.

In the gastric antrum electrical activity generated by both the ICC\textsubscript{MY} and ICC\textsubscript{IM} is thought to be composed of elementary Ca\textsuperscript{2+} signaling events, termed unitary potentials. These events are considered to be dependent on Ca\textsuperscript{2+} released from the IP\textsubscript{3} sensitive intracellular store, since both pacemaker potentials recorded in ICC\textsubscript{MY} and slow waves are inhibited by blockade or mutation of the receptors that release Ca\textsuperscript{2+} from these stores (Suzuki et al., 2000; Ward et al., 2000; Hirst & Edwards, 2001; Fukuta et al., 2002; Hennig et al., 2004). Ca\textsuperscript{2+} release from this store results in the activation of ion channels in the membrane, producing a small transient depolarization (Van Helden et al., 2000; Hirst & Edwards, 2001; Kito et al., 2002b). Like slow waves, pacemaker potentials recorded from ICC\textsubscript{MY} are typically characterized by two components, an initial rapid depolarization,
followed by a secondary, long lasting plateau phase (Dickens et al., 1999). In the antrum the rapid initial component is thought to occur when underlying unitary potentials sum to activate a number of membrane ion channels, the opening of which causes a significant membrane depolarization (Hirst & Edwards, 2001). The generation of this inward current has been suggested to depend on the activation of voltage dependent Ca\textsuperscript{2+} permeable channels (Kito et al., 2002a). Others have proposed that the membrane depolarization is due to Ca\textsuperscript{2+} uptake by mitochondria, causing a localized decrease in [Ca\textsuperscript{2+}]i near the plasma membrane and activation of non-selective cation channels (Sanders et al., 2000; Ward et al., 2000). The slower secondary component, or plateau phase consists of a high frequency discharge of unitary potentials (Hirst & Edwards, 2001) and activation of Cl\textsubscript{Ca} channels (Kito et al., 2002a). The pacemaker potential enters a refractory period when Ca\textsuperscript{2+} release from the IP\textsubscript{3} sensitive store is decreased following the voltage dependent inhibition of IP\textsubscript{3} formation (Nose et al., 2000). As recovery from inactivation takes place, unitary Ca\textsuperscript{2+} release from the IP\textsubscript{3} sensitive intracellular store increases in frequency until the threshold for the initial component is once again reached and a significant membrane depolarization occurs. It is thought that spread of the depolarizing current across the ICCMY network, synchronizes the pacemaker potentials so that coordinated activity takes place (Hirst & Edwards, 2001; Kim et al., 2002). Regenerative potentials arising from the ICC\textsubscript{IM}, occur when depolarization, initiated by the ICC\textsubscript{MY}, is passively propagated to the circular smooth muscle layer. Under these conditions, the frequency of discharge of unitary potentials is increased, resulting in the recruited activation of Cl\textsubscript{Ca} channels and the membrane is depolarized (Hirst et al., 2002). In this regard, regenerative potentials are analogous to the plateau component recorded in the ICC\textsubscript{MY}, which together, appear to augment the secondary component of the slow wave recorded from the circular smooth muscle. Coordinated contraction takes place when the resulting depolarization reaches threshold and activates VDCCs and Ca\textsuperscript{2+} influx in the smooth muscle (Dickens et al., 1999). The importance of intracellular Ca\textsuperscript{2+} release for the generation of the underlying electrical activity is highlighted by the fact that inhibitors of the VDCCs, abolish the rhythmical contractions, but appear to have little effect on either the amplitude or time course of the slow waves recorded from smooth muscles of the gastrointestinal tract (Hirst et al., 2002).

Since pacemaker cells with similar morphological properties to these described in the gut have been recently observed in the rabbit portal vein (Povstyan et al., 2003), it raises an intriguing hypothesis that ICC like cells may be responsible for initiating the
synchronizing depolarizing current thought to be responsible for coordinating intracellular Ca\(^{2+}\) release/influx in blood vessels undergoing vasomotion. Such a proposal gains relevance in light of the fact that rhythmical contractions and depolarizations, recorded in the rat iris arteriole appear to occur independently of the activation of VDCCs (Hill et al., 1999).

1.10 THESIS AIMS

The aim of this thesis was to investigate the basic ionic mechanisms and intracellular signaling pathways that underlie spontaneous vasomotion in two apparently different vascular beds of juvenile Wistar rat, the arterioles of the iris and the basilar artery and its major branches. Rhythmical contractions recorded from iris arterioles have previously been shown to be unaffected by blockade of VDCCs, but reduced by drugs which interfere with intracellular Ca\(^{2+}\) release (Hill et al., 1999). On the other hand, spontaneous vasomotion recorded \textit{in vivo} in the rat basilar artery has been described to rely on voltage dependent mechanisms (Fujii et al., 1990a). Thus, this study has compared and contrasted the mechanisms underlying two different models of spontaneous vasomotion, one relying on apparently voltage independent mechanisms and the other, on voltage dependent events.

Previous studies in rat mesenteric arteries have indicated an important role for both the endothelium and for cellular coupling via gap junctions in the generation of agonist induced vasomotion (Peng et al., 2001). Since regulation of the cerebral circulation varies in several respects from that of systemic vessels (Faraci & Heistad, 1998), the present study has also explored the influence of the endothelium and the role of gap junctions in the generation of spontaneous rhythmical activity recorded from the basilar artery. Experiments have focused on the use of connexin mimetic peptides as selective blockers of arterial gap junctions.

The thesis has been divided into the following chapters:

\textbf{Chapter 2.} An outline of the methods and materials used in this thesis.
Chapter 3. Voltage independence of vasomotion in isolated irideal arterioles of the rat.

Chapter 4. Voltage dependence of vasomotion in rat basilar artery.

Chapter 5. Role for the endothelium in rat basilar artery vasomotion.


Chapter 7. General discussion arising from the results of thesis.
2.1 ANIMALS

During the course of this thesis, standard physiological and immunohistochemical procedures have been used to investigate the mechanisms underlying vasomotion in the iris arteriolar and basilar artery vascular beds. All experiments were conducted on male wistar rats, aged 14–17 days postnatal, weighing 30-40g, anaesthetized with ether and killed by decapitation. In those experiments which required perfusion prior to dissection, rats were deeply anaesthetized with an intraperitoneal injection of ketamine (44 mg kg$^{-1}$) and xylazine (8 mg kg$^{-1}$). Data were obtained from a minimum of four preparations each from a different animal ($n = 4$). Electrophysiological data were collected from a different animal, while measurements of [Ca$^{2+}$]$_i$ in the arterial wall and in individual SMCs were recorded from the same preparation. All experiments have been performed in accordance with the guidelines of the Animal Experimentation Ethics Committee of the Australian National University.

2.2 ELECTROPHYSIOLOGY

2.2.1 In vitro preparations

Iris arterioles: Following decapitation, both eyes were removed from the cranium and placed in cold (5-7°C) dissection buffer containing (mM): 3 3-(N-morpholino)propanesulphonic acid (MOPS); 1.2 NaH$_2$PO$_4$; 4.6 glucose; 2 pyruvate; 0.02 EDTA(Na); 0.15 albumin; 145 NaCl; 4.7 KCl; 2 CaCl$_2$; 1.2 MgSO$_4$. The iris was isolated from the sclera, lens and cornea and cut in half. Each section containing, on average, two arterioles embedded in the dilator muscle layer, and bordered by the ciliary and sphincter muscle edges was pinned flat with tungsten wire pins on to the base of a Sylgard silicone resin (Dow Corning Corporation, USA) coated coverslip, which formed the base of the recording chamber (Fig 2.1).
Caudal cerebellar arteries: The brain, composed of the cerebrum, cerebellum and brainstem was removed from the cranium and placed into cold dissection buffer. A rectangular section of the meninges, containing the basilar artery, caudal cerebellar arteries (CCA) and a short section of the cerebellar labyrinthine (secondary CCA) was isolated from the surface of the brain. The basilar artery preparations attached to the base of the recording chamber with tungsten wire pins through the surrounding pia (Fig 2.2).

2.2.2 Experimental protocol

The recording chamber, containing either the iris arteriolar or basilar artery preparations, was mounted on an inverted Olympus IX70 microscope (Olympus Optical, Japan) and perfused at a constant flow (3 ml min⁻¹) with Krebs solution (mM): 120 NaCl; 5 KCl; 25 NaHCO₃; 1 NaH₂PO₄; 2.5 CaCl₂; 2 MgCl₂; 11 glucose; gassed with 95% O₂ and 5% CO₂, maintained at 33 – 34°C. All preparations were equilibrated for 20 min or until spontaneous activity was constant in frequency and amplitude. Following equilibration, a section of the iris arteriole or CCA, was visualized using video microscopy (Hamamatsu Performance Vidicon camera) and vessel diameter was continuously measured using the Diamtrak computer tracking program (Neild, 1989).

Since previous studies had shown that iris arterioles undergo spontaneous rhythmical contractions with no significant change in contraction frequency or amplitude during the first hour of incubation (Hill et al., 1999), experiments were completed within this time frame. On the other hand, consistent spontaneous activity could be recorded from the basilar artery for over 2 hours. In this preparation, experiments were usually conducted within the first hour, after the onset of activity. Preparations were incubated in drug solutions for a maximum of 20 min or until the preparation became stable. Reversibility was determined following return to control Krebs solution.

2.2.3 Measurement of membrane potential

Cells in the arterial wall were impaled with sharp microelectrodes (borosilicate glass capillaries, GC 120-F-10, Havard Apparatus, Ltd, US), which were back filled with either 0.5 M KCl or with the nuclear marker, propidium iodide (0.2 %), dissolved in 0.5 M KCl, so that impaled cells could be identified (Fig 2.3A). Electrode resistances were typically between 100 and 200 MΩ (Flaming Brown micropipette puller, Sutter Instrument Co.).
**Figure 2.1**
Schematic diagram showing a typical iris wholemount preparation. Boxed area represents the general experimental site from which data was recorded.

**Figure 2.2**
Schematic diagram of cerebral blood vessels positioned on the ventral surface of the brain. Boxed area indicates section of the meninges, containing the basilar artery, caudal cerebellar arteries, and the caudal cerebellar artery $2^{\circ}$ branches which were isolated and pinned into the recording chamber for data collection.
Propidium iodide appeared to have no significant effect on electrode resistance or resting membrane potential.

Measurement of membrane potential was made using an Axoclamp 2B (Axon Instruments, 0.1 x headstage) with a AgCl reference electrode. All membrane potential records were lowpass filtered (cut-off frequency 1 kHz). Penetration of smooth muscle cells with the electrode tip was achieved by gentle tapping of the microelectrode manipulator. Successful impalements were generally associated with a rapid downward deflection in membrane voltage. Deflections of less than 40 mV in iris arterioles and less than 35 mV in the basilar artery preparation, were considered to be poor impalements. The criteria for a successful impalement were: 1) an abrupt change in potential upon penetration, 2) recordings stabilizing within 30 s with drift of less than 10 mV, and 3) return to baseline potentials with less than 15 mV voltage offset when the electrode was withdrawn. In the basilar artery preparation, stable intracellular recordings could be maintained for up to 1.5 hrs.

Resting membrane potential was determined as the difference between the potential of the electrode in the cell and the voltage offset recorded upon removal of the electrode from the cell. Simultaneous changes in membrane potential and changes in vessel diameter in the region where the cell was impaled were acquired with sample rates of greater than 100 Hz and stored for analysis using pClamp 8 software (Axon Instruments, Inc, USA).

Recordings were also made from short isopotential segments of the iris arteriole and from isolated segments of the CCA, which were cut to lengths of about 300 µm (mean 328 ± 19 µm, n = 10) and 1 mm (1190 ± 80 µm, n = 12) respectively using a microsurgical knife (Sharpoint, USA). In these studies, microelectrodes were dipped in silicone and the fluid level of the organ bath was reduced to a minimum to reduce electrode tip capacitance. When recordings were made in discontinuous current clamp mode (DCC), the electrode capacitance was compensated by monitoring the head stage voltage. Typically, switching frequencies of 200 to 500 Hz were employed and the head-stage voltage was continuously monitored when current was passed to change the membrane potential, ensuring that the settling of tip capacitance did not distort the voltage recording. When the voltage clamp mode was used, using a single electrode, the same precautions were taken. In short segments of iris arterioles, the membrane potential was hyperpolarised or depolarised over a range of potentials (-40 to -99 mV) by injecting positive or negative current, while in the
basilar artery preparation, input resistance was determined by injection of hyperpolarizing current pulses (0.1, 0.2-1.0 nA, duration: 30-45 s).

### 2.2.4 Analysis of contraction and membrane potential

Membrane potentials of spontaneously oscillating arterioles were defined as the most negative values recorded. The frequency of spontaneous contractions or depolarizations in both control and drug solutions was determined over a 5 min time period. The amplitude of spontaneous contractions was expressed as a percentage of resting vessel diameter (RVD) and was the average of 15 consecutive contractions, while the amplitude of the rhythmical depolarisations was an absolute voltage value. Measurements were made over the entire course of the experiment and data was analysed at 20 min, immediately following the equilibration period, and at 45 min, the time point at which drugs would have been present in the tissue bath for 20 min. The effects of drugs were determined by expressing the amplitude of spontaneous contractions in drug solution as a percentage of the contractions in control Krebs solution. Results are given as mean ± S.E.M of \( n \) preparations where each preparation was from a different rat. Statistical analysis was determined using 95\% confidence limits \((P < 0.05)\) and one-way ANOVA followed by pairwise \( t \) tests using Bonferroni correction for multiple groups. Paired or unpaired \( t \) tests were used when comparing two data groups. I-V relationships were fitted using non-linear regression analysis and input resistance \((I)\) was calculated from Ohm’s Law \((I = V / R)\). Data analysis and production of graphs were performed using the scientific statistical package, GraphPad Prism.

### 2.3 MEASUREMENT OF INTRACELLULAR CALCIUM

#### 2.3.1 in vitro preparations

Measurements of changes in \([\text{Ca}^{2+}]_i\) in the smooth muscle were made using the ratiometric fluorescent \(\text{Ca}^{2+}\) indicator, Fura-2 acetoxymethyl ester (Fura-2 AM).

In iris arteriolar preparations, preliminary experiments showed that the Fura-2 AM did not penetrate the cells of the arteriolar wall when the vessel remained embedded within the dilator muscle. Furthermore, arterioles did not contract if dissected entirely free from the dilator muscle. Consequently, the iris hemisection was pinned to the bottom of the recording chamber, as previously described for electrophysiological experiments, and a
small area of dilator muscle and stroma surrounding the arteriole was gently removed to expose the arteriole to the Fura solution. Similarly, a small area of meninges surrounding the CCA was removed to expose the artery. Removal of non vascular tissue from the vessels ensured adequate uptake of the calcium indicator into the smooth muscle cells and reduced the possible interference of \([Ca^{2+}]_i\) changes in surrounding tissues.

Following mounting in the recording chamber, preparations were incubated at room temperature (22°C) in modified Krebs solution containing reduced \(Ca^{2+}\) (0.5 mM \(CaCl_2\)), the detergent pluronic F-127 (0.01 %) and Fura-2 AM (5 mM) for 40 min. After loading, the recording chamber was mounted on an inverted Olympus IX70 microscope (Olympus Optical, Japan) and superfused with warmed (~34°C) Krebs solution, containing 2.5 mM \(CaCl_2\), for 20 min. No autofluorescence was observed when the tissue was not loaded with dye and only vessels that exhibited stable spontaneous contractions were studied further. The endothelium appeared to be impermeable to dye loading from the adventitial surface, thus changes in \([Ca^{2+}]_i\) were considered to be smooth muscle specific.

Data was first recorded in control solutions, using both the photomultiplier tube to record global \(Ca^{2+}\) changes in the arterial wall, followed by the intensified cooled CCD camera to resolve changes in individual SMC \(Ca^{2+}\), and then the data collection was repeated in the presence of drug solutions.

2.3.2 Measurement of calcium in the arterial wall

In order to study global changes in \([Ca^{2+}]_i\), within the arterial wall (Fig 2.3B), preparations loaded with Fura-2 AM were illuminated with alternating excitation wavelengths of 340 and 380 nm (75 W Xenon source) at a frequency of 30 Hz (Polychrome II illumination system) and emitted light at 510 nm measured (T.I.L.L Photonics GMBH, Germany). Simultaneous measurements of vessel diameter were made using the DIAMTRAK computer tracking program (Nield, 1989) by illuminating the preparation with infrared light (775 nm). Fluorescence signals and diameter changes were recorded and analysed using pClamp 8 software (Axon Instruments, Inc., USA). In all cases, care was taken to limit exposure to the fluorescent light. Changes in \([Ca^{2+}]_i\) are expressed as the ratio of the fluorescence emission recorded at 510 nm in response to excitation at 340 nm and 380 nm \((F_{340/380})\) excitation.
Figure 2.3
Figure 2.3
An illustration of the physiological techniques used to study the mechanisms underlying spontaneous vasomotion in the primary branch of the basilar artery. A, Simultaneous changes in smooth muscle cell membrane potential and vessel diameter were recorded using sharp microelectrodes which were backfilled with propidium iodide (0.2%) and a vessel edge tracking program. B, Global changes in arterial wall calcium were measured using a photometry system while simultaneously tracking changes in vessel diameter. C, Changes in individual smooth muscle cell calcium were imaged using an ICCD camera. Photometry and imaging data were recorded from the same preparation.
A

Recording electrode in smooth muscle cell

1° Branch

Camera

SMC filled with PI

Recording of vessel diameter

B

Measures Ca\(^{2+}\) indicator Fura-2 AM

Wall Calcium

Diameter

Region of interest

C

Images Ca\(^{2+}\) indicator Fura-2 AM

SMC calcium (n=4 cells)

Diameter

Region of interest
2.3.3 Measurement of calcium in individual smooth muscle cells

In order to resolve changes in \([\text{Ca}^{2+}]_\text{i}\) of individual smooth muscle cells (Fig 2.3C), preparations loaded with Fura 2-AM were imaged with an intensified cooled CCD camera (Princeton Instruments, USA). A region of interest, containing on average 20 smooth muscles cells were imaged and from within this region, 6-10 contiguous cells were selected for analysis. Care was taken to select cells from the region in which global calcium changes were previously recorded. Preparations were excited with alternating 340 and 380 nm light at a frequency of 3.9 ± 0.07 Hz. Data was collected and analysed using Axon Imaging Workbench software (Axon Instruments, Inc. USA). Simultaneous measurements of vessel diameter were made using the Diamtrak computer tracking program and analysed using pClamp 8 software (Axon Instruments, Inc., USA). Changes in \([\text{Ca}^{2+}]_\text{i}\) are expressed as the ratio of the fluorescence emission recorded in response to 340 nm and 380 nm (F$_{340}$/F$_{380}$) excitation.

2.3.4 Analysis of changes in calcium
Changes in \([\text{Ca}^{2+}]_\text{i}\) due to drug intervention were expressed as a percentage of the fluorescence ratio recorded in control, which was defined as 100 %. Control measurements were made for 1 min immediately following the equilibration period and at the time when drugs would have been present in the tissue bath for 20 min. In the majority of cases, responses to drugs were observed within a 5 min exposure period. If responses to drugs had not stabilized by 20 min, measurements were continued until drugs had been present for 30 min. Basal \([\text{Ca}^{2+}]_\text{i}\) levels were determined from the minimum values recorded. Statistical analysis was determined using 95% confidence limits (P < 0.05). Student’s paired t test was used when comparing drug effects to their respective control measurements. A ratio of the amplitude of the calcium oscillations in the arterial wall to the amplitude of the calcium oscillations in individual smooth muscle cells was used as a measure of cell coupling. Data analysis was performed using the scientific statistical package GraphPad Prism (GraphPad Software Inc., USA).

2.4 IMPAIRMENT OF ENDOTHELIAL FUNCTION

In order to assess the importance of the endothelium in the generation of vasomotion, we mechanically disrupted the endothelial cell layer of both the iris arteriole
and the primary branch of the basilar artery. Because of the technical limitations associated with removing the endothelial cell layer from the small arterioles, without significant damage to adjacent smooth muscle, two distinct methods were employed to remove the endothelium from either preparation.

2.4.1 Iris arteriole

Rats were deeply anaesthetized with ketamine (44 mg kg\(^{-1}\)) and xylazine (8 mg kg\(^{-1}\)). The thoracic cavity was opened and the descending aorta was exposed and clamped with artery forceps so that blood flow to the abdominal cavity and hindlimbs was occluded. Animals were then perfused (1.25 ml min\(^{-1}\)) via the innominate artery, which supplies the right upper extremity, with Krebs solution containing 1% sodium nitrite, to cause dilation of blood vessels, and 1% bovine serum albumin (BSA) and 0.1% heparin (5U ml\(^{-1}\)) to prevent blood clot formation. The right atrium was cut to allow blood to escape. When the exudate from the atrium was clear, the endothelium was disrupted by switching the perfusion fluid to distilled water for 4 min. Following this, the animal was re-perfused with Krebs solution for a final 4 min. The right eye was then removed, the iris dissected free and set up as previously described.

Following the 20 min incubation period, arteriolar activity was first recorded in control Krebs solution. The tissue was then incubated in the \(\alpha_2\)-adrenoceptor agonist UK14304-18 (100 nM) for 4 min to increase vessel tone, before acetylcholine (ACh; 1 \(\mu\)m, 10 \(\mu\)m), an endothelium dependent vasodilator, was added to the bath to assess the effectiveness and selectivity of the endothelial cell damage. Control preparations containing vessels with intact endothelium were also prepared in the same manner, except for perfusion with water. The effect of ACh (1 \(\mu\)m, 10 \(\mu\)m) was also assessed in these preparations in the presence of UK14304-18 (100 nM).

Removal of the endothelium was considered successful if there was no relaxation following application of ACh (<5% control response to ACh). In order to confirm that abolition of the relaxation due to ACh was due to removal of the endothelium and not simply due to damage of the underlying smooth muscle, we compared the maximal contraction amplitude to UK14304-18 in both the endothelium intact and endothelium denuded preparations. If the contraction amplitude in an endothelium denuded preparation was less than 90% of the maximum contraction to UK14304-18 observed in an endothelium intact preparation, preparations were discarded. In addition, the NO donor,
SNAP (10 μM), a smooth muscle cell vasodilator, was also used to assess smooth muscle integrity. Smooth muscle was considered to be intact if the relaxation to SNAP (10 μM) in an endothelium denuded preparation was ≥ 90% of the maximum relaxation observed in the presence of SNAP (10 μM) in an endothelium intact preparation.

2.4.2 Basilar artery

In those experiments investigating the functional role of the endothelium in the basilar artery, rats were anaesthetised with ether and the basilar artery and its branches were isolated and loosely pinned into the recording chamber. A small incision was made in the main basilar artery at the site of origin of the CCA and a segment of tungsten wire (0.05mm) was gently inserted through the basilar artery into the lumen of the CCA. The endothelium was removed by gently rubbing the intimal surface with the tungsten wire. The segment of artery, in which the endothelium had been removed, was then isolated from the vascular tree using a microsurgical knife and firmly pinned. In some experiments, removal of the endothelium was performed by perfusion of the artery with water, as previously described for iris arterioles. There appeared to be no significant difference between the outcome of the two techniques. Preparations were subsequently either loaded with Fura 2-AM in order to record changes in $[\text{Ca}^{2+}]_i$, or prepared so that the SMCs could be impaled with microelectrodes.

Experimental protocols were similar to those described above for the iris arterioles except that a preconstricting agent was not required as removal of the endothelium appeared to have no significant effect on basal tone.

2.5 DRUGS AND SOLUTIONS

The following drugs were used: (1,2-bis(o-aminophenoxy) ethane-$N,N,N',N''$-tetraacetic acid tetra (acetoxymethyl) ester (BAPTA-AM), 1-(6-((17b-3-methoxyestra-1, 3, 5(10)-trien-17-yl) amino)hexyl)-1H-pyrrole-2, 5-dione (U73122), 1-(6-((17b-3-methoxyestra-1, 3, 5 (10)-trien-17-yl) amino) hexyl)-2,5-pyrrolidine-dione (U73343), myristoylated protein kinase C (18-28) (MPK-C), ryanodine, 1H-[$1,2,4$]oxadiazo[4,3-a]quinoxalin-1-one (ODQ) (BIOMOL, USA); 4,4,-diisothiocyanatostilbene-2,2,-disulphonic acid (DIDS), nifedipine, tetraethylammonium (TEA), 2-hydroxyethanesulfonic acid (sodium isethionate), niflumic acid (NFA), charybdotoxin (CbtX), apamin, 4,
aminojpyridine (4-AP), 5-hydroxytryptamine (5-HT), chelerythrine chloride, indomethacin, nordihydroguaretic acid (NDGA), acetylsalicylic acid (aspirin), acetylcholine (ACH), S-nitroso-N-acetylpenicillamine (SNAP), (Sigma Chemical Co., USA); 17-octadecynoic acid (17-ODYA), arachidonyl trifluoromethyl ketone (AACOCF₃), 5,8,11 eicosatrienoic adipic acid (ETI), (Fluka Chemie, Switzerland); 2-aminoethylidiphenyl borate (2-APB), thapsigargin (Calbiochem, Germany); iberotoxin (IbTX) (Tocris, UK); caffeine (Alomone Labs Ltd, Israel); Fura-2 AM, pluronic acid (Molecular Probes, USA); UK14304-18, (Pfizer Central Research, England); 2,2'-O-Dibutyrylguanosine 3',5'-cyclic monophosphate. sodium salt (DBcGMP.Na), (Alexis Biochemicals); Cyclopiazonic Acid (CPA) and N⁵-nitro-L-arginine methyl ester (L-NAME) (Sapphire Bioscience Pty Ltd., Australia); TRAM-34 (kindly supplied by Dr Heike Wulff) was synthesized from clotrimazole (Sigma) as described previously (Wulff et al., 2000).

Connexin (Cx) mimetic peptides were made using previously identified sites with known peptide sequences in the first and second extracellular loops of the vascular Cxs (Table 1, Fig 2.4) (Chaytor et al., 1997; Kwak & Jongsma, 1999; Li & Simard, 2001; Dhein, 2002). Peptides were synthesised and purified by the Biomolecular Resource Facility, John Curtin School of Medical Research, ANU.

Stock solutions of U73122, U73343, chelerythrine chloride, BAPTA-AM, DIDS, indomethacin, NFA, TRAM-34, thapsigargin and aspirin were dissolved in DMSO (0.0001%); NDGA, 17-ODYA, SNAP, 2-APB, nifedipine and ETI were dissolved in ethanol; UK-14304-18 in 2% acetic acid and CbTX was dissolved in PBS containing 0.1% BSA. All other drugs were made up as x 1000 stock solutions in distilled water and diluted into Krebs solution, except for AACOCF₃ which was purchased as a solution in ethanol and diluted directly into Krebs solution.

Control experiments showed no significant effects of DMSO (0.001 %, 0.0001 %) or ethanol (0.001 %, 0.0001%) on spontaneous activity or resting vessel diameter. Light-sensitive drugs were protected through the use of long wavelength (> 610 nm) light.

2.6 PROTEIN EXPRESSION FOR CONNEXINS IN THE BASILAR ARTERY

2.6.1 Wholemount tissue preparation

Immunohistochemistry was used to investigate the distribution and expression of Cx 37, Cx40, Cx43 and Cx45 in the basilar artery preparation. Male wistar rats, aged 14-17
Table 1

Amino acid sequences of Cx mimetic peptides used to investigate spontaneous vasomotion in the rat basilar artery (Dhein, 2002; Chaytor et al., 2001; Li & Simard, 2001; Kwak & Jongsma, 1999).

<table>
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<th>Peptide</th>
<th>Cx</th>
<th>Topograpical position</th>
<th>Residue No.</th>
<th>Sequence</th>
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<td>37, 43</td>
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<td>201-211</td>
<td>SRPTEKTIFII</td>
</tr>
<tr>
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<td>40</td>
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<td>178-188</td>
<td>SRPTEKNVFIV</td>
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<tr>
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<td>45</td>
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<td>223-233</td>
<td>SRPTEKTIFLL</td>
</tr>
<tr>
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<td>VCYDQAFPISHIR</td>
</tr>
<tr>
<td>43 Gap26</td>
<td>43</td>
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<td>64-76</td>
<td>VCYDKSFPISHVR</td>
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<td>Gap 45</td>
<td>45</td>
<td>Extracellular loop 2</td>
<td>203-216</td>
<td>VHPFYVCSRLPCPH</td>
</tr>
</tbody>
</table>

Figure 2.4

Connexin topology at the plasma membrane. Connexin mimetic peptides are designed to target specific amino acid sequences in extracellular loops 1 (EL1) and 2 (EL2).
days postnatal were anaesthetised with intraperitoneal injection of 44 mg kg$^{-1}$ ketamine and 8 mg kg$^{-1}$ xylazine and perfused via the right atrium at a pressure of 60 mmHg. The initial perfusate composed of saline (0.9 % NaCl), also contained 0.1% sodium nitrite, 0.1% (5U ml$^{-1}$) heparin and 0.1% bovine serum albumin (BSA). When the exudate from the atrium was clear, the perfusate was replaced with 2% paraformaldehyde in 0.1 M L$^{-1}$ sodium phosphate buffer for 10 mins. The arteries were removed, placed into PBS and stripped of the meninges and pinned onto Sylgard silicone resin (Dow Corning, USA).

### 2.6.2 Antibodies

Primary antibodies were raised in sheep against amino acids 266-281 of the C-terminus of rat Cx37, amino acids 254-270 of the C-terminus of rat Cx40, and amino acids 354-367 of human Cx45. The human Cx45 sequence is 93% homologous to the mouse sequence, whereas the rat sequence is unknown (Rummery et al., 2002a). Specificity of these antibodies has been previously demonstrated (Coppen et al., 1998; Yeh et al., 1998; Yeh et al., 2000; Rummery et al., 2002b). Antibodies against rat Cx43 were raised in rabbits and purchased commercially (Zymed).

### 2.6.3 Immunohistochemistry

To block non-specific staining, wholemount tissues were pre-incubated in 2% BSA and 0.2% Triton-X (Tx) in PBS at room temperature for 1 hr. Tissues were then incubated at 37°C for 2hrs (or 37°C for 1 hr and then overnight at room temperature) with the primary antibody solutions (1:100 for Cx37, Cx40, Cx43 and Cx45) prepared in 2% BSA and 0.2% Tx in PBS. After 3 washes with PBS, the tissues were incubated for 1 hr at room temperature with either the secondary antibody (Cy3-conjugated anti-goat or Cy3-conjugated anti-rabbit immunoglobulins, 1:100; Jackson Immunoresearch Laboratories Inc, USA) in 0.01% Tx in PBS. Tissues were again washed a minimum of 3 times with PBS and mounted in buffered glycerol. Specificity of the antibodies has previously been tested, whereby tissues were pre-incubated in the appropriate immunizing peptides (10-fold excess by weight, 1 h, RT) before the application of primary antibodies or by omission of the primary antibody (Rummery et al., 2002b).
2.6.4 Confocal microscopy

Following staining, all preparations were examined using an inverted microscope (40x and 100x oil immersion objectives coupled to a confocal laser-scanning microscope (TCS 4D, Leica Instruments, Austria) and excited by an argon/krypton laser fitted with filters appropriate for the detection of Cy3 and FITC fluorescence. Optical sections were collected at 1 μm intervals and digitized images were obtained with line averaging. Each series of images was recombined to create a single image incorporating all smooth muscle or endothelial cell labelling (ScanWare, Leica, Germany). Care was taken to maintain similar gain settings for comparisons of antibody staining between preparations. For each preparation, images were collected from at least 3 different fields and each artery was sampled from at least 4 different animals.

2.7 ELECTRON MICROSCOPY: MYOENDOTHELIAL GAP JUNCTIONS

2.7.1 Tissue preparation

Fourteen day old male Wistar rats were anaesthetized with intraperitoneal injection of ketamine (44 mg kg⁻¹) and xylazine (8 mg kg⁻¹) and perfused via the left ventricle, at 60 mmHg, with saline (0.9% NaCl) containing 0.1% NaN₃, 0.1% BSA and 5U ml⁻¹ heparin at 25°C. When the exudate from the atrium was clear, animals were perfused with 3% glutaraldehyde and 1% paraformaldehyde in 0.1 mM sodium cacodylate with 0.2 mM CaCl₂.6H₂O, 0.15 M sucrose and 10 mM betaine (pH 7.35) for 10 mins at 25°C. The basilar arteries were removed and immersion fixed in the same solution at 4°C for a further 2 hrs. Arteries were subsequently post-fixed in 4% osmium tetroxide in 0.1 M sodium cacodylate buffer for 2 hrs, stained with saturated aqueous uranyl acetate and lead citrate for 2 hrs each and embedded in Alraldite 502 according to conventional procedures.

2.7.2 Serial section electron microscopy

50 transverse serial sections were cut from the basilar artery (n = 3-4 animals). Each series totalled approximately 5 μm of vessel length, assuming a section thickness of 100 nm. Low-magnification micrographs (x 2500) of the central section of each series were taken on plate film on a Hitachi 7100 transmission electron microscope. The vessel circumference was estimated as the length of the internal elastic lamina (IEL) and the number of medial smooth muscle layers from each vessel, was determined by counting the number of
individual smooth muscle cell profiles, from the outer edge of the IEL to the inner edge of the external elastic lamina along each of four linear plots 90° apart.

To detect myoendothelial gap junctions (MEGJs), the IEL in each serial section was examined at x 10000. All MEGJs through serial sections were photographed on plate film at high magnification (x 20000 to x 40000), and the numbers of MEGJs, characterized by a pentalaminar membrane structure, were recorded. Photographed MEGJ profiles and their surrounding endothelial and smooth muscle cell regions were digitized from contact prints using a flatbed scanner (HP Scanjet 4c), at a resolution of 300 dots per inch.

The number of MEGJs per endothelial cell was estimated from the surface area of the 5 μm long segment at the level of the IEL, the number of endothelial cells in this region as detected from connexion immunohistochemistry and the number of MEGJs present in this region.
CHAPTER 3
VOLTAGE INDEPENDENCE OF VASOMOTION IN ISOLATED IRIDEAL ARTERIOLES OF THE RAT

3.1 INTRODUCTION

As discussed in the general introduction, the mechanisms underlying vasomotion appear to be heterogenous, depending on the vascular bed or species studied, and have been described to result from at least two different mechanisms. One, based entirely on voltage-dependent events (Omote & Mizusawa, 1995) and the other relying on both voltage dependent and independent intracellular events (Gustafsson & Nilsson, 1993; Van Helden, 1993; Hashitani & Edwards, 1999).

Recent studies by Hill et al., (1999), however, have demonstrated that spontaneous contractions, recorded in developing iris arterioles, appear to be voltage independent, since they were not blocked by dihydropyridines, antagonists of voltage-dependent L-type calcium channels. Concurrently, a new class of voltage-dependent calcium channels, which are not blocked by dihydropyridines, were also shown to be present in arterioles (Morita et al., 1999).

Since spontaneous contractions in iris arterioles were preceded by large spontaneous depolarizations (Hill et al., 1999), we wanted to readdress the issue of their voltage independence. Additional factors have also been implicated in the generation or modulation of rhythmical contraction in other vessels, such as the influence of the endothelium through the release of a number of vasoactive factors, arachidonic acid metabolites produced by the phospholipase A₂ signal transduction pathway, blood pressure/stretch and local metabolic conditions, such as hyperoxygenation (Auer & Gallhofer, 1981; Fujii et al., 1990b; Chemtob et al., 1992; Gustafsson, 1993; Bertuglia et al., 1994; Omote & Mizusawa, 1995; von der Weid et al., 1996; Verbeuren et al., 1997; Hudetz et al., 1998; Hill et al., 1999; Smith et al., 2003). Thus, the particular aims of this chapter were; firstly, to confirm that rhythmical contractions are indeed voltage independent through the use of voltage and current clamp studies; secondly, to further investigate the role of intracellular calcium stores and the intracellular signal transduction
pathway involving phospholipase C; thirdly, to investigate the role of the phospholipase A2 pathway and finally, to study the role of the endothelium in iridal arteriolar spontaneous contractions.

3.2 RESULTS

3.2.1 General observations

3.2.1.1 Contractions.

In the juvenile rat, rhythmical contractions, or vasomotion, are routinely recorded throughout the iris arteriolar network in all preparations (see Fig 2.1). These small vessels are considered true arterioles, being composed of a single layer of endothelial cells and a single layer of smooth muscle cells (Rhodin, 1967; Hirst et al., 1997). Contractions are spontaneous and have previously been shown to be unaffected by tetrodotoxin and a variety of neuroreceptor antagonists (Hill et al., 1999).

In the present study, vasomotion was characterised by pulsatile movements of the arteriole wall and significant movement of red blood cells remaining in the vessel lumen. Contractions were typically observed within 15-20 min of incubation in control Krebs solution, whereby the average frequency was $3.2 \pm 0.1$ min$^{-1}$ ($n = 64$). The average amplitude of spontaneous contractions for all preparations in control Krebs solution was $14.7 \pm 0.6$ % resting vessel diameter (RVD) ($n = 64$), where the mean resting vessel diameter was $26.3 \pm 0.7$ μm ($n = 64$). The percentage of preparations exhibiting rhythmical activity was similar to that previously described by Hill et al., (1999).

Contractions showed no significant change in frequency or amplitude during the first hour, however, vessel tone showed a trend to increase over time, as evidenced by a decrease in resting vessel diameter (Fig. 3.1A; $t = 20$ min: $25.5 \pm 3.1$ μm, $t = 40$ min: $22.6 \pm 2.6$ μm; $n = 4$). After this period, spontaneous activity became irregular and finally ceased. Although all experiments were carried out in non-perfused, non-pressurised vessels, the appearance, form and cessation of spontaneous contractions and the gradual increase in tone is similar to that previously described in isolated pressurised arterioles, such as those found in the hamster cheek pouch (Duling et al., 1981). The degree of tone exhibited by spontaneously contracting arterioles was ascertained by the addition of the vasodilator, SNAP (10 μM) and determined to be $13.0$ % RVD ($n = 4$).
3.2.1.2 Iris arteriolar membrane potential.

When intracellular recordings were made from the vessels, spontaneous rhythmical depolarizations were found to precede the spontaneous contractions (Fig 3.1B). The average peak amplitude of the depolarizations was 31 ± 1.8 mV \((n = 24)\) and the most negative membrane potential of contracting arterioles was \(-60 ± 1.1 \text{ mV} \(n = 24\)\).

3.2.1.3 Measurements of arteriolar wall calcium.

In those preparations loaded with the ratiometric \([\text{Ca}^{2+}]_i\) indicator, Fura 2-AM \([\text{Ca}^{2+}]_i\) oscillations could be recorded from the arterial wall (Fig 3.1C). These oscillations always preceded the spontaneous contractions. The mean delay between the peak of the \([\text{Ca}^{2+}]_i\) oscillations and the peak of the contraction was \(1.4 ± 0.07\text{ s} \(n = 20\)\). With time \textit{in vitro}, the basal level of \([\text{Ca}^{2+}]_i\) showed a trend to increase (t = 20 min: \(0.51 ± 0.02 \text{ F}_{340/380}, t = 40 \text{ min: } 0.53 ± 0.02 \text{ F}_{340/380}; n = 4\) and the resting vessel diameter to decrease. The average \([\text{Ca}^{2+}]_i\) oscillation amplitude was \(0.023 ± 0.001 \text{ F}_{340/380}\) in control Krebs solution \((n = 4)\).

Approximately 50% of the preparations loaded with Fura 2-AM failed to initiate spontaneous contractile activity. This may have been due to vessel damage that occurred during the procedure to isolate the arterioles, or alternatively because Fura itself had buffered \([\text{Ca}^{2+}]_i\) to sufficiently low levels that contraction could not be initiated.

3.2.2 Effect of stretch on rhythmical contractions

Since measurements were recorded from the same region of the arteriole in all preparations, then the diameter of arterioles would be expected to be similar, with variations in rhythmical activity being attributed to age of the donor animal and degree of stretch. Consequently, arterioles taken from the same aged rats (14 days) exhibiting significantly different resting diameters might be considered to be experiencing different degrees of stretch. In arterioles with significantly different resting vessel diameters (38.8 ± 1.3 \(\mu\text{m}\) and 21.4 ± 0.9 \(\mu\text{m}\); \(n = 6\) and \(n = 8\) respectively; \(P <0.05\), paired t-test), there was no significant difference in the frequency (3.2 ± 0.3, and 3.3 ± 0.2 \text{ min}^{-1}) or amplitude (14.3 ± 2.2 and 13.9 ± 1.7\% RVD) of spontaneous contractions. Therefore differential stretch of the vessels during pinning of the iris had no effect on spontaneous contractions.
Figure 3.1

Rhythmical activity in iris arterioles recorded in control Krebs solution over time. A, Recordings of rhythmical contractions from the iris arteriole over time, showed a trend for the vessel diameter to decrease. B, Intracellular recordings show that spontaneous depolarizations precede spontaneous contractions. C, Using photometry, [Ca^{2+}]_i oscillations were recorded from the arterial wall and these also preceded spontaneous contractions. With time, basal [Ca^{2+}]_i levels showed a trend to increase. Constriction is represented by a downward deflection (A), while depolarization of the membrane potential (B) and increases in smooth muscle cell [Ca^{2+}]_i (C) are represented by an upward deflection. Control resting vessel diameters were 28.9 μm, 20.3 μm and 24.1 μm in A, B and C respectively.
3.2.3 Effect of oxygen on rhythmical contractions

Preparations were routinely perfused with Krebs solution which was gassed with 5% CO₂:95% O₂. In order to determine whether the 95% O₂ had any effect on contractions, some experiments were performed in Krebs solution gassed with 5% CO₂ in air (n = 4). No significant differences were found in either the frequency or amplitude of spontaneous contractions, compared to vessels in 5% CO₂:95% O₂ when tested either early or late in the experimental period (5% CO₂:95% O₂, t = 20 min: 3.8 ± 0.4 min⁻¹ / 15.7 ± 1 % RVD, t = 45 min: 3.7 ± 0.5 min⁻¹ / 16.0 ± 1.1 % RVD; 5% CO₂ in air, t = 20 min: 3.5 ± 0.1 min⁻¹ / 15.2 ± 1.1 % RVD, t = 45 min: 3.3 ± 0.5 min⁻¹ / 15.6 ± 1.1 % RVD). At t = 20 min, resting vessel diameters were 22.6 ± 2.6 and 18.5 ± 3.4 μm for the 5% CO₂:95% O₂ and 5% CO₂ in air groups respectively; and at t = 45 min, were 18.4 ± 4.0 μm and 17.4 ± 3.3 μm, respectively.

3.2.4 Effect of nifedipine on rhythmical contractions, membrane potential and calcium

The voltage dependent calcium channel antagonists, nifedipine (1 μM) and felodipine (1 nM), have previously been shown to have no effect on iris arteriole rhythmical contractions (Hill et al., 1999). In the present study, nifedipine (1 μM, n = 4) was also shown to have no effect on rhythmical contractions (Fig 3.2B; Control: 3.2 min⁻¹ / 10.8 ± 0.7 % RVD, nifedipine: 3.0 ± 0.3 min⁻¹ / 9.5 ± 0.7 % RVD) or on [Ca²⁺]ᵢ oscillations (Fig 3.2A; Control: 0.023 ± 0.001 Fᵢ₃₄₀/₃₈₀, nifedipine: 0.022 ± 0.001 Fᵢ₃₄₀/₃₈₀), basal [Ca²⁺]ᵢ levels (Fig 3.2A; Control: 0.51 ± 0.02 Fᵢ₃₄₀/₃₈₀, nifedipine: 0.51 ± 0.02 Fᵢ₃₄₀/₃₈₀) or vessel diameter (Fig 3.2B; Control: 26.9 ± 1.9 μm; nifedipine: 27.3 ± 2.1 μm).

3.2.5 Current clamp of short segments

Isolated segments of arteriole (328 ± 19 μm, n = 10), also generated rhythmical contractions and depolarizations like those recorded from intact arteriolar trees. These segments had resting membrane potentials of -58.9 ± 2.0 mV (n = 12) which was not significantly different from that of intact arterioles. The preparations had input resistances in the range of 27 to 110 M (55.6 ± 8.0 M ; n = 10). If the preparations had similar electrical properties to those determined previously (Hirst et al., 1997), their electrical lengths would be less than half of a length constant. Since the electrode was placed in the centre of the preparation, the maximum attenuation along the preparations would be less
Figure 3.2

Inhibition of L-type voltage dependent calcium channels and calcium-dependent chloride channels. Nifedipine (1 μM) had no effect on spontaneous [Ca^{2+}]_i oscillations (A) or rhythmical contractions (B). The addition of DIDS (100 μM), in the presence of nifedipine, abolished rhythmical activity (A, B) and caused the arteriole to relax (B). The F_{340/380} was 0.48 and the vessel diameter was 26.2 μm in A and B, in nifedipine respectively.
Effect of calcium-dependent chloride channel inhibition on spontaneous depolarisations and contractions. Addition of the calcium-dependent chloride channel inhibitor, DIDS (100 μM) abolished rhythmical depolarisations (A) and contractions (B), relaxed the vessel (B) and hyperpolarised the membrane potential (A) (n = 5; P<0.05). The mean frequency of contractions in control solution was 3.2 ± 1.3 min⁻¹ and the mean amplitude was 13.2 ± 1.1 % RVD (n = 5). In B, the control resting vessel diameter was 28.5 μm.
than 5% and the preparations will approach isopotentiality except for very rapidly rising transients (Hirst & Neild, 1980).

In eight preparations, taken from five different animals, the effect of changing the membrane potential on the frequency of rhythmical depolarizations was examined. In each preparation, depolarizing or hyperpolarizing the preparation from its resting membrane potential failed to abolish the rhythmical depolarizations (Fig 3.4). Furthermore, while the amplitudes of the rhythmical depolarizations appeared to be reduced at more positive membrane potentials and increased at more negative membrane potentials, no consistent change in their frequency was observed. At normal resting membrane potential the frequency of slow waves was 4.9 ± 0.2 waves min⁻¹ (n = 8). After hyperpolarizing the membrane potential by about 20 mV (21.3 ± 2.4 mV), the frequency of slow waves was 5.0 ± 0.3 waves min⁻¹.

3.2.6 Voltage clamp of short segments

When preparations were voltage clamped at their peak negative membrane potentials, rhythmical inward currents were detected (Fig 3.5B). Before applying the voltage clamp, the rate of occurrence of spontaneous depolarizations was 5.5 ± 0.6 waves min⁻¹ (n = 7), their mean amplitude was 20.5 ± 4.1 mV. After applying the voltage clamp, currents occurred at 5.5 ± 0.6 waves min⁻¹ and they had peak amplitudes of 0.35 ± 0.03 nA. When the current records were compared with the membrane potential recordings (Fig 3.5A) it was apparent that not only did they occur at the same frequency but also that they had very similar time courses. Visual observation of the preparation indicated that rhythmical movements of the preparations continued whilst the voltage clamp was applied.

3.2.7 Effect of BAPTA-AM on rhythmical contractions and membrane potential

The intracellular calcium chelator, BAPTA-AM (20 μM) was used to investigate the role of intracellular calcium stores and intracellular signalling pathways in spontaneous contractions. Spontaneous depolarizations (Fig 3.6A) and contractions (Fig 3.6B) were rapidly abolished within 2-3 min of BAPTA-AM being present in the bath (n = 6; P<0.05, paired t-test). Although the vessels relaxed (Control: 27.8 ± 2.5 μm, BAPTA: 34.2 ± 1.7 μm; P<0.05, paired t-test), no significant change in resting membrane potential was observed (Control: -61.0 ± 1.8 mV, BAPTA: -63.0 ± 1.4 mV). In the presence of BAPTA-
Figure 3.4
Figure 3.4
Lack of effect of membrane potential of a myogenically active short segment of irideal arterial on spontaneous oscillations. When the membrane potential was either depolarized or hyperpolarized over a range of potentials from -40 mV to -99 mV, rhythmical depolarizations persisted without changing their frequency of occurrence. The resting membrane potential of the arteriolar segment was -55 mV. The time and voltage scale bars apply to all traces.
**Figure 3.5**

Rhythmical discharge of inward current recorded from a segment of irideal arteriole. The upper pair of traces (A) shows the rhythmical changes in membrane potential before; and the error signal detected after the application of a voltage clamp using a single electrode voltage clamp circuit. It can be seen that when the membrane potential change was controlled to less than 2.5% of the control responses, a rhythmical inward current was detected (B). This current had the same frequency of occurrence as the rhythmical depolarization. The time bar applies to all traces.
Figure 3.6
Effect of BAPTA-AM on spontaneous depolarisations and contractions of iris arterioles. Spontaneous depolarisations (A) and contractions (B) are recorded in control Krebs solution. In the presence of BAPTA-AM (20 μM), both depolarisations (A) and contractions (B) are rapidly abolished. The mean frequency of contractions in Control solution was $3.5 \pm 0.3 \text{ min}^{-1}$ and the mean amplitude was $13.8 \pm 1.1 \% \text{ RVD}$ ($n = 6$). Voltage scale bar applies to the top two traces, the diameter bar to the bottom two traces, while the time scale bar applies to all records. The control resting vessel diameter in B was 26.7 μm.
AM, an increase in the extracellular concentration of K⁺ (50 mM) caused a large depolarization and contraction, as previously described (Hill & Gould, 1997).

3.2.8 Effect of ryanodine and caffeine on rhythmical contractions

Ryanodine (10 μM, n = 5) was used to investigate the role of the ryanodine sensitive intracellular calcium stores, or calcium induced calcium release, in arteriolar vasomotion. Incubation in this inhibitor, for up to 30 min, had no effect on spontaneous contractions or vessel diameter. In the presence of both ryanodine and caffeine (1 mM), which was used to deplete ryanodine-sensitive calcium stores, spontaneous contractions were rapidly abolished (Fig 3.7B, P<0.05, paired t-test) and the vessel relaxed (111.1 ± 1.5 % RVD in ryanodine; P<0.05, paired t-test). Spontaneous contractions reappeared after the caffeine was washed out and the vessel diameter returned to control diameters (Fig 3.7A; Control: 24.8 ± 1.6 μm), even though ryanodine continued to be present in the physiological saline (Fig 3.7C; ryanodine: 24.9 ± 1.6 μm).

3.2.9 Effect of DIDS on rhythmical contractions, membrane potential and calcium

The calcium-dependent chloride channel inhibitor, DIDS (100 μM, n = 4) was used to investigate the role of calcium-dependent chloride channels in spontaneous activity. Consequently, DIDS abolished the rhythmical depolarizations (Fig 3.3A; P<0.05, paired t-test) and contractions (Fig 3.3B; P<0.05, paired t-test), and caused a small but significant hyperpolarization (Control: -68.2 ± 2.5 mV, DIDS: -68.2 ± 2.5 mV; n = 5; P<0.05, paired t-test) and relaxation of the arteriole (Control: 28.1 ± 0.9 μm, DIDS: 32.5 ± 1.1 μm; n = 5; P<0.05, paired t-test).

In the presence of nifedipine, DIDS abolished the spontaneous contractions (Fig 3.2B; n = 4; P<0.05, paired t-test), [Ca²⁺]ᵢ oscillations and decreased basal [Ca²⁺]ᵢ levels (Fig 3.2A; nifedipine: 0.51 ± 0.02 F₃₄₀/₃₈₀, nifedipine & DIDS: 0.48 ± 0.0005 F₃₄₀/₃₈₀; n = 4; P<0.05, paired t-test).

3.2.10 Effect of phospholipase C pathway inhibition on rhythmical contractions and calcium

In five out of six preparations, the phospholipase C inhibitor, U73122 (10 μM; n = 10) completely abolished rhythmical contractions (Fig 3.8A and B) and oscillations in [Ca²⁺]ᵢ (Fig 3.8C; n = 4; P<0.05, paired t-test). U73122 also significantly increased the
Figure 3.7
Effect of ryanodine on spontaneous contractions. In the presence of ryanodine (10 μM) and caffeine (1 mM), spontaneous contractions were rapidly abolished (B). When caffeine was washed out (C), spontaneous contractions returned in spite of the continued presence of ryanodine. In ryanodine, the resting vessel diameter was not significantly different from control (A). The mean frequency and amplitude of spontaneous contractions in control solution was 3.7 ± 0.2 min⁻¹ and 15.2 ± 3.8 % RVD (n = 5), while the mean frequency and amplitude in ryanodine was 3.9 ± 0.4 min⁻¹ and 14.7 ± 3.8 % RVD (n = 5). Control resting vessel diameters were 28 μm, 32 μm and 28.8 μm in A, B, and C, respectively. Scale bars apply to all records.
resting vessel diameter (Control: 29.4 ± 1.9 µm; U73122: 33.5 ± 1.7 µm; n = 10; P<0.05, paired t-test). On the other hand, the inactive isomer of U73122, U73343 (10 µM) which was used to confirm the specificity of U733122, had no effect on rhythmical contractions or vessel diameter (n = 6). Non-specific effects of U73122 were seen at 100 µM when the inactive U73343 also inhibited vasomotion. Incubation in the protein kinase C inhibitors, chelerythrine chloride (CC; 5 µM, n = 4) or the peptide myristoylated protein kinase C (MPK-C; 10 µM, n = 7) reduced both the frequency and amplitude (Fig 3.8A and B, respectively; P<0.05, paired t-test) of spontaneous contractions but had no effect on resting vessel diameter (Control: 29.6 ± 1.5 µm, CC: 31.9 ± 1.4 µm; Control: 29.9 ± 1.9 µm, MPK-C: 30.7 ± 2.4 µm). [Ca^{2+}]_i oscillations were abolished in the presence of MPK-C (Fig 3.8D; n = 4; P < 0.05, paired t-test).

3.2.11 Effect of 2-APB on rhythmical contractions

2-APB has previously been demonstrated to inhibit IP_3 receptor mediated calcium release and capacitive calcium re-entry (Maruyama et al., 1997, Ma et al., 2000; Potocnik and Hill, 2001). In the present study, 2-APB (60 µM, n = 4) caused a rapid but transient (3.0 ± 0.6 min) abolition of spontaneous contractions (P<0.05, paired t-test), without significant loss of vessel tone (Control: 26.4 ± 2.7 µm, 2-APB: 26.9 ± 1.3 µm). After this inhibition, spontaneous contractions were significantly increased in frequency (Control: 3.6 ± 0.2 min^{-1}, 2-APB: 4.3 ± 0.3 min^{-1}; P < 0.05, paired t-test) and decreased in amplitude (Control: 13.2 ± 0.5 % RVD, 2-APB: 8.2 ± 0.1 % RVD; P<0.05, paired t-test), but no significant effects were observed on resting vessel diameter (Control: 26.4 ± 2.7 µm, 2-APB: 24.1 ± 2.2 µm).

3.2.12 Effect of phospholipase A_2 pathway inhibition on rhythmical contractions and calcium

The phospholipase A_2 inhibitor, arachidonyl trifluoromethyl ketone (AACOCF_3; 30 µM, n = 6), the dual lipoxygenase and cyclo-oxygenase inhibitor, nordihydroguaiaretic acid (NDGA; 10 µM, n = 10) and the lipoxygenase inhibitor, 5, 8, 11 –eicosatrienoic acid (ETI; 1 µM, n = 7) all produced significant decreases in the frequency and amplitude of spontaneous contractions (Fig 3.9A and B; P<0.05, paired t-test), but had no effect on vessel diameter (Control: 24.7 ± 0.6 µm, AACOCF_3: 26.3 ± 1.9 µm; Control: 22.3 ± 1.2 µm, NDGA: 22.1 ± 1.3 µm; Control: 25.2 ± 1.2 µm, ETI: 22.3 ± 1.2 µm). The
Figure 3.8
Figure 3.8
Role of the phospholipase C pathway on \([Ca^{2+}]_i\) and vasomotion. The effect of phospholipase C pathway inhibition on the frequency (A) and amplitude (B) of spontaneous contractions was analysed 20 min after incubation in the presence of the phospholipase C inhibitor, U73122 (10 \(\mu\)M), its inactive isomer, U73343 (10 \(\mu\)M), the protein kinase C peptide, myristoylated protein kinase C (MPK-C, 10 \(\mu\)M) and the protein kinase C inhibitor, chelerythrine chloride (CC, 5 \(\mu\)M). Rhythmical contractions and \([Ca^{2+}]_i\) oscillations recorded from the vessel wall were abolished in the presence of (C) U73122 (10 \(\mu\)M) and (D) MPK-C (10 \(\mu\)M). Traces are representative of four experiments using tissues from different animals. Control resting vessel diameters were 20.4 \(\mu\)m and 18.7 and the basal smooth muscle cell \([Ca^{2+}]_i\) was 0.44 F_{340/380} and 0.68 F_{340/380} in C and D, respectively. Values represent means and S.E.M. * significantly different from control (P<0.05).
A. 

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<tr>
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<td>4.0 ± 0.5</td>
</tr>
<tr>
<td>U73122</td>
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</tr>
<tr>
<td>MPK-C</td>
<td>2.0 ± 0.5</td>
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<tr>
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B. 

<table>
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C. Control  
Wall Calcium

D. Control  
Wall Calcium

U73122 (10 µM)  

MPK-C (10 µM)  

Wall Calcium

Diameter

340/380

2 µm

10 s
cyclooxygenase inhibitors, indomethacin (10 μM, n = 8) and aspirin (10 μM, n = 4) and the cytochrome P450 inhibitor, 17-ODYA (10 μM, n = 4) had no effect on the frequency of spontaneous contractions (Fig 3.9A) or resting vessel diameter (Control: 25.2 ± 2.5 μm, indomethacin: 26.1 ± 1.5 μm; Control: 27.9 ± 1.4 μm, Aspirin: 27.7 ± 1.4 μm; Control: 24.8 ± 2.5, 17-ODYA: 26.4 ± 2.4 μm), however the amplitude of spontaneous contractions was reduced in the presence of 17-ODYA (Fig 3.9B; P<0.05, paired t-test). Parallel experiments using Fura 2-AM loaded arterioles showed that inhibition of the phospholipase A2 pathway with AACOCF3 (30 μM; Fig 3.9C, n = 4) abolished [Ca2+]i oscillations and associated contractions, but had little effect on basal [Ca2+]i levels (Control: 0.49 ± 0.01 F340/380, AACOCF3: 0.49 ± 0.02 F340/380).

3.2.13 Effect of phospholipase A2 pathway agonists on rhythmical contractions

Since metabolites of the lipoxygenase arm of the phospholipase A2 pathway appear to be important in the generation of rhythmical contractions in the iris arteriole, an active metabolite of this pathway, leukotriene D4 (LTD4) was added to the superfusing solution. LTD4 (20 nM, n = 8) produced a transient inhibition (2.2 ± 0.4 min⁻¹) of the rhythmical contractions. Upon re-establishment of rhythmical activity, contractions showed a trend to be increased in frequency (Control: 3.6 ± 1.2 min⁻¹, LTD4: 4.7 ± 1.2 min⁻¹), but no significant effects were observed on the amplitude of the spontaneous contractions (Control: 13.5 ± 1.1 % RVD, LTD4: 12.3 ± 1.1 % RVD) or resting vessel diameter (Control: 27.1 ± 2.8 μm, LTD4: 25.6 ± 2.5 μm). In the presence of the phospholipase A2 inhibitor, AACOCF3 (30 μM), which abolished the rhythmical contractions, LTD4 did not significantly re-initiate activity (AACOCF3: 0.2 ± 0.1 min⁻¹, AACOCF3 & LTD4: 1.3 ± 0.8 min⁻¹; n = 4) and had no further effects on vessel diameter (AACOCF3: 26.3 ± 1.9 μm, AACOCF3 & LTD4: 25.3 ± 2.1 μm). A similar result was obtained in the presence of the phospholipase C inhibitor, U733122 (10μM, n = 4), in that LTD4 failed to re-initiate rhythmical activity (U73122: 0 min⁻¹; U73122 & LTD4: 0 min⁻¹) nor were any further effects on vessel diameter observed (U73122: 26.2 ± 3 μm, U73122 & LTD4: 30.2 ± 2.9 μm).

Addition of the agonist, arachidonic acid (1 μM, n = 4), to the superfusing solution abolished (P<0.05, paired t-test) rhythmical contractions (Control: 3.3 ± 0.5 min⁻¹ / 13.6 ± 1.7 % RVD) and caused the vessel to relax (Control: 27.9 ± 1.5 μm, A.A: 32.0 ± 0.7 μm; P<0.05, paired t-test).
Figure 3.9
Figure 3.9

Effect of phospholipase A<sub>2</sub> pathway inhibitors on rhythmical activity. The phospholipase A<sub>2</sub> inhibitor, AACOCF<sub>3</sub> (30 μM), the dual lipoxygenase and cyclo-oxygenase inhibitor, NDGA (10 μM), the lipoxygenase inhibitor, ETI (1 μM), the cyclo-oxygenase inhibitors, indomethacin (Indo, 10 μM) and aspirin (10 μM) and the cytochrome P<sub>450</sub> inhibitor, 17-ODYA (10 μM) were tested for their effects on the frequency (A) and amplitude (B) of spontaneous contractions. Values represent means and S.E.M. * indicates a significant difference from control. C, AACOCF<sub>3</sub> (30 μM) abolished spontaneous [Ca<sup>2+</sup>]<sub>i</sub> oscillations and contractions. Traces are representative of four experiments using tissues from different animals. The control resting vessel diameter was 19.9 μm and basal smooth muscle [Ca<sup>2+</sup>]<sub>i</sub> was 0.54 F<sub>340/380</sub>. 
A. 

# of spontaneous contractions (min⁻¹)

B. 

Amp. of spontaneous contractions (% Control)

C. Control

AACOFC₃ (30 μM)

Wall Calcium

Diameter

[Graph and data showing]
3.2.14 Role of the vascular endothelium in rhythmic contractions

In order to determine whether rhythmic contractions in iridal arterioles could result from the spontaneous release of endothelium-derived substances, spontaneous activity was studied in the presence and absence of the vascular endothelium (Fig 3.10). Disruption of the endothelium (*n* = 9) had no significant effect on the frequency (Fig 3.10 A; 3.1 ± 0.1 min⁻¹) or amplitude (Fig 3.10B; 9.9 ± 1.1 % RVD) of spontaneous contractions when compared to those vessels with intact endothelium (Fig 3.10A, 3.0 ± 0.2 min⁻¹; Fig 3.10B, 11.2 ± 1.4 % RVD, respectively; *n* = 14). Resting vessel diameters did not significantly differ between endothelium intact (28.4 ± 2.2 μm) and endothelium denuded preparations (26.4 ± 1.5 μm).

3.2.15 Confirmation of endothelial removal

The endothelium dependent vasodilator ACh (1 μM, 10 μM) was used to assess the effectiveness and selectivity of endothelial cell damage in preparations both with and without endothelium (Fig 3.11). In the first instance, preparations were incubated in the α₂-adrenoceptor antagonist UK-14304 (100 nM) to increase vessel tone. The time course of this constriction was studied in preparations with intact endothelium in the absence of ACh (Fig 3.11A, *n* = 5). While some relaxation of the vessel occurs over time, no significant differences were found in the amplitude of the UK-14304 constriction at time points where ACh (1 μM, 10 μM) would be present in the bath, when compared to the peak amplitude of the contraction. In endothelium denuded preparations, UK-14304 caused a large constriction (Fig 3.11C; 39.2 ± 5.3 % RVD; *n* = 9) which was not significantly different from that observed in endothelium intact preparations (Fig 3.11A and B; 40.6 ± 2.9 % RVD; *n* = 14). Application of ACh (1 μm, 10 μM) to vessels with intact endothelium produced a significant reduction in the amplitude of the UK-14304 constriction (Fig 3.11B; 88.5 ± 3.1 % and 99.3 ± 2.5% reduction, respectively; *n* = 9; P<0.05). In endothelial denuded preparations, results confirmed that the perfusion with water had successfully removed the endothelium since vasodilation following the addition of ACh (1 μM, 10 μM) was not significantly different from that occurring with time in intact preparations in the absence of ACh (Fig 3.11B; 15.1 ± 5.5 %, 25.3 ± 5.5%; *n* = 9 and Fig 3.11C; 15.4 ± 6.2%, 27.3 ± 8.3%; *n* = 5, respectively).

The smooth muscle dependent vasodilator, SNAP (10 μM), was used to assess smooth muscle integrity after removal of the endothelium (*n* = 9). Only those preparations
Figure 3.10
Figure 3.10
Effect of the endothelium of spontaneous contractions. Disruption of the endothelial cell layer in iris arterioles had no significant effects on the frequency (A) or amplitude (B) of spontaneous contractions. C, Shows a representative trace of spontaneous contractions in vessels with intact endothelium (24.2 µm) compared with a preparation where the endothelium has been destroyed (25.8 µm). Bars represent mean and S.E.M.
A.  
# Spontaneous Contractions / 5 min

B.  
Amplitude of spontaneous contraction (%RVD)

C.  
+ ENDO
- ENDO

30 s  5 μm
Figure 3.11
Figure 3.11
Confirmation of endothelial disruption. A, Time course of UK-14304 (100 nM) constriction in vessels with intact endothelium. t1 and t2 represent the point at which ACh (1 μM and 10 μM), would be present in the bath, respectively. B, shows the vasodilation in response to ACh (1μM, 10 μM) in vessels with intact endothelium. C, Shows the lack of response to ACh (1 μM, 10 μM) in those preparations in which the endothelium was considered to be successfully destroyed. Bars represent mean and S.E.M. * indicates a significant difference from control, whereby the initial contraction in UK-14304 is defined as 100 %. Representative traces show the time course of the UK-14304 induced constriction in an endothelium intact preparation (A), where the vessel diameter in control is 34.2 μm; the effect of ACh in an endothelium intact preparation (B), where the vessel diameter in control is 32.9 μm; and the lack of effect of ACh in an endothelium denuded preparation (C), where the resting vessel diameter in control is 26.5 μm.
A. Amplitude of contraction (% maximal UK 14304 contraction)

B. Amplitude of contraction (% maximal UK 14304 contraction)

C. Amplitude of contraction (% maximal UK 14304 contraction)

UK-14304 (100 nM)

ACh (1 μM) (10 μM)

15 μm

4 min
in which the application of SNAP resulted in a vasodilation (114.9 ± 1.3% RVD) comparable to the relaxation to SNAP observed in endothelium intact preparations (113.0 ± 1.6% RVD) were used in the present study.

3.3 DISCUSSION

The present study has investigated the mechanisms underlying spontaneous rhythmical contractions in isolated rat iris arterioles. The data would indicate that vasomotion is independent of voltage changes, but instead appears to result from the cyclical activation of the phospholipase C and phospholipase A2 pathways and the cyclical release of intracellular calcium from the IP3-sensitive calcium store. Furthermore, rhythmical contractions appear to occur independently of the vascular endothelium.

3.3.1 Spontaneous vasomotion in rat iris arterioles

Rhythmical contractions of blood vessels have been observed both in vivo in vessels of the microcirculation, as well as in larger vessels such as the basilar artery (Auer & Gallhofer, 1981; Hundley et al., 1988; Fujii et al., 1990b; Morita-Tsuzuki et al., 1992; Bertuglia). In vitro, vasomotion has been shown to occur spontaneously, when activated by an agonist, or following short duration electrical stimulation (Gustafsson et al., 1993; Hill et al., 1999; Peng et al., 2001). In the present study, rhythmical contractions occur spontaneously and are preceded by oscillations in both membrane potential and arterial wall [Ca2+]. An association between oscillations in smooth muscle membrane potential, [Ca2+], and rhythmical contractions has previously been reported in other vessels (Segal & Beny, 1992; Gustafsson et al., 1993; von der Weid & Beny, 1993; Peng et al., 2001; Oishi et al., 2002).

The cells from which the spontaneous rhythmical depolarizations were recorded displayed similar electrophysiological characteristics to that previously identified in the iris arteriole as smooth muscle (Hirst et al., 1997). Furthermore, the average resting membrane potential of these cells lies within the range previously reported for arterial smooth muscle (Hirst & Edwards, 1989; Kuriyama et al., 1998; Davis & Hill, 1999). On the other hand, in those preparations from which changes in cell calcium were studied, definitive identification of the individual cells loaded from the adventitial surface with the fluorescent calcium indicator Fura 2-AM was difficult. A number of previous studies, however, have shown that
the transfer of dyes between the endothelial and smooth muscle cell layers does not occur following incubation periods similar to that used in the present study (Dora et al., 1997; Yashiro & Duling, 2000; Oishi et al., 2001). Therefore, in the iris arteriole while it is assumed that measurements of calcium movements were from the smooth muscle cell layer, loading of endothelial cells with Fura cannot be discounted.

Although all experiments were carried out in non-perfused, non-pressurised vessels, the appearance, form, frequency and cessation of spontaneous contractions and the gradual increase in tone is similar to that previously described in isolated pressurised arterioles (Duling et al., 1981). In contrast, in the rat mesenteric artery, a vessel commonly used to study vasomotion, no development of basal tone or spontaneous contractions are observed over time or following increases in luminal pressure (Gustafsson et al., 1993). This supports the suggestion that heterogeneities exist in the mechanisms underlying vasomotion between vascular beds. In the iris arteriole, increases in vascular tone were accompanied by an increase in basal calcium levels. This elevated basal calcium level may be due to the summation of unsynchronised calcium oscillations in individual smooth muscle cells as previously proposed (Iino et al., 1994; Shimamura et al., 1999; Rottingen & Iversen, 2000; Mauban et al., 2001). While imaging calcium movements within individual irideal arteriolar smooth muscle cells would assist in resolving these issues, at this time this procedure remains outside the technical limitations of this study.

Mechanical stress has been reported to alter smooth muscle cell resting membrane potential and intracellular calcium levels through the activation and influx of calcium directly through stretch-activated cation channels (Setoguchi et al., 1997; Wu & Davis, 2001). In rabbit mesenteric arterioles and rat cerebral arteries a stretch activated chloride conductance has been suggested to be responsible for initiating depolarization, although the existence of this current in cerebral vessels remains controversial (Nelson et al., 1997; Remillard et al., 2000; Doughty & Langton, 2001). Increases in smooth muscle calcium have also been described to occur through stretch induced phospholipase C activity and release of calcium from intracellular stores, independently of nifedipine sensitive L-type voltage dependent calcium channels in the rabbit aorta (Matsumoto et al., 1995). In the present study, while smooth muscle cell stretch had no significant effects on the frequency or amplitude of spontaneous contractions, it cannot be discounted that stretch of the preparation during pinning, may contribute to the initiation of cyclical activity through stretch induced increases in basal calcium levels.

64
In the hamster cheek pouch arteriole, bath solutions with high oxygen concentrations have been shown to suppress rhythmical activity (Bouskela & Grampp, 1992), while in the rat pulmonary artery, vasomotion is induced by hypoxic conditions (Bonnet et al., 2001). In the iris arteriole, however, variations in the oxygen concentration of solutions had no effect on rhythmical contractions or vessel tone. Since alterations in pH have been shown to affect calcium handling within vascular smooth muscle (Heppner et al., 2002), these differences may be attributable to changes in oxidative metabolism and the ability of individual vascular beds to buffer subsequent changes in pH (Ellis & Noireaud, 1987).

3.3.2 Spontaneous contractions are voltage independent.

Like the rhythmical activity recorded in hamster cheek pouch arterioles in vivo and pig coronary artery strips in vitro (Segal & Beny, 1992; von der Weid & Beny, 1993), the spontaneous rhythmical contractions developed by isolated irideal arterioles were always preceded by rhythmical membrane potential fluctuations. However, the voltage dependent calcium channel antagonist, nifedipine, had no effect on rhythmical contractions, calcium oscillations, basal calcium levels or vessel tone. Previous studies have also shown that nifedipine has no effect on rhythmical depolarizations (Hill et al., 1999). Thus in the iris arteriole, increases in intracellular calcium are not due to the influx of extracellular calcium through nifedipine sensitive voltage dependent calcium channels. However, nifedipine insensitive voltage dependent calcium channels are not ruled out. This is in direct contrast to the rabbit femoral artery and rat thoracic aorta where rhythmical contractions have been described to result entirely from voltage dependent events (O mote & Mizusawa, 1995).

In tissues where rhythmical depolarizations are considered dependent on the activation of voltage dependent ion channels in the cell membrane, for example, in cardiac muscle, activity can be prevented by hyperpolarization or by applying a voltage clamp (Brown et al., 1984). In contrast, when short isopotential segments of iris arterioles were examined, neither the frequency of spontaneous depolarizations nor the associated contractions were altered by membrane depolarization or hyperpolarization. Furthermore, under voltage clamp conditions, spontaneous inward currents were recorded at frequencies identical to those of the original voltage oscillations, indicating that the underlying currents were independent of voltage changes. This voltage independence of the contractions in these vessels suggests that they would be little altered by pressurization which results in depolarization of the resting membrane potential.
As the preparations consisted of a monolayer of smooth muscle cells, apparently unconnected to the endothelium (Hirst et al., 1997), complicating factors related to the control of the membrane potentials of complex syncytia do not apply. Furthermore, since the segments of arteriole were cut to have physical lengths much shorter than their electrical length constants (Hirst et al., 1997), then the changes in membrane potential imposed at the centre of the arteriolar segment would be little if at all attenuated at the ends of the segments. Thus the experiments show that the rhythmical activity of iris arterioles results from the cyclical activation of sets of ion channels in the virtual absence of a potential change. Since the intracellular calcium chelator, BAPTA-AM, abolished both spontaneous rhythmical depolarizations and contractions, vasomotion in the iris arteriole would appear to depend on the cyclical release of \( \text{Ca}^{2+} \) from an internal store.

3.3.3 Absence of a role for the ryanodine sensitive intracellular calcium store

Ryanodine had no effect on spontaneous contractions confirming that calcium induced calcium release from the ryanodine sensitive intracellular store is not significantly contributing to the rhythmical activity observed in the iris arteriole (Hill et al., 1999). Activation of the ryanodine sensitive store has been reported to be dependent on calcium influx through VDCCs in intact rat cerebral arteries and in isolated rat portal vein and cerebral artery myocytes (Nelson et al., 1995; Arnaudeau et al., 1997; Jaggar et al., 1998a). Since vasomotion in the present study is not inhibited by nifedipine this interaction between VDCCs and ryanodine stores may not occur in iris arterioles.

Caffeine, which is commonly used to activate calcium release through ryanodine receptors (Rousseau et al., 1987), rapidly abolished spontaneous contractions and relaxed the vessel. This is in direct contrast with findings in the rat afferent arteriole and rat portal vein whereby incubation in caffeine increased both the vessel tone and frequency of rhythmical contractions, suggesting a role for the ryanodine sensitive intracellular calcium store in these vessels (Burt, 2003; Takenaka et al., 2003). Since ryanodine had no effect on irideal rhythmical contractions, it would appear unlikely, that in the iris arteriole, caffeine is depleting ryanodine sensitive intracellular calcium stores. While caffeine has been reported to directly inhibit L-type voltage dependent calcium channels in rat ventricular myocytes (Zahradnik & Palade, 1993), this action is unlikely in iris arterioles since did not abolish spontaneous vasomotion. Caffeine has also been shown to inhibit calcium release from the IP\(_3\) sensitive calcium store (Ehrlich & Watras, 1988; Zhao & van Helden, 2003) as well as
to increase cAMP levels through phosphodiesterase inhibition (Arnaud, 1987; Beavo & Reifsnyder, 1990). Since BAPTA abolished rhythmical activity, suggesting the involvement of an intracellular calcium store, and ryanodine had no effect on contractions, caffeine may indeed be acting on IP$_3$ receptors to inhibit spontaneous activity in the iris arteriole. On the other hand, since the adenylate cyclase inhibitor 2’5 dideoxyadenosine (10 μM) increased the frequency and amplitude of spontaneous contractions and decreased vessel diameter (data not shown), it cannot be excluded that caffeine may be acting at the level of phosphodiesterase activity to decrease or modify spontaneous contractions.

3.3.4 A role for phospholipase C and the IP$_3$ sensitive intracellular calcium store

Calcium released from the IP$_3$ sensitive calcium store, through activity of the phospholipase C pathway, has been shown to be important in the generation of rhythmical activity in other smooth muscle tissues, such as the guinea-pig gastric pylorus and porcine urinary bladder smooth muscle (Van Helden et al., 2000) as well as in the generation of calcium oscillations in the rat tail artery and canine pulmonary smooth muscle cells (Iino et al., 1994; Hamada et al., 1997; Sward et al., 2002). In irideal arterioles, phospholipase C inhibition with U73122 rapidly abolished the spontaneous contractions and calcium oscillations, while the inactive isomer of this inhibitor, U73343, had no effect. U73122 also decreased basal calcium levels and significantly increased the vessel diameter. This data would indicate that basal calcium levels, calcium oscillations and subsequent contractions require phospholipase C activity for their maintenance. Moreover, it is possible that phospholipase C is tonically activated while the vessel is at rest and that its activity is low until a stimulus boosts the signal and spontaneous contractions are initiated through the additional release of calcium from the IP$_3$ sensitive calcium store.

To further establish a role for the IP$_3$ sensitive calcium store in rhythmical contractions we used the putative IP$_3$ receptor channel inhibitor, 2-APB (Maruyama et al., 1997). Surprisingly, 2-APB had little effect on the rhythmical contractions. It is possible that the lack of a significant effect may be due to the involvement of a subtype of IP$_3$ receptor with reduced sensitivity to 2-APB, as has recently been reported (Kukkonen et al., 2001). However, 2-APB has also been demonstrated to be involved in both the inhibition of capacitive calcium re-entry (Ma et al., 2000; Potocnik & Hill, 2001) and the SR Ca$^{2+}$-ATPase (Bilmen et al., 2002). Thus, an alternative explanation for the transient inhibition of rhythmical activity caused by 2-APB may be due to differing effects on the various
subtypes of TRP channels involved in calcium influx (Putney & McKay, 1999). It would
seem unlikely that 2-APB is acting at the level of the Ca\(^{2+}\) ATPase as both thapsigargin and
cyclopiazonic acid have previously been shown to completely abolish rhythmical activity
and relax the iris arteriole (Hill et al., 1999). Interestingly, preliminary experiments using
an alternate IP\(_3\) receptor inhibitor, Xestospongin C (1 \(\mu\)M), had no effect on rhythmical
activity. The lack of specificity of this antagonist has previously been described (De Smet
et al., 1999; Bootman et al., 2002; Solovyova et al., 2002). Regardless, the finding that
U73122 abolished spontaneous vasomotion is consistent with the view that calcium release
from the IP\(_3\)-sensitive calcium stores is required for vasomotion.

In addition to IP\(_3\), activation of PLC generates diacylglycerol which leads to the
translocation and activation of protein kinase C (see Orallo, 1996 for review). In the present
study, inhibition of protein kinase C abolished the spontaneous contractions. Simultaneous
abolition of the underlying [Ca\(^{2+}\)], oscillations suggests that the effects of PKC are at the
level of the calcium stores, although the possibility of additional modulation at the level of
phosphorylation of myosin light chain cannot be excluded. In cat renal and cerebral
arterioles, diacylglycerol is thought to activate calcium channels (Harder et al., 1997) and
release arachidonic acid through the activation of DAG lipase, for conversion by COX,
LOX and cytochrome P450 enzymes (McGiff & Quilley, 1999). Further studies are
required in order to examine the role of DAG in spontaneous contractions in rat irideal
arterioles.

### 3.3.5 Chloride channel inhibition abolishes rhythmical activity

In vascular smooth muscle cells, the estimated equilibrium potential for chloride
\((E_{Cl})\) lies between -20 and -40 mV, which is some 15-30 mV more positive than the smooth
muscle cell resting membrane potential in any given vascular bed (Hogg et al., 1994b;
Lamb & Barna, 1998). Thus, in blood vessels, activation of a chloride channel is thought to
provide an important mechanism through which depolarization of the membrane potential
and ultimately vessel tone is controlled (Large & Wang, 1996; Lamb et al., 2000). In the
iris arteriole, spontaneous rhythmical contractions are preceded by large rhythmical
depolarizations, in the magnitude of 30 mV. While these depolarizations do not appear to
be essential for vessel contraction, depolarizations of this amplitude would appear
indicative of a role for Cl\(^-\) channels in this activity.
Two major chloride currents have previously been described in vascular smooth muscle cells, namely the calcium dependent chloride channel and the volume (or swell) regulated chloride channel (Remillard et al., 2000; Greenwood et al., 2001; Piper & Large, 2003). More recently, a third channel, the cGMP-activated calcium-dependent chloride channel has also been described (Matchkov et al., 2004a; Piper & Large, 2004). In the iris arteriole, rhythmical depolarizations have previously been suggested to be due to the activation of calcium dependent chloride channels following increases in intracellular calcium (Gould & Hill, 1996; Hill et al., 1999). Depolarization of the smooth muscle cell membrane potential following the activation and opening of calcium dependent chloride channels due to increases in intracellular calcium have been previously described in a number of vascular and non-vascular smooth muscle tissues (Byrne & Large, 1987; Van Helden, 1988; Amedee et al., 1990; Klockner & Isenberg, 1991; Daniel et al., 1993; Van Helden, 1993; Lamb et al., 1994; Large & Wang, 1996; Hashitani & Edwards, 1999; Hirakawa et al., 1999; Hirst et al., 2002).

In the present study, the oscillating depolarizations were abolished by the putative calcium dependent chloride channel antagonist, DIDS, suggesting at first that Cl− channels may be activated by the cyclical increases in [Ca2+]. However, DIDS also abolished rhythmical changes in [Ca2+] and associated contractions. Thus, while data would support a role for calcium dependent chloride channels, it would appear that DIDS has additional effects on Ca2+ handling within arteriolar smooth muscle cells. One explanation for the data then, is that DIDs directly exerts a non-selective action on intracellular calcium stores or, alternatively, blocks chloride channels in the sarcoplasmic reticulum which may function to maintain electrical neutrality across the store (Kawano & Hiraoka, 1993). Chloride channels have been reported to be present on the SR of both skeletal and cardiac muscles, where they are thought to play an essential role in excitation-contraction coupling by balancing charge movement during calcium release and reuptake (Kourie, 1997). Overall, this result implies that DIDS is not a particularly successful agent in elucidating the exact role of spontaneous depolarizations that precede spontaneous contractions. The lack of specific chloride channel inhibitors has previously been reported (Doughty et al., 1998; Piper et al., 2002) and further research will be required to establish the specific role of chloride channels in spontaneous rhythmical contractions of the iris arteriole.
3.3.6 A role for the phospholipase A\textsubscript{2} pathway in spontaneous vasomotion

Like the effects of PLC inhibition, the specific cytosolic phospholipase A\textsubscript{2} inhibitor AACOCF\textsubscript{3} (LaBelle & Polyak, 1998) abolished the spontaneous contractions, as did antagonists of the lipoxygenase pathway. On the other hand, spontaneous contractions were resistant to cyclo-oxygenase inhibitors, while inhibition of the cytochrome P450 pathway, by 17-ODYA, caused a small reduction in the amplitude, but not the frequency, of contractions. The effect of 17-ODYA is consistent with a minor contractile effect of HETEs in smooth muscle cells rather than a relaxant effect mediated by EET production from endothelium (Campbell & Harder, 1999). The lipoxygenase pathway is reported to produce heterogeneous responses in vascular smooth muscle, with metabolites demonstrating both potent contractile (Scriabine \textit{et al.}, 1990) and relaxant effects (Barlow \textit{et al.}, 2000; Faraci \textit{et al.}, 2001). Such divergent responses may arise because lipoxygenase metabolites can activate receptors on either smooth muscle cells to produce contraction (Back \textit{et al.}, 2000) or on endothelial cells to produce relaxation (Walch \textit{et al.}, 1999). Since phospholipase A\textsubscript{2} inhibition produced an abolition of the intracellular $[\text{Ca}^{2+}]_i$ oscillations, as did inhibition of the PLC pathway, then it appears that this pathway is also integral to the generation of the calcium oscillations which underly the depolarizations and contractions. Lipoxygenases convert arachidonic acid into HPETEs that can be metabolized into a variety of potent signalling molecules including leukotrienes, HETEs and THETEs, all of which have vasoactive properties that are mediated at the cell surface on receptors or channels (Brash, 1999). In a variety of cell types (Mong \textit{et al.}, 1988; Bouchelouche \textit{et al.}, 1990; Sjolander \textit{et al.}, 1990; Oliva \textit{et al.}, 1994; Bouchelouche \textit{et al.}, 2001), leukotrienes have been shown to bind to G protein coupled membrane receptors that stimulate the phospholipase C/IP\textsubscript{3} cascade and the mobilization of $[\text{Ca}^{2+}]_i$. It is possible that a similar mechanism, involving the production of leukotrienes, is occurring in the iris arteriole.

Since data indicates metabolites of the lipoxygenase arm of the phospholipase A\textsubscript{2} pathway appear to be important in the generation of rhythmical contractions in the iris arteriole, it might have been expected that agonists of this pathway would upregulate rhythmical activity. However, while an active metabolite of this pathway, leukotriene D\textsubscript{4} appeared to have bimodal effects on vasomotion, in that it failed to re-initiate activity in the presence of antagonists of either the phospholipase C or A\textsubscript{2} signalling pathways. Oscillations in force and intracellular calcium due to leukotriene D\textsubscript{4} have previously been
observed in human detrusor myocytes (Bouchelouche et al., 2003). On the other hand, the precursor molecule, arachidonic acid, completely abolished spontaneous contractions and relaxed the vessel. Indeed, abolition of calcium oscillations in the presence of arachidonic acid has previously been reported (Sergeeva et al., 2003). Thus while the present results appear consistent with receptor activation on both smooth muscle and endothelial cells, it also highlights the apparent difficulties associated with bath application of agonists to mimic the compartmental activation of a pathway within a cell. Since recent evidence suggests that heterogeneity exists within the leukotriene receptor population (Labat et al., 1992; Coleman et al., 1995; Back et al., 2000) characterization of the active lipoxygenase metabolite must await future studies.

3.3.7 Rhythmical contractions occur independently of the vascular endothelium

Disruption of the vascular endothelium in the present study, failed to eliminate arteriolar contractions, suggesting that the spontaneous rhythmical contractions observed in irideal arterioles do not require vasoactive substances of endothelial origin for their generation. Moreover, it would suggest that under physiological conditions, the lipoxygenase metabolites are active in smooth muscle cells and not in the endothelial cell layer. In many vessels, vasodilatory and vasocontractile mechanisms are endothelium dependent (Drexler & Hornig, 1999; Mombouli & Vanhoutte, 1999; Shimokawa, 1999). On the other hand, and in support of the present finding, in some vascular beds both vasomotion and normal myogenic responsiveness have been demonstrated to occur independently of the endothelium (Meininger & Davis, 1992; Omote et al., 1992; Omote & Mizusawa, 1994). The success of the procedure for damaging the endothelium was demonstrated by the absence of ACh induced vasodilation.

Although the endothelium itself does not appear to be essential to the generation of spontaneous contractions, modulation of smooth muscle contractile activity by the diffusion of endothelial derived factors through the extracellular space is still possible. Indeed, removal of the endothelium would be expected to result in a loss of endogenous nitric oxide. It is surprising then that disruption of the endothelium did not potentiate the contraction evoked by the adrenergic agonist, UK14304, as has been documented in other vessels after treatment with L-NAME (Kuriyama et al., 1998; Van Helden et al., 2000). Similarly, there was no increase in frequency and amplitude of spontaneous contractions in endothelium
denuded vessels, as described above for intact vessels in the presence of L-NAME (Hill et al., 1999). These latter observations may suggest that the treatment involved in endothelial disruption may have caused some damage to the single layer of smooth muscle cells which make up the remainder of the wall of these arterioles (Hirst et al., 1997). Indeed, prolonged exposure to water may damage smooth muscle cell function (Rosenblum, 1986), a method commonly used to disrupt endothelial integrity.

Without a doubt, the difficulty of removing the endothelium was highlighted during the course of the experiments as a majority of preparations, approximately 60 %, exhibited a dilatory response to ACh, thus demonstrating endothelial integrity. Such experiments were therefore discarded. Indeed, such technical difficulties associated with endothelial removal are not restricted to the rat iris arterioles. In hamster cheek pouch arterioles, for example, the endothelium was destroyed by fluorescence tagging and light illumination. When using this technique, exposure to light for extended periods of time caused damage to the underlying smooth muscle (Bartlett & Segal, 2000). Therefore, although dilation to the endothelium independent vasodilator, SNAP, was not significantly different in either the endothelium intact or denuded preparations, it is possible that some subtle damage to the smooth muscle cell layer goes undetected.

3.3.8 Proposed mechanism underlying spontaneous contractions of iris arterioles

Since inhibitors of phospholipase C or phospholipase A\(_2\) independently abolished \([Ca^{2+}]_i\) oscillations and vasomotion, it appears that cross talk must occur between the two pathways. The possibility exists that this may be brought about through protein kinase C, since up-regulation of phospholipase A\(_2\) activity can occur through protein kinase C activation (Garcia et al., 1992). Based on the data presented here, we propose a mechanism by which intracellular calcium is integral to the spontaneous, rhythmical contractions generated by the irideal arterioles (Fig 3.12). Constitutive activity of the PLC pathway leads to a basal level of calcium, intracellular tone and up-regulation of the PLA\(_2\) pathway through activation of protein kinase C. Metabolism of arachidonic acid through the lipoxygenase pathway produces vasoactive substances which can augment the release of calcium from the IP\(_3\)-sensitive calcium store. The calcium oscillations produce oscillating contractions and coincidentally activate calcium dependent channels in the cell membrane to produce oscillatory voltage changes. The mechanism by which lipoxygenase metabolites contribute to the intracellular calcium oscillations is at present unknown. Constitutive
activity of the phospholipase C pathway may result from stretch during the pinning procedure or alternatively this pathway may show increased activity during development.
Figure 3.12
Figure 3.12

Proposed mechanisms underlying spontaneous contractions of iris arterioles. Constitutive activity of phospholipase C (PLC) leads to the production of IP$_3$ and basal release of calcium (Ca$^{2+}$) from the IP$_3$ sensitive calcium store, which contributes to arteriolar tone. Simultaneous activation of protein kinase C (PKC) in turn activates phospholipase A$_2$ (PLA$_2$) and breakdown of arachidonic acid via the lipoxygenase (LOX) pathway. The resultant metabolites further stimulate the PLC pathway resulting in cyclical oscillations of the cell membrane through an unknown ion channel.
4.1 INTRODUCTION

As discussed in the general Introduction, spontaneous vasomotion is commonly observed in small vessels of the microcirculation, including the rat iris arteriole (Auer & Gallhofer, 1981; Hundley et al., 1988; Morita-Tsuzuki et al., 1992; Bertuglia et al., 1994; Hill et al., 1999). Rhythmical oscillations in vessel diameter have also been shown to occur both in vivo and in vitro in larger vessels either spontaneously, or in response to agonist stimulation (Kawasaki et al., 1981; Fujii et al., 1990b; Gustafsson et al., 1994). Since evidence suggests that vasomotion is both physiologically and pathophysiologically relevant (Hudetz et al., 1988; Shimamura et al., 1999; Rucker et al., 2000; Gratton, 2002), studies of the underlying mechanisms in functionally different vessels are required.

Recent studies in rat mesenteric arteries in vitro, have shown that agonist induced vasomotion was due to the synchronization of calcium oscillations in individual smooth muscle cells (Mauban et al., 2001; Peng et al., 2001). It was further hypothesized that these calcium oscillations result from the intermittent release of calcium from ryanodine-sensitive intracellular calcium stores, while synchronicity occurs following the activation of voltage dependent calcium channels presumably after depolarization (Peng et al., 2001).

Vasomotion is widely observed throughout the cerebral circulation. Spontaneous rhythmical contractions recorded in the adult rat basilar artery in vivo, have been shown to be dependent on voltage activated mechanisms involving calcium activated potassium channels (Fujii et al., 1990a). Paradoxically, studies of cultured smooth muscle cells and of pressurized cerebral arteries have previously demonstrated an important role for intracellular ryanodine sensitive calcium stores and calcium activated potassium channels in relaxation, rather than contraction of cerebral vessels (Nelson et al., 1995; Bonev et al., 1997; Gollasch et al., 1998; Jaggar & Nelson, 2000; Jaggar, 2001; Perez et al., 2001). A role for potassium channels in modulating vasomotion has also been demonstrated in the rat mesenteric artery (Gustafsson & Nilsson, 1994).
Since control of blood flow through cerebral vessels varies in several respects from that in systemic vessels (Faraci & Heistad, 1998), we were interested to investigate the mechanisms underlying vasomotion in cerebral vessels. Thus the aims of the present study were to investigate the role of voltage dependent and independent mechanisms by correlating changes in membrane voltage with changes in intracellular calcium and determining the role of specific intracellular calcium stores and ion channels.

4.2 RESULTS

4.2.1 General observations

In the juvenile rat, spontaneous rhythmical contractions are routinely recorded in the rat basilar artery (BA) and its branches (caudal cerebellar arteries: CCA). Vasomotion was typically observed throughout the entire basilar artery network within 15-20 min of incubation in control Krebs solution and could be reliably recorded for more than 2 hours. No differences were observed in the frequency of contractions between the BA and the CCAs.

4.2.1.1 Contractions.

In those preparations used for electrophysiology, the mean resting vessel diameter was larger (P<0.05) due to the tighter stretching required for long term impalements in actively contracting vessels (58.2 ± 1.7 μm, n = 100 animals), compared to those preparations used for measuring intracellular calcium (40.4 ± 1.2 μm, n = 140 animals). In addition, the frequency of contractions was increased, compared to those used for imaging (18.2 ± 0.9 and 14.4 ± 0.7 min⁻¹, respectively; P<0.05). However, no significant differences in the amplitude of contractions between either of the two methodological approaches used were observed (6.4 ± 0.7 and 6.1 ± 0.2 % resting vessel diameter (RVD), respectively). Since preparations set up as described for intracellular calcium protocols showed no significant difference in the frequency (16.4 ± 1.4 min⁻¹; n = 4) or amplitude (7.0 ± 1.1 % RVD, n = 4) of contractions in the absence of Fura loading, data would suggest that the discrepancies in contraction frequency between the two methodological approaches used can be attributed to the degree of vessel stretch, and not to Fura itself buffering [Ca²⁺] if lower levels.

Importantly, since no differences in the responses to drug solutions were observed between the two procedures, data from both groups have been pooled for clarity. Thus, in
the present study, spontaneous contractions occurred at a rate of $16.5 \pm 0.3 \text{ min}^{-1}$, the average amplitude for all preparations in control Krebs’ solution was $6.2 \pm 0.6 \% \text{ RVD} (n = 240)$, and the average RVD was $47.7 \pm 1.1 \mu\text{m} (n = 240)$.

4.2.1.2 Measurement of SMC membrane potential.

When intracellular recordings were made from SMCs, as identified by the nuclear marker propidium iodide, spontaneous rhythmical depolarizations were found to precede the spontaneous contractions by $1.6 \pm 0.1 \text{ s}$ (Fig 4.1A; peak depolarization to peak contraction). Depolarizations typically ranged from 5 to 25 mV in amplitude ($10.5 \pm 0.7 \text{ mV}, n = 100$) and the most negative membrane potential reached was $-41.3 \pm 0.4 \text{ mV} (n = 100)$.

4.2.1.3 Measurements of SMC $[\text{Ca}^{2+}]_i$

Using photometry, $[\text{Ca}^{2+}]_i$ oscillations were recorded from the vessel wall and these oscillations preceded the spontaneous contractions by $0.9 \pm 0.1 \text{ s}$ (Fig 4.1B; $n = 5$). Calcium oscillations typically ranged from 0.04 to 0.14 $F_{340/380}$ in amplitude ($0.087 \pm 0.013 F_{340/380}, n = 140$). Imaging of individual SMCs recorded after the onset of rhythmical contractions, revealed that calcium propagated in a wave like manner from one end of the cell to the other (Fig 4.2). When averaged over the entire cell, these waves were recorded as oscillations (Fig 4.1C) similar to those recorded with photometry from the arterial wall (Fig 4.1B).

4.2.1.4 Rhythmical activity over time.

An hour after the onset of rhythmical activity, no change was observed in the frequency of spontaneous contractions (onset: $22.4 \pm 2.6 \text{ min}^{-1}$, 60 min: $23.0 \pm 2.7 \text{ min}^{-1}$), depolarizations or $[\text{Ca}^{2+}]_i$ oscillations in the arterial wall ($n = 14$). Although the amplitude of contractions (onset: $5.3 \pm 0.6 \% \text{ RVD}, 60 \text{ min: } 5.9 \pm 0.7 \% \text{ RVD}; n = 14$), depolarizations (onset: $6.6 \pm 0.3 \text{ mV}, 60 \text{ min: } 7.7 \pm 0.6 \text{ mV}; n = 6$) and $[\text{Ca}^{2+}]_i$ oscillations in the arterial wall (60 min: $107.2 \pm 2.2 \% \text{ of amplitude of control } F_{340/380} \text{ oscillation}; n = 8$) showed a trend to increase, this was not significantly different to that recorded immediately following the onset of activity. With time, vessel tone was increased, as evidenced by a significant decrease in resting vessel diameter (onset: $40.6 \pm 3.9 \mu\text{m}, 60 \text{ min: } 37.5 \pm 3.4 \mu\text{m}; n = 14; P<0.05$, paired t-test). This was accompanied by a significant increase in basal $[\text{Ca}^{2+}]_i$ levels in the arterial wall (107.1 $\pm 2.5 \% F_{340/380}; n = 8; P<0.05$, paired t-test). No significant changes were observed in resting membrane potential (onset: $-41.3 \pm 1.8 \text{ mV}, 60$
Figure 4.1
Figure 4.1
Spontaneous rhythmical contractions recorded in control Krebs’ solution. A, Intracellular recordings show that spontaneous depolarizations precede spontaneous contractions. Using photometry and calcium imaging, $[\text{Ca}^{2+}]_i$ oscillations are recorded from the arterial wall and from individual SMCs and these precede spontaneous contractions (B and C, respectively). Constriction is represented by a downward deflection while increases in $[\text{Ca}^{2+}]_i$ are represented by an upward deflection. D, Image of SMCs (arrows) loaded with Fura-2 AM, illuminated with 380 nm light. The black line highlights the vessel edge from which changes in vessel diameter are recorded. The scale bar is 10 $\mu$m.
During spontaneous vasomotion, $[\text{Ca}^{2+}]_i$ waves were recorded in individual SMCs and investigated using an intensified CCD camera and appropriate software for line scan mode. Sequential images (A), and corresponding traces of $[\text{Ca}^{2+}]_i$ movements in an individual SMC (B) shows that $[\text{Ca}^{2+}]_i$ is first increased at one end of the cell, propagating in a wave-like manner along its length (a-c). Images show individual SMCs in the intact artery loaded with Fura 2-AM and illuminated with 380 nM light. Arrow indicates SMC from which $[\text{Ca}^{2+}]_i$ movements were recorded.
min: -39.8 ± 3.3 mV; \( n = 6 \). [Ca\(^{2+}\)]\(_i\) oscillations in individual SMCs showed a trend to become more synchronized.

### 4.2.1.5 Variations in vasomotion and synchronicity of Ca\(^{2+}\) oscillations.

In a proportion of preparations (Fig 4.3; \( n = 4 \)), large regular contractions (11.9 ± 0.7 % RVD) and [Ca\(^{2+}\)]\(_i\) oscillations in the arterial wall (0.0971 ± 0.006 F\(_{340/380}\)) were recorded (Fig 4.3Aa). In these preparations, [Ca\(^{2+}\)]\(_i\) oscillations in individual SMCs were well synchronized (Fig 4.3Ab) and had an average amplitude of 0.053 ± 0.002 F\(_{340/380}\). In others (Fig 4.3Ba; \( n = 4 \)), contractions were smaller and irregular (5.1 ± 0.4 % RVD). Accordingly, [Ca\(^{2+}\)]\(_i\) oscillations in the arterial wall were found to be reduced (0.0761 ± 0.002 F\(_{340/380}\); \( P < 0.05 \), unpaired t-test) compared to those preparations exhibiting more robust contractions. Although [Ca\(^{2+}\)]\(_i\) oscillations in individual cells were of similar amplitudes to those seen in the previous group (0.530 ± 0.004 F\(_{340/380}\)), oscillations in adjacent cells appeared to be asynchronous (Fig 4.3Bb).

Thus, in the present study, both the amplitude of the [Ca\(^{2+}\)]\(_i\) oscillations in individual SMCs, and in the vessel wall have been used to as a measure of the degree of SMC coupling. In those preparations in which SMC [Ca\(^{2+}\)]\(_i\) oscillations were synchronized, the ratio of the amplitude of [Ca\(^{2+}\)]\(_i\) oscillations in individual SMCs, to the amplitude of the [Ca\(^{2+}\)]\(_i\) oscillations in the arterial wall is less than 1 (0.56 ± 0.06, \( n = 4 \)). On the other hand, in those preparations in which SMC [Ca\(^{2+}\)]\(_i\) oscillations were more asynchronous this ratio is increased (Fig 4.3C; 0.70 ± 0.06; \( n = 4 \); \( P < 0.05 \), unpaired t-test).

[Ca\(^{2+}\)]\(_i\) oscillations recorded from adjacent SMCs, in both synchronized and unsynchronized preparations, showed variations in amplitude and in temporal characteristics. Variations in the amplitude of sequential [Ca\(^{2+}\)]\(_i\) oscillations in individual SMCs were also observed (Fig 4.4).

In a number of preparations, periodic variations in the amplitude of the spontaneous contractions, depolarizations (Fig 4.5A) and oscillations in arterial wall calcium (Fig 4.5B) were observed. In these preparations the amplitude of the contractions tended to be similar to the amplitude of the underlying depolarizations and [Ca\(^{2+}\)]\(_i\) oscillations. Imaging of individual SMCs in preparations displaying this periodic pattern, showed that [Ca\(^{2+}\)]\(_i\) oscillations in individual cells were synchronized within small groups of 2-3 adjacent cells, while [Ca\(^{2+}\)]\(_i\) oscillations in adjacent groups of cells behaved in an unsynchronized manner (Fig 4.5C).
Figure 4.3
Figure 4.3

Preparations show differences in synchronicity of smooth muscle cell calcium oscillations. A, Adjacent SMC profiles during vasomotion in which \([\text{Ca}^{2+}]_i\) oscillations in individual cells were well synchronized (Ab). The amplitude of the arterial wall \([\text{Ca}^{2+}]_i\) oscillations is large (Aa) and rhythmical contractions are robust (Aa and Ab), compared to (B) preparations in which \([\text{Ca}^{2+}]_i\) oscillations were more asynchronous (Bb). In these preparations, the amplitude of the \([\text{Ca}^{2+}]_i\) oscillations in the arterial wall (Ba) and the amplitude of rhythmical contractions is reduced (Ba and Bb). C, Ratio of the amplitude of individual SMC \([\text{Ca}^{2+}]_i\) oscillations to the amplitude of the \([\text{Ca}^{2+}]_i\) oscillations in the arterial is increased in “less coupled” preparations compared to “coupled” preparations. Increases in \([\text{Ca}^{2+}]_i\) are represented as an upward deflection. Basal \text{Ca}^{2+} levels in the representative traces were 0.64 \text{F}_{340/380} (Aa) and 0.58 \text{F}_{340/380} (Ba). Values represent mean and S.E.M. * significantly different from control (P<0.05).
A. "Coupled"

a. Wall calcium

b. SMC calcium (n=4 cells)

B. "Less coupled"

C.

"Coupled" "Less coupled"
Figure 4.4
Calcium oscillations in individual SMCs. A, [Ca^{2+}]_i oscillations recorded from groups of adjacent cells were well synchronized, but showed variations in amplitude and in temporal characteristics (eg. cell 2). Variations in the amplitude of [Ca^{2+}]_i oscillations were also observed amongst the cells (boxed area). Increases in [Ca^{2+}]_i are represented by an upward deflection.
Figure 4.5
Figure 4.5
Spontaneous depolarizations (A) and oscillations in arterial wall $[\text{Ca}^{2+}]_i$ (B) show periodic variations in amplitude. The amplitude of the voltage changes and the $[\text{Ca}^{2+}]_i$ oscillations is proportional to the amplitude of the following contraction (A and B). Under these conditions, (C) $[\text{Ca}^{2+}]_i$ oscillations in individual SMCs are synchronized within small groups of adjacent cells, while oscillations in adjacent groups of cells behave in an unsynchronized manner. Increases in $[\text{Ca}^{2+}]_i$ are represented as an upward deflection. The basal $\text{Ca}^{2+}$ level in the representative trace was 0.59 $F_{340/380}$. 
A. Membrane potential

B. Wall calcium

C. SMC calcium (n=5 cells)
4.2.2 Role of extracellular calcium in rhythmical contractions and $[\text{Ca}^{2+}]_i$

Superfusion with Ca$^{2+}$-free Krebs’ led to a loss of spontaneous contractions and $[\text{Ca}^{2+}]_i$ oscillations in the arterial wall in about 6 min ($n = 4$; $P<0.05$, paired t-test). Basal $[\text{Ca}^{2+}]_i$ levels were decreased (Ca$^{2+}$-free: 84.3 ± 3.8 % F$_{340/380}$; $n = 4$; $P<0.05$, paired t-test) and the vessel relaxed (Control: 52.0 ± 5.3 μm, Ca$^{2+}$-free: 57.2 ± 5.6 μm; $n = 4$; $P<0.05$, paired t-test). On washout, uncoordinated contractions were observed within 3 min and coordinated contractions were recorded by 6 min. Vessel diameter (51.0 ± 3.3 μm, $n = 4$) and basal $[\text{Ca}^{2+}]_i$ levels (99.2 ± 4.3 % F$_{340/380}$; $n = 4$) returned to pre-incubation levels by 15 min. $[\text{Ca}^{2+}]_i$ oscillations in SMCs were abolished.

To assess the involvement of extracellular calcium entering the SMCs via L-type VDCCs, preparations were exposed to the antagonist nifedipine (1 μM; Fig 4.6). Within 4 min of the drug entering the recording chamber, nifedipine produced a significant reduction in the frequency (Control: 16.3 ± 1.1 min$^{-1}$, nifedipine: 3.6 ± 1.2 min$^{-1}$; $n = 19$; $P<0.05$, paired t-test) and amplitude (Control: 6.5 ± 1.1 % RVD, nifedipine: 2.0 ± 0.4 % RVD; $n = 19$; $P<0.05$, paired t-test) of rhythmical contractions, resulting in the appearance of uncoordinated movements without apparent loss of vessel tone (Fig 4.6A and B; Control: 37.2 ± 2.8 μm, nifedipine: 36.5 ± 2.9 μm; $n = 19$). Spontaneous depolarizations were abolished ($P<0.05$, paired t-test) and the membrane potential depolarized (Fig 4.6A; control: -42.0 ± 1.6 mV; nifedipine: -34.2 ± 1.5 mV; $P<0.05$, $n = 8$, paired t-test).

Measurement of arterial wall $[\text{Ca}^{2+}]_i$ revealed the presence of irregular $[\text{Ca}^{2+}]_i$ oscillations and basal calcium levels were unaltered (Fig 4.6B; nifedipine: 103.0 ± 9.5 % F$_{340/380}$; $n = 11$). Imaging of SMCs showed that small $[\text{Ca}^{2+}]_i$ oscillations were present in individual cells, but these were unsynchronized (Fig 4.6C). Further analysis showed that in the presence of nifedipine, the frequency (Fig 4.7A) and amplitude (Fig 4.7B) of the unsynchronized SMC $[\text{Ca}^{2+}]_i$ oscillations was reduced ($n = 11$, $P<0.05$, paired t-test). In addition, the ratio of the amplitude of $[\text{Ca}^{2+}]_i$ oscillations in individual SMCs, to the amplitude of the $[\text{Ca}^{2+}]_i$ oscillations in the arterial wall increased (Fig 4.7C; $n = 11$, $P<0.05$, paired t-test). When a hyperpolarizing current (0.1-1.0 nA) was applied to short segments of 4 arteries (~300 μm, see chapter 2), the membrane potential hyperpolarized (4.8A). Input resistance ($R_m$) was calculated from the current voltage relationship and was shown to be significantly increased in the presence of nifedipine (Fig 4.8B; $n = 4$; $P<0.05$, paired t-test).

4.2.3 Role of intracellular calcium stores
Figure 4.6

The effect of voltage dependent calcium channel antagonist, nifedipine (1 μM) on rhythmical activity. A, Nifedipine abolished rhythmical depolarizations and contractions and depolarized SMCs (trace 4 min in drug). B, [Ca^{2+}]_i oscillations recorded from the arterial wall became irregular (trace 10 min in drug). (C) [Ca^{2+}]_i oscillations recorded in individual, adjacent SMCs were reduced in amplitude and became asynchronous. Increases in [Ca^{2+}]_i are represented as an upward deflection. The basal Ca^{2+} level in the representative control trace was 0.72 F_{340/380}. 

<table>
<thead>
<tr>
<th>Control</th>
<th>Nifedipine (1 μM)</th>
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<tr>
<td><strong>A. Membrane potential</strong></td>
<td>![Graph showing membrane potential changes with and without nifedipine]</td>
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<td><strong>B. Wall calcium</strong></td>
<td>![Graph showing wall calcium changes with and without nifedipine]</td>
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<td>![Graph showing wall calcium changes with and without nifedipine]</td>
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<td><strong>C. SMC calcium (n=5 cells)</strong></td>
<td>![Graph showing SMC calcium changes with and without nifedipine]</td>
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<td>![Graph showing SMC calcium changes with and without nifedipine]</td>
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Figure 4.7
Figure 4.7
Effect of nifedipine (1 μM) on Ca\(^{2+}\) oscillations. The effect of voltage dependent calcium channel inhibition on the frequency (A) and amplitude (B) of [Ca\(^{2+}\)]\(_i\) oscillations recorded from individual SMCs after the addition of nifedipine. Points represent values for individual SMCs, whereby 6 adjacent cells were analyzed from 11 different preparations and the cell with the frequency or greatest amplitude designated as 100% in each preparation. Mean frequency and amplitude of SMC [Ca\(^{2+}\)]\(_i\) oscillations in control was 13.1 ± 0.9 min\(^{-1}\) and 0.049 ± 0.000 F\(_{340/380}\), respectively. Straight lines indicate mean values. C, Ratio of the amplitude of individual SMC [Ca\(^{2+}\)]\(_i\) oscillations to the amplitude of the [Ca\(^{2+}\)]\(_i\) in the arterial wall is increased in the presence of nifedipine. Values represent mean and S.E.M. * significantly different from control (P<0.05).
Smooth muscle cell

A. Freq. of [Ca^{2+}] oscillation % of the max. freq.

B. Amp. of [Ca^{2+}] oscillation % max. amplitude

Control | Nifedipine (1 µM)

C. SMC/wall calcium oscillation amplitude

Control | Nifedipine (1 µM)
Figure 4.8

I/V relationship in the basilar artery during spontaneous vasomotion. A, Membrane potential (mV) was recorded from a SMC during a 30 s hyperpolarizing current pulse in (□) control Krebs’ solution and in the presence of (▲) nifedipine (1 μM). Straight lines indicates linear I/V portion (Non-linear regression analysis). B, Input resistance (R$_{in}$) calculated before and after the addition of nifedipine. Values represent mean and S.E.M. * significantly different from control (P<0.05).
The calcium ATP-ase inhibitors, thapsigargin (2 μM; Fig 4.9) and cyclopiazonic acid (CPA: 3μM; Fig 4.10) and the intracellular calcium chelator BAPTA-AM (10 μM; Fig 4.11), were used to examine the role of intracellular calcium stores. Both thapsigargin and BAPTA abolished spontaneous contractions, rhythmical depolarizations and oscillations in both arterial wall calcium and in individual SMCs (P<0.05, paired t-test). Furthermore, the membrane potential hyperpolarized (Fig 4.9A, control: -40.0 ± 2.0, thapsigargin: -50.5 ± 0.5 mV; Fig 4.11A, control: -42.7 ± 3.7 mV, BAPTA-AM: -64.2 ± 3.1 mV; n = 4, P<0.05, paired t-test), calcium levels across the vessel wall decreased (Fig 4.9B, thapsigargin: 93.1 ± 1.7 % F340/380; Fig 4.11B, BAPTA: 88.6 ± 4.5 % F340/380; n = 4; P<0.05, paired t-test) and the artery relaxed (Fig 4.9A and B, Control: 47.1 ± 6.4 μm, thapsigargin: 50.7 ± 7.1 μm; Fig 4.11A and B, Control: 45.5 ± 7.2 μm, BAPTA 51.5 ± 8.5 μm; n = 8, P<0.05 P<0.05).

The respective effects of thapsigargin and BAPTA-AM on rhythmical activity were observed within 10 and 6 min of the drugs entering the recording chamber.

On the other hand, CPA increased both the frequency (control:12.7 ± 1.2 min⁻¹, CPA: 15.8 ± 0.9 min⁻¹; n = 8; P<0.05, paired t-test) and amplitude (control: 4.5 ± 0.6 % RVD, CPA: 6.4 ± 0.9 % RVD; n = 8; P<0.05, paired t-test) of the rhythmical contractions and depolarizations (Fig 4.10A, n = 4), [Ca²⁺]ᵢ oscillations in the arterial wall (Fig 4.10B, n = 4) and in individual smooth muscle cells (Fig 4.10C). The membrane potential depolarized (control: -41.2 ± 1.1 mV, CPA: -38.2 ± 0.8 mV; n = 4; P<0.05, paired t-test) but no significant differences were observed in vessel diameter (control: 45.4 ± 5.5 μm, CPA: 48.5 ± 4.9 μm; n = 8). Basal calcium levels in the arterial wall were also not significantly different to that recorded in control solution (Fig 4.10B; CPA: 105.1 ± 6.7 % F340/380; n = 4). The effects of CPA on rhythmical activity were observed within 15 min of the drug entering the bath.

4.2.3.1 Effect of phospholipase C pathway inhibition on spontaneous rhythmical activity.

The role of phospholipase C and the IP₃ pathway was tested using U73122 (10 μM; Fig 4.12), which completely abolished the spontaneous contractions (n = 9, P<0.05, paired t-test) and caused a relaxation of the vessel (control: 45.7 ± 0.5 μm, U73122: 50.3 ± 1.2 μm; n = 9; P<0.05, paired t-test) within 5 min. Electrophysiogical recordings revealed that the membrane potential hyperpolarized significantly (Fig 4.1A; Control: -43 ± 6.1 mV; U73312: -50.0 ± 8.1 mV; n = 4; P<0.05, paired t-test). Measurements of arterial wall calcium showed that the [Ca²⁺]ᵢ oscillations were abolished (P<0.05, paired t-test) and the
Thapsigargin (2 μM) abolishes spontaneous rhythmical activity. A, Ca²⁺-ATPase inhibition by thapsigargin hyperpolarized SMCs and abolished spontaneous depolarizations and contractions. B, [Ca²⁺]ᵢ oscillations in the vessel wall were inhibited and basal calcium levels decreased. C, [Ca²⁺]ᵢ oscillations in individual SMCs were also abolished. Increases in [Ca²⁺]ᵢ are represented as an upward deflection. The basal Ca²⁺ level in the representative control trace is 0.86 F₃₄₀/₃₈₀.
Figure 4.10

Effect of CPA (3 μM) on spontaneous rhythmical activity. The Ca^{2+}-ATPase inhibitor, CPA increased the frequency and amplitude of (A) rhythmical depolarizations, (B) [Ca^{2+}]_i oscillations in the arterial wall and in (C) individual SMCs. Increases in [Ca^{2+}]_i are represented as an upward deflection. The representative basal Ca^{2+} level in control is 1.0 F_{340/380}.
Figure 4.11

BAPTA-AM (10 μM) abolishes spontaneous rhythmical activity. A, Following intracellular calcium chelation with BAPTA-AM, rhythmical depolarizations and contractions were abolished and SMCs were hyperpolarized. B, [Ca^{2+}]_i oscillations in the arterial wall were inhibited and basal [Ca^{2+}]_i levels were decreased. C, [Ca^{2+}]_i oscillations in individual SMCs were also abolished. Increases in [Ca^{2+}]_i are represented as an upward deflection. The basal Ca^{2+} level in the representative control trace is 0.67 F_{340/380}. 
basal level of calcium was decreased (Fig 4.12B; U73122: 90.7 ± 1.6 % F340/380; n = 5; P<0.05, paired t-test). [Ca^{2+}]_{i} oscillations in individual SMCs were also abolished (Fig 4.12C). The inactive isomer of U73122, U73343 (10 μM) which was used to confirm the specificity of U73122, had no effect on spontaneous contractions, [Ca^{2+}]_{i} oscillations in the arterial wall or in individual SMCs (n = 4). In addition, no effect was observed on basal levels (U73343: 106.5 ± 1.3 F340/380; n = 4) or vessel diameter (Control: 23.5 ± 1.5 μm, U73343: 22.7 ± 1.7 μm; n = 4).

In the present study, the peptide, myristoylated protein kinase C (MPK-C, 10 μM) was used to investigate the role of protein kinase C (PKC) in spontaneous vasomotion of the juvenile rat basilar artery. MPK-C significantly increased the frequency (Control: 13.5 ± 0.9 min^{-1}, MPK-C: 17.2 ± 1.1 min^{-1}; n = 4; P<0.05, paired t-test) but decreased the amplitude of rhythmical contractions (Control: 5.8 ± 0.7 % RVD, MPK-C: 1.8 ± 0.9 % RVD; n = 4; P<0.05, paired t-test). [Ca^{2+}]_{i} oscillations in the arterial wall were also increased in frequency (P<0.05) but significantly decreased in amplitude (MPK-C: 34.4 ± 10 % of amplitude of control F340/380 oscillation; n = 4; P<0.05, paired t-test). No effect was observed on vessel tone (30.2 ± 4.2 μm, MPK-C: 32.5 ± 5.2 μm; n = 4) or on basal [Ca^{2+}]_{i} levels in the arterial wall (MPK-C: 110.9 ± 4.6 % F340/380; n = 4). Imaging of individual SMCs showed that [Ca^{2+}]_{i} oscillations were increased in frequency but decreased in amplitude.

### 4.2.3.2 Effect of ryanodine on spontaneous rhythmical activity.

In the majority of preparations, ryanodine (10 μM), which acts by inhibiting ryanodine receptors, abolished rhythmical contractions (P<0.05, paired t-test) but did not alter vessel tone (Fig 4.13; Control: 49.1 ± 5.5 μm, ryanodine: 47.5 ± 5.5 μm; n = 9). However, in some preparations, small contractions were still observed (Fig 4.13B). The spontaneous depolarizations were increased in frequency, but decreased in amplitude, due to a reduction in the hyperpolarizing phase of the voltage oscillations (Fig 4.13A; control:-41.0 ± 3.0 mV; ryanodine: -32.7 ± 2.8 mV; n = 5; P<0.05, paired t-test). Measurements of arterial wall calcium, mirrored the effect of ryanodine on rhythmical depolarizations in that the oscillations were also increased in frequency (control: 21.0 ± 1.2 min^{-1}, ryanodine: 29 ± 1.0 min^{-1}, P<0.05, paired t-test) but significantly decreased in amplitude (ryanodine: 38.1 ± 3.3% of amplitude of control F340/380 oscillation; n = 4, P<0.05, paired t-test) although the basal level of calcium was not different from control (Fig 4.13B; ryanodine: 109.2 ± 4.9 %
Figure 4.12

The effect of U73122 (10 μM) on spontaneous rhythmical activity. Phospholipase C inhibition by U73122 hyperpolarized SMCs and abolished spontaneous depolarizations and contractions (A), inhibited [Ca^{2+}]_{i} oscillations in the vessel wall (B) and in individual SMCs (C). Increases in [Ca^{2+}]_{i} are represented as an upward deflection. The basal Ca^{2+} level in the representative control trace is 1.40 F_{340/380}.
Figure 4.13
Effect of ryanodine (10 µM) on spontaneous rhythmical activity. Ryanodine (A) depolarized the SMCs and abolished rhythmical contractions. Spontaneous depolarizations and calcium oscillations recorded from the vessel wall were increased in frequency but decreased in amplitude (A and B). Ryanodine generally had no effect on vessel tone and in some preparations small contractions could also be recorded (B). C, Calcium oscillations in individual SMCs were not recorded. Increases in [Ca\textsuperscript{2+}]\textsubscript{i} are represented as an upward deflection. The basal Ca\textsuperscript{2+} level in the representative control trace is 0.97 F\textsubscript{340/380}. 
The response to ryanodine was observed within a 5 min period after exposure to the drug. \( [\text{Ca}^{2+}] \), oscillations in individual SMCs were not present (Fig 4.13C), despite the fact that weak calcium waves could be observed directly from the ICCD file. Addition of ryanodine, after nifedipine, led to the abolition of the asynchronous calcium waves \((n = 4; P<0.05)\), which were seen in nifedipine in individual cells (Fig 4.6C), but had no further effect on arterial wall calcium levels \((112.1 \pm 3.0 \% \frac{F_{340/380}}{F_{380}}; n = 4, P<0.05, \text{paired t-test})\) or on vessel tone \((95.0 \pm 1.5 \% \text{RVD}; n = 4, P<0.05, \text{paired t-test})\).

### 4.2.3.3 Spatial distribution of intracellular calcium stores.

In order to determine whether the IP\(_3\)- and ryanodine-sensitive SR calcium stores spatially overlap or represent separate discrete calcium pools, preparations were exposed to 5-HT \((1 \mu M)\), to release calcium from the IP\(_3\)-sensitive calcium stores, in the presence of both nifedipine \((1 \mu M)\) and caffeine \((1 mM)\), to prevent influx of calcium through VDCCs and to cause depletion from the ryanodine-sensitive store, respectively. In the presence of both nifedipine and caffeine, spontaneous contractions were abolished \((P<0.05)\) and the vessel relaxed \((\text{Control: } 33.0 \pm 5.0 \mu m, \text{nif/caff: } 37.9 \pm 4.7 \mu m; n = 4; P<0.05, \text{paired t-test})\). Addition of 5-HT caused the vessel to constrict \((86.7 \pm 4.1 \% \text{RVD recorded in nifedipine and caffeine}; n = 4; P<0.05, \text{paired t-test})\) but did not initiate vasomotion.

### 4.2.4 Effect of phospholipase A\(_2\) pathway

In the rat iris arteriole, inhibition of the phospholipase A\(_2\) pathway with AACOCF\(_3\) abolished spontaneous, rhythmic contractions. In the present study, however, incubation in AACOCF\(_3\) \((30 \mu M)\) for up to 20 min, had no effect on the frequency \((\text{Control: } 13.5 \pm 1.5 \text{ min}^{-1}, \text{AACOCF}_{3}: 12.5 \pm 2.5 \text{ min}^{-1}; n = 4)\) or amplitude of spontaneous contractions \((\text{Control: } 4.2 \pm 0.3 \% \text{RVD}, \text{AACOCF}_{3}: 4.2 \pm 0.7; n = 4), [\text{Ca}^{2+}]_i \text{ oscillations in the arterial wall (AACOCF}_{3}: 95.9 \pm 6.3 \% \text{ of amplitude of control } F_{340/380} \text{ oscillation}; n = 4), \text{ vessel tone (Control: } 58.1 \pm 2.1 \mu m, \text{AACOCF}_{3}: 59.5 \pm 2.5 \mu m; n = 4)\) or basal \([\text{Ca}^{2+}]_i\) levels \((\text{AACOCF}_{3}: 96.4 \pm 6.6 \% \frac{F_{340/380}}{F_{380}}; n = 4)\). Imaging of SMCs showed that AACOCF\(_3\) had no effect on \([\text{Ca}^{2+}]_i\) in individual cells.

### 4.2.5 Role of ion channels

#### 4.2.5.1 Involvement of calcium dependent chloride channels.

Chloride substitution, in which NaCl \((120 \text{ mM})\) was replaced by equimolar sodium isethionate \((120 \text{ mM} + 3.3 \text{ mM CaCl}_2)\) was used to investigate the role of chloride channels
in spontaneous contractions (Fig 4.14). Spontaneous depolarizations (Fig 4.14A), contractions and \([\text{Ca}^{2+}]_i\) oscillations in the arterial wall (Fig 4.14B) were abolished (P<0.05) within 4 min of sodium isethionate entering the recording chamber. Furthermore, the vessel constricted (Control: 44.1 ± 4.4 µm, isethionate: 37.5 ± 2.1 µm; n = 10; P<0.05, paired t-test), the membrane potential depolarized (Fig 4.14A; Control: -40.6 ± 2.5 mV; isethionate: -30.6 ± 0.5 mV; n = 5; P< 0.05, paired t-test) and the basal calcium levels in the arterial wall increased significantly (Fig 4.14B; isethionate: 121.0 ± 6.6 % \(F_{340/380}\); n = 5; P<0.05, paired t-test). \([\text{Ca}^{2+}]_i\) oscillations in individual SMCs were also inhibited (Fig 4.14C).

In the presence of the calcium dependent chloride channel inhibitors DIDS (100 µM) and NFA (50 µM), spontaneous contractions and depolarizations were abolished (Fig 4.15 A and 4.16A, respectively; P<0.05, paired t-test). DIDS hyperpolarized the membrane potential (Control: -42.7 ± 1.6 mV, DIDS: -55.2 ± 5.7 mV; n = 4; P<0.05, paired t-test) and caused the vessel to relax (Fig 4.17 A; Control: 48.2 ± 5.9 µm, DIDS: 53.6 ± 6.3 µm; n = 8; P<0.05, paired t-test). On the other hand, while NFA also hyperpolarized the membrane potential (Control: -39.5 ± 2.0 mV, NFA: -53.8 ± 1.9 mV; n = 4; P<0.05, paired t-test), there was no significant loss of vessel tone (Fig 4.16A; Control: 43.1 ± 6.0 µm, NFA: 46.3 ± 7.2 µm; n = 9). Both inhibitors abolished the \([\text{Ca}^{2+}]_i\) oscillations in the arterial wall (Fig 4.15B and Fig 4.16B; P<0.05, paired t-test) and in individual SMCs (Fig 4.15C and Fig 4.16C). NFA had no significant effect on the basal calcium levels (Fig 4.16B; NFA: 106.4 ± 5.8 % \(F_{340/380}\); n = 5) while DIDS significantly decreased basal calcium levels in the arterial wall (Fig 4.15B; DIDS: 86.8 ± 3.1 % \(F_{340/380}\); n = 4; P<0.05, paired t-test). The effects of these inhibitors were observed 3-5 min after the drugs entered the recording chamber.

### 4.2.5.2 Involvement of calcium dependent potassium (\(K_{\text{Ca}}\)) channels.

Incubation in the putative large conductance calcium activated potassium (\(B_{\text{KCa}}\)) channel inhibitor, TEA (1 mM), had no effect on the frequency of rhythmical contractions, depolarizations, \([\text{Ca}^{2+}]_i\) oscillations in the arterial wall (Fig 4.17A and B; Control: 15.6 ± 1.4 min\(^{-1}\), TEA: 16.0 ± 1.2 min\(^{-1}\), n = 9). The resting membrane potential also remained unaffected (Fig 4.17A; Control: -46.2 ± 2.4 mV, TEA: -48.2 ± 2.4 mV; n = 4). On the other hand, TEA increased the amplitude of arterial wall \([\text{Ca}^{2+}]_i\) oscillations (122.6 ± 3.7 % of amplitude of control \(F_{340/380}\) oscillation; n = 5, P<0.05, paired t-test) and rhythmical
Control  Isethionate (120 mM)

A. Membrane potential

B. Wall calcium

C. SMC calcium (n= 4 cells)

Figure 4.14

A, Chloride substitution with sodium isethionate (120 mM) depolarized SMCs and abolished rhythmical depolarizations and contractions. B, [Ca$^{2+}$]$_i$ oscillations in the arterial wall were abolished and the basal [Ca$^{2+}$]$_i$ level increased. C, [Ca$^{2+}$]$_i$ oscillations in individual SMCs were also abolished. Increases in [Ca$^{2+}$]$_i$ are represented as an upward deflection. The basal Ca$^{2+}$ level in the representative control trace is 0.62 F$_{340/380}$. 
The effect of $\text{Cl}_{\text{Ca}}$ channel inhibition on spontaneous rhythmical activity.

A. Addition of the calcium dependent chloride channel inhibitor, DIDS (100 $\mu$M) abolished rhythmical depolarizations, hyperpolarized and relaxed the vessel. B, $[\text{Ca}^{2+}]_{i}$ oscillations in the vessel wall were inhibited and basal $[\text{Ca}^{2+}]_{i}$ levels decreased. C, $[\text{Ca}^{2+}]_{i}$ oscillations in individual SMCs were also abolished. Increases in $[\text{Ca}^{2+}]_{i}$ are represented as an upward deflection. The basal $\text{Ca}^{2+}$ level in the representative control trace is 0.54 $F_{340/380}$. 

Figure 4.15
Figure 4.16

The effect of Niflumic acid (50 μM) on spontaneous rhythmical activity. Calcium dependent chloride channel inhibition by niflumic acid hyperpolarized SMCs and abolished spontaneous depolarizations and contractions (A), inhibited [Ca^{2+}] oscillations in wall calcium (B) and in individual SMCs (C). Increases in [Ca^{2+}], are represented as an upward deflection. The basal Ca^{2+} level in the representative control trace is 0.40 F_{340/380}. 
contractions (Fig 4.17B; Control: 4.1 ± 0.8 % RVD, TEA: 5.2 ± 0.7 % RVD; n = 9; P<0.05, paired t-test). No significant effects were observed on basal [Ca\(^{2+}\)]_i levels (TEA: 112.4 ± 2.9 % F\(_{340/380}\); n = 5) or on vessel diameter vessel diameter (Fig 4.17B; Control: 47.9 ± 5.6 µm, TEA: 45.8 ± 6.3; n = 9). Finally, in the presence of TEA, [Ca\(^{2+}\)] oscillations in individual cells became more synchronized (Fig 4.17C; n = 5).

While the selective BK\(_{Ca}\) channel inhibitor iberiotoxin (IbTX, 100 nM) also had no effect on the frequency of vasomotion (Fig 4.18; Control: 17.4 ± 2.1 min\(^{-1}\), IbTX: 17.7 ± 2.5 min\(^{-1}\), n = 10), unlike TEA, it did not appear to significantly affect the amplitude of rhythmical contractions (Control: 4.8 ± 0.9 % RVD, IbTX: 5.1 ± 1.1 % RVD, n = 10), depolarizations (Fig 4.18A; n = 4) or [Ca\(^{2+}\)] oscillations in the arterial wall (Fig 4.18B; IbTX: 111.0 ± 19.1 % of amplitude of control F\(_{340/380}\) oscillation, n = 6). In addition, no differences were observed in the resting membrane potential (Fig 4.18A; Control: -41.3 ± 2.3 mV, IbTX: -40.7 ± 2.4 mV, n = 4). However, Iberiotoxin increased the basal calcium levels (Fig 4.18B; IbTX: 126.3 ± 2.7 % F\(_{340/380}\); n = 6; P<0.05, paired t-test) and decreased the vessel diameter (Fig 4.18A and B; Control: 51.5 ± 5.1 µm, IbTX: 48.4 ± 4.5 µm; n = 10; P<0.05, paired t-test). No changes were observed in the degree of synchronicity between individual SMCs (Fig 4.18C).

Inhibition of BK\(_{Ca}\) and IK\(_{Ca}\) channels with charybdotoxin (60 nM; Fig 4.19) and IK\(_{Ca}\) channels with TRAM-34 (50 nM; Fig 4.20) rapidly decreased the vessel diameter (control: 46.3 ± 6.5 µm, CbTX: 40.0 ± 4.8 µm, n = 8 ; control: 33.5 ± 2.5 µm, TRAM-34: 31.0 ± 2.7 µm; n = 5, P<0.05, paired t-test), depolarized the membrane potential (Fig 4.19A, control:-41.7 ± 2.1 mV, CbTX: -30.5 ± 1.2 mV; Fig 4.20A, control: 40.0 ± 1.7 mV, TRAM-34: -31.0 ± 1.9 mV; n = 4, P<0.05, paired t-test) and increased basal calcium levels in the arterial wall (Fig 4.19B, CbTX:123.9 ± 3.6 % F\(_{340/380}\), n = 4, P<0.05; Fig 4.20B, TRAM-34: 123.3 ± 5.0 % F\(_{340/380}\), n = 5; P<0.05, paired t-test). Rhythmical contractions were increased in frequency (control: 16.5 ± 2.2 min\(^{-1}\); CbTX: 24.7 ± 3.9 min\(^{-1}\), n = 8, P<0.05, paired t-test; control: 12.2 ± 0.7 min\(^{-1}\), TRAM-34: 17.1 ± 1.0 min\(^{-1}\), n = 8; P<0.05, paired t-test), but decreased in amplitude (Control: 5.2 ± 0.9 % RVD, CbTX: 2.4 ± 0.2 %RVD, n = 8, P<0.05, paired t-test; Control: 5.5 ± 1.0 % RVD, TRAM-34: 2.5 ± 0.4 % RVD; n = 8, P<0.05, paired t-test) and rhythmical depolarizations (Fig 4.19A and Fig 4.20A), and [Ca\(^{2+}\)] oscillations in both the arterial wall (Fig 4.19B and Fig 4.20B) and in individual SMCs (Fig 4.19C and Fig 4.20C) mirrored this effect (P<0.05, paired t-test). The
Control

A. Membrane potential

Diameter

TEA (1 mM)

Diameter

B. Wall calcium

C. SMC calcium (n=4 cells)

Figure 4.17

Effect of K⁺ channel inhibition on spontaneous activity. TEA (1 mM) had no effect on the presence of spontaneous depolarizations, contractions, membrane potential or vessel diameter. B, [Ca²⁺]ᵢ oscillations in the arterial wall were increased in amplitude and [Ca²⁺]ᵢ oscillations in individual SMCs were more synchronized. Increases in [Ca²⁺]ᵢ are represented as an upward deflection. The basal Ca²⁺ level in the representative control trace is 0.57 F₃₄₀/₃₈₀.
Figure 4.18

The effect of BK_{Ca} inhibition on spontaneous rhythmical activity. A, Iberiotoxin (100 nM) had no effect on the most negative membrane potential, nor on the frequency or amplitude, of rhythmical depolarizations. Rhythmical contractions were increased in amplitude and the vessel was constricted. B, Basal [Ca^{2+}]_{i} levels in the arterial wall were increased. Increases in [Ca^{2+}]_{i} are represented as an upward deflection. The basal Ca^{2+} level in the representative control trace is 0.73 F_{340/380}. 
Effect of BK$_{Ca}$ and IK$_{Ca}$ inhibition on spontaneous activity. A, Charybdotoxin (60 nM) increased the frequency of the rhythmical contractions and depolarizations, and depolarized the SMCs (trace 5 min in drug). B, [Ca$^{2+}$]$_i$ oscillations in the arterial wall were also increased in frequency and the basal [Ca$^{2+}$]$_i$ level increased (trace 10 min in drug). C, [Ca$^{2+}$]$_i$ oscillations in individual SMCs were increased in frequency. Increases in [Ca$^{2+}$]$_i$ are represented as an upward deflection. The basal Ca$^{2+}$ level in the representative control trace is 0.52 F$_{340/380}$. 
Control | TRAM-34 (50 nM)
---|---
A. Membrane potential | ![Graph showing membrane potential changes]

B. Wall calcium | ![Graph showing wall calcium changes]

C. SMC calcium (n=8 cells) | ![Graph showing SMC calcium changes]

**Figure 4.20**
Effect of TRAM-34 (50 nM) on rhythmical activity. A, Inhibition of IKCa with TRAM-34 depolarized the SMCs and constricted the vessel. Spontaneous depolarizations, contractions (B) and calcium oscillations in the arterial wall (B) as well as in individual SMCs (C) were increased in frequency but decreased in amplitude. The basal Ca2+ level in the representative control trace is 0.61 F340/380.
effect of charybdoxin and TRAM-34 on rhythmical activity was observed by 5 min after exposure to drug.

Apamin (0.5 μM, n = 4), an inhibitor of small conductance calcium activated potassium (SK_{Ca}) channels, had no significant effect on the frequency (Control: 12.0 ± 0.7 min^{-1}, apamin: 12.2 ± 0.9 min^{-1}) or amplitude (Control: 5.0 ± 0.9 % RVD, apamin: 4.7 ± 0.7 % RVD) of rhythmical contractions, depolarizations (Fig 4.21A) or on [Ca^{2+}]_i oscillations in the arterial wall (Fig 4.21B) or in individual SMCs (Fig 4.21C). Vessel diameter (Control: 31.6 ± 3.5 μm, apamin: 32.9 ± 2.3 μm), membrane potential (Fig 4.21A, Control: -43 ± 1.2 mV, apamin: -43 ± 1.1 mV), and basal [Ca^{2+}]_i levels in the arterial wall (Fig 4.21B, apamin:106.6 ± 2.2 % F_{340/380}) were also unaltered.

Similarly, the voltage gated potassium (K_v) channel inhibitor 4-aminopyridine (100 μM; Fig 4.22; n = 8) and the K_{ATP} channel inhibitor, glibenclamide (10μM; n = 8) had no significant effect on the contraction frequency (Control: 15.0 ± 1.4 min^{-1}, 4-AP: 14.4 ± 1.2 min^{-1}; Control:13.0 ± 1.1 min^{-1}, Glib: 13.2 ± 1.9 min^{-1}) or amplitude (Control: 5.8 ± 0.8 % RVD, 4-AP: 6.2 ± 1.1 % RVD; Control: 6.0 ± 0.6 % RVD, Glib: 6.6 ± 0.6 % RVD). In addition, no effect of either inhibitor was observed on the rhythmical depolarizations (Fig 4.22A; 4-AP; n = 4), [Ca^{2+}]_i oscillations in the arterial wall (Fig 4.22B, 4-AP; n = 4) or in individual SMCs (Fig 4.22C, 4-AP; n = 4). Vessel diameter (Control: 33.6 ± 5.5 μm, 4-AP: 33.1 ± 5.7 μm; Control: 65.0 ± 3.3 μm, Glib: 63.5 ± 2.7 μm), membrane potential (Control: - 42.0 ± 1.7 mV, 4-AP: - 41.0 ± 2.2 mV; Control: -40.0 ± 1.7 mV, Glib: -39.1 ± 1.2 mV) and basal [Ca^{2+}]_i levels in the arterial wall (4-AP: 110.5 ± 6.1 % F_{340/380}; Glib: 103.3 ± 3.3 % F_{340/380}) were also unaffected.

4.3 DISCUSSION

During spontaneous vasomotion of juvenile rat basilar arteries both rhythmical oscillations in membrane potential and in SMC calcium were found to precede the rhythmical contractions. We have further shown that calcium release from intracellular IP_{3}-sensitive stores is primarily responsible for the vasomotion since it leads to depolarization of the membrane. The depolarization in turn produces a cyclical voltage oscillation through calcium influx via VDCCs, calcium-induced calcium release from ryanodine stores and subsequent hyperpolarization through the activation of IK_{Ca} channels. The selectivity of the
Figure 4.21

Effect of Apamin (0.5 μM) on rhythmical activity. A, Inhibition of SK$_{Ca}$ with apamin had no significant effects on rhythmical depolarizations, contractions, membrane potential or vessel diameter. Apamin also had no effect on basal calcium levels, calcium oscillations in the arterial wall (B) and in individual SMCs (C). Increases in [Ca$^{2+}$]$_i$ are represented as an upward deflection. The basal Ca$^{2+}$ level in the representative control trace is 0.53 F$_{340/380}$. 
Figure 4.22

A. Effect of $K_v$ channel inhibition on rhythmical activity. Spontaneous depolarizations, contractions, membrane potential and vessel diameter were unaffected in the presence of 4-Aminopyridine (100 µM). 4-Aminopyridine also had no significant effects on $[Ca^{2+}]_i$ oscillations in the arterial wall (B) or in individual SMCs (C). Increases in $[Ca^{2+}]_i$ are represented as an upward deflection. The basal $Ca^{2+}$ level in the representative control trace is 0.73 $F_{340/380}$. 
initial phase with IP₃-sensitive stores and chloride channels and the secondary phase involving ryanodine-sensitive stores, VDCC and IK₉Ca provides support for compartmentalization and co-localization of different calcium stores with particular types of ion channels.

4.3.1 Spontaneous vasomotion in the juvenile rat basilar artery

In the present study, rhythmical contractions were consistently observed to occur throughout the rat basilar and caudal cerebellar arteries in the absence of external stimuli. In all cases, these spontaneous contractions were preceded by oscillations in both membrane potential and in arterial wall calcium in a manner similar to that described in Chapter 3 in rat iris arterioles. Rhythmical depolarizations were shown to be recorded from smooth muscle cells as identified by the nuclear marker propidium iodide. The resting membrane potential of these cells was comparable to that observed in pressurized cerebral arteries (Knot & Nelson, 1998) but was significantly more depolarized than that recorded in the irideal arterioles. In those preparations from which changes in cell calcium were studied, individual cells loaded with Fura 2-AM were identified as smooth muscle cells. Since endothelial cells did not appear to become loaded with Fura, measurements of changes in arterial wall calcium were considered to be due to smooth muscle cells alone.

4.3.2 Variation in cellular coupling during spontaneous vasomotion

During spontaneous vasomotion we have been able to record calcium waves in individual SMCs similar to those observed in small mesenteric arteries following agonist stimulation (Mauban et al., 2001). When averaged for a single cell, these waves appeared as an oscillation. The oscillations in adjacent cells frequently showed variations in amplitude and in temporal characteristics, suggesting that movement of calcium through SMC gap junctions is not occurring in this preparation, as suggested in other vessels (Miriel et al., 1999). Calcium oscillations within individual cells during vasomotion appeared to be approximately synchronized, although the absolute degree was variable. This is consistent with findings by (Shaw et al., 2004) who showed that complete synchronization of calcium oscillations between smooth muscle cells was not prerequisite for agonist induced vasomotion in pressurized rat mesenteric arteries. On the other hand, in those arteries exhibiting more robust contractions, calcium oscillations between adjacent cells appeared to be well synchronized suggesting that a proportion of preparations are tightly coupled.
the present study, a number of preparations showed periodic variations in the amplitude of the rhythmical contractions and depolarizations. In these preparations, similar oscillatory patterns of arterial wall calcium were recorded and calcium oscillations in individual SMCs appeared to be synchronised only within small groups of adjacent cells, while calcium oscillations in adjacent groups of cells were out of phase. Thus, in the present study, there appear to be variations in the degree of cell coupling, presumably through differences in the regulation of gap junctions, which affect the spread of current along the vessel, resulting in differential patterns of calcium release, and the appearance of coordinated contraction.

4.3.3 Essential role for phospholipase C and the IP$_3$ sensitive intracellular calcium store

In the present study, the intracellular calcium chelator BAPTA-AM and the Ca$^{2+}$-ATPase inhibitor, thapsigargin, abolished the spontaneous depolarizations, calcium oscillations and contractions, hyperpolarized and relaxed the arteries, suggesting that the rhythmical events depend on intracellular signaling pathways and the release of calcium from an internal store. Calcium waves in individual SMCs were also abolished, supporting previous data that calcium waves result from regenerative calcium release from the SR network (Ino et al., 1994; Ruehlmann et al., 2000; Peng et al., 2001). Since inhibition of phospholipase C with U73122, mimicked the effects of BAPTA-AM and thapsigargin, we suggest that IP$_3$ and subsequent calcium release from the IP$_3$-sensitive store is responsible for the initiation and maintenance of vasomotion. In juvenile rats, this pathway may be constitutively active in a manner similar to described in juvenile rat iris arterioles (see Chapter 3) or may be activated by the stretch imposed during preparation of the vessels for the in vitro experiments, similar to that described in canine cerebral arteries (Tanaka et al., 1994). It is interesting then, that CPA, another Ca$^{2+}$-ATPase antagonist, caused spontaneous rhythmical activity to be augmented suggesting that CPA and thapsigargin must be targeting different calcium stores. At present, however, it is not possible to speculate the mechanisms by which CPA augments the basilar artery rhythmical activity and thus future experiments are required to address the effects of CPA in rat basilar artery spontaneous contractile events.

4.3.4 Protein kinase C inhibition modulates rhythmical activity
In the present study, inhibition of protein kinase C resulted in an increase in the frequency, but a decrease in the amplitude of spontaneous contractions and calcium oscillations without an apparent effect on vessel tone or basal calcium levels. While this suggests that in the juvenile rat basilar artery, spontaneous vasomotion may be modulated by activity of protein kinase C, its precise role remains difficult to elucidate.

In vascular smooth muscle tissues, protein kinase C has been reported to exert a wide range of cellular effects. In intact rabbit inferior vena cava, rat cerebral, rabbit mesenteric and femoral arteries PKC has been shown to be important in regulating vascular tone through inhibition of myosin light chain phosphatase or phosphorylation of actin binding proteins, independently of alterations in smooth muscle cell calcium (i.e. calcium sensitization) (Nishimura et al., 1990; Masuo, 1994; Walsh et al., 1994; Rokolya et al., 1998; Gokina et al., 1999). Conversely, protein kinase C induced calcium sensitization has been shown to be reduced in fetal, but not adult sheep cerebral arteries (Longo et al., 2000), while in the adult rat basilar artery in vivo, PKC activity does not appear to be involved in the maintenance of vessel tone (Chrissobolis, 2001). The findings of the present study would appear to support the latter, suggesting that during spontaneous vasomotion in the juvenile rat basilar artery, PKC has little effect on vascular tone. On the other hand, since protein kinase C inhibition resulted in an increase in the frequency, but a decrease in the amplitude of spontaneous contractions and \([\text{Ca}^{2+}]_i\) oscillations, data would suggest PKC may activate ryanodine or \(\text{IKca}\) channels. In the rat basilar artery in vivo, rat renal arterioles in vitro, and in isolated rat tail and cerebral artery smooth muscle cells, PKC has been shown to directly inhibit these channels (Bonev et al., 1997; Kirton & Loutzenhiser, 1998; Schubert & Mulvany, 1999; Chrissobolis & Sobey, 2002).

In the rat iris arteriolar preparation, activity of PKC was shown to be critical to the activation of phospholipase A\(_2\) (see chapter 3). In the present study, however, inhibition of phospholipase A\(_2\) had no effect on spontaneous activity or vessel tone. Thus it would be reasonable to suggest that PKC does not upregulate PLA\(_2\) activity and the precise role of PKC in spontaneous vasomotion of the juvenile rat basilar artery must await future examination.

### 4.3.5 Rhythmical contractions require extracellular calcium

In the present study, removal of calcium from the superfusing solution rapidly abolished the rhythmical contractions and caused the artery to relax, suggesting that influx of extracellular calcium is critical to the development of rhythmical activity in the juvenile
rat CCA. These results are similar to those previously described in the rat basilar artery in vivo and in human pial arteries in vitro, whereby removal of calcium from the bathing solutions also led to the abolition of spontaneous rhythmical activity (Fujii et al., 1990a; Gokina et al., 1996). In contrast, Gustafsson and Nilsson (1993) reported that a reduction in the extracellular calcium concentration did not significantly alter the rhythmical activity recorded in the rat mesenteric artery, while Peng et al., (2001) recently demonstrated that omission of extracellular calcium caused the loss of synchronized oscillations and the appearance of asynchronous waves. Together these results would support the proposal that in the mesenteric artery rhythmical activity relies on the intermittent release of calcium from an internal store, while in cerebral vessels, extracellular calcium appears to be essential for the maintenance of the rhythmical contractions. Thus, cerebral vessels appear to be more dependent on extracellular calcium for contraction than peripheral arteries (McCalden & Bevan, 1981).

4.3.6 **Voltage dependent calcium channels synchronize rhythmical activity**

Inhibition of calcium influx through VDCCs abolished the rhythmical contractions, rhythmical depolarizations and calcium oscillations in the arterial wall and depolarized the cell membrane. On the other hand, vessel tone and basal calcium levels in the arterial wall were not affected. Calcium oscillations in individual SMCs, although reduced in amplitude, were no longer synchronized. This would suggest that calcium entering via VDCCs is essential for coordinating vasomotion, but is not essential for the individual intracellular calcium oscillations. Similarly in rat mesenteric arteries, Peng et al., (2001) reported that inhibition of VDCCs led to a loss of synchronicity of intracellular calcium waves in SMCs. It was therefore proposed that the spread of a depolarizing current through gap junctions enables the simultaneous activation of VDCCs and coordinated contractions occur. Data from the present study would also suggest that calcium which has entered the SMCs through voltage dependent calcium channels also contributes to amplifying the calcium signal.

In contrast to the effects of U73122, BAPTA-AM and thapsigargin, ryanodine caused an increase in frequency and decrease in amplitude of both the spontaneous depolarizations and calcium oscillations, and depolarized the cell membrane. These results suggest that calcium released from the ryanodine-sensitive store is not involved in the initiation of vasomotion but rather is involved in a negative feedback loop of the oscillatory
cycle by activating a hyperpolarizing current. Such negative feedback has been described in cultured SMCs and pressurized cerebral arteries where ryanodine receptor channels are co-localised with VDCCs and BKCa channels, forming a functional unit to control levels of intracellular calcium and promote relaxation (see Jaggar et al., 2000 for review). Therefore, the depolarization seen in the presence of nifedipine, like that seen in ryanodine, suggests that calcium influx through VDCC is responsible for the ryanodine activation of a hyperpolarizing current, while the maintenance of basal calcium levels and vessel tone supports the suggestion that it is calcium from the IP3-sensitive store which contributes to basal tone. Voltage dependence in the activation of ryanodine stores has been reported previously in cerebral vessels (Jaggar et al., 1998a).

4.3.7 Organization of intracellular calcium stores

In the present study, loss of the asynchronous calcium oscillations in individual cells following the addition of ryanodine, after VDCC influx has been blocked by nifedipine, further suggests that a component of calcium released from the IP3-sensitive store is involved in activating calcium induced calcium release from ryanodine-sensitive stores, while the maintenance of tone under these conditions further supports the contribution of IP3 receptor calcium to this phenomenon. Compartmentalization of different ion channels or intracellular calcium stores may be responsible for these apparent functional microdomains. Release of calcium from the IP3-sensitive store by 5-HT, in the presence of both nifedipine to block voltage dependent calcium influx and caffeine to deplete ryanodine-sensitive stores, resulted in vessel constriction. Thus, supporting the existence of spatially separate IP3-sensitive and ryanodine sensitive calcium stores as have been described in isolated rat mesenteric and canine pulmonary artery smooth muscle cells (Golovina & Blaustein, 1997; Janiak et al., 2001).

4.3.8 Chloride channel inhibition abolishes rhythmical activity

Under physiological conditions, cerebral arteries are subjected to intraluminal pressure which has been shown to cause SMC membrane depolarization to approximately –40 mV, activation of VDCCs, increased intracellular calcium levels and vasoconstriction (Harder, 1987; Knot & Nelson, 1998). Activation of a calcium dependent chloride channel
has been implicated in the depolarization of the membrane potential and opening of VDCCs in response to agonist stimulation (Large & Wang, 1996) and in the depolarization associated with myogenic contraction to pressurization (Doughty & Langton, 2001). In the present study, in which the SMCs were depolarized to a similar value, chloride (Cl\(^-\)) substitution by channel impermeant isethionate abolished all rhythmical activity and produced vasoconstriction in association with further membrane depolarization and increased arterial wall calcium levels. Given that Cl\(^-\) substitution would move the equilibrium potential for Cl\(^-\) toward more positive values, we suggest that the results are consistent with the depolarization resulting from the activation of a calcium dependent chloride channel. In support of this finding, the calcium dependent chloride channel antagonists, NFA and DIDS, abolished spontaneous depolarizations, calcium oscillations in the arterial wall and rhythmical contractions and hyperpolarized the cell membrane. Interestingly, exposure to DIDS caused the basal calcium levels in the arterial wall to decrease, suggesting that this drug may have additional effects on intracellular calcium stores (see chapter 3). On the other hand, NFA had no effect on basal calcium levels suggesting that its effects were selective on calcium dependent chloride channels as described previously in vascular smooth muscle cells (Hogg et al., 1994a; Criddle et al., 1997).

4.3.9 A role for intermediate conductance calcium activated potassium (IK\(_{\text{Ca}}\)) channels

Previous studies in cerebral vessels have shown that calcium release from ryanodine-sensitive stores can directly activate BK\(_{\text{Ca}}\) channels to cause membrane hyperpolarization and vessel relaxation (Benham & Bolton, 1986; Nelson et al., 1995). However, in the present study, despite an increase in the contraction amplitude in the presence of TEA and an overall increase in the basal calcium levels and vessel tone in the presence of iberiotoxin, blocking BK\(_{\text{Ca}}\) channels had little effect on rhythmical activity or membrane potential. In fact, it has been proposed that TEA can non-specifically activate VDCCs (Gustafsson & Nilsson, 1994; Hirst et al., 1996), which is suggested in the present study by the synchronization of calcium oscillations in individual SMCs. Inhibition of SK\(_{\text{Ca}}\) channels or Kv channels also had no effect on membrane potential or rhythmical activity. On the other hand, charybdotoxin, an antagonist of both BK\(_{\text{Ca}}\) and IK\(_{\text{Ca}}\) channels (Neylon et al., 1999) caused rapid depolarization of the membrane potential and vessel constriction.
Charybdotoxin also increased the frequency, but decreased the amplitude of both spontaneous depolarizations, calcium oscillations and contractions in a manner similar to that observed in the presence of ryanodine, suggesting that the residual oscillations result from the continued opening and closing of VDCCs. As the calcium oscillations were larger after inhibition of IK\textsubscript{Ca} channels than after ryanodine, it appears that the voltage dependent calcium influx is amplified by calcium induced calcium release from the ryanodine-sensitive store.

The results above suggested that IK\textsubscript{Ca} channels, but not BK\textsubscript{Ca} channels, were involved in the basilar artery rhythmic activity. Moreover, this data provided support for a sequential relationship between the release of calcium from the ryanodine-sensitive calcium store and activation of this channel. To confirm the involvement of IK\textsubscript{Ca} channels, we tested TRAM-34, a potent and selective blocker of IK\textsubscript{Ca} channels. This drug has been derived from clotrimazole but lacks the inhibitory effect on cytochrome P450 enzymes (Wulff et al., 2000). As TRAM-34 mimicked the effect observed in the presence of charybdotoxin, our results show that activation of IK\textsubscript{Ca}, following release of calcium from the ryanodine-sensitive stores and subsequent membrane hyperpolarization, is essential to negatively modulate both vessel tone and rhythmical activity of the juvenile rat basilar artery. It is interesting to note that smooth muscle IK\textsubscript{Ca} channels, but not BK\textsubscript{Ca} channels, have recently been shown to play a role in regulating the functional properties of immature smooth muscle cells (Neylon et al., 1999).

4.3.10 Absence of a role of the phospholipase A\textsubscript{2} signaling pathway

In arterioles of the rat iris, metabolites derived from the lipoxygenase arm of the phospholipase A\textsubscript{2} pathway were shown to be essential to the generation of spontaneous rhythmical contractions. In the present study, however, inhibition of phospholipase A\textsubscript{2} by AACOCF\textsubscript{3} had no effect on vasomotion, vessel tone or on basal calcium levels in the arterial wall, thus demonstrating that spontaneous activity recorded in the juvenile rat basilar artery occurs independently of this pathway. Since metabolites of arachidonic acid pathway have previously been shown to be important in controlling vascular tone in cerebral vessels from a number of different species (Jancar et al., 1987; Harder et al., 1994; Miyamoto et al., 1998; Faraci et al., 2001), it is possible that in the juvenile rat, the apparent lack of effect observed following phospholipase A\textsubscript{2} inhibition may be attributable to developmental differences between the animal models studied.
4.3.11 Proposed mechanisms underlying spontaneous vasomotion in the rat basilar artery

Based on the data presented in the present study, we propose a mechanism by which spontaneous vasomotion in the juvenile basilar artery results from an interaction between voltage independent and voltage dependent events (Fig 4.23). Constitutive or stretch-induced activity of the phospholipase C pathway leads to an increase in basal calcium levels, intracellular tone and a depolarizing current perhaps through the opening of $\text{Cl}_{\text{Ca}}$ channels in the membrane. The depolarization in turn leads to the influx of extracellular calcium through VDCCs activating ryanodine receptors to cause further release of calcium and contraction takes place. Calcium released from the ryanodine sensitive calcium store also results in the activation of $\text{IK}_{\text{Ca}}$ channels. The subsequent hyperpolarizing current inactivates VDCCs promoting relaxation. The coordinate loss of activation of ryanodine-sensitive stores leads to closure of the $\text{IK}_{\text{Ca}}$ channels and reopening of VDCCs to repeat the cycle.
Figure 4.23
Figure 4.23

Proposed mechanisms underlying spontaneous vasomotion in the rat basilar artery. Constitutive activity of phospholipase C (PLC) leads to the production of IP₃ and release of calcium (Ca²⁺) from the IP₃ sensitive intracellular calcium store. This calcium then activates a calcium dependent chloride channel (ClᵥCa) which depolarises the membrane potential and opens voltage dependent calcium channels (VDCC) causing extracellular calcium influx into the cell. The calcium that has entered the cell through these channels, then activates the ryanodine receptor (RyR) channels causing calcium induced calcium release from the ryanodine sensitive intracellular calcium store. The calcium within the cell is now amplified, a threshold is reached and contraction takes place. Calcium released from the ryanodine sensitive store also activates an intermediate conductance calcium activated potassium (IKᵥCa) channel causing the membrane to hyperpolarise. This in turn inactivates the VDCCs and promotes relaxation. Closure of the VDCCs ensures down regulation of the cycle in preparation for it to begin again.
5.1 INTRODUCTION

The endothelial cell layer of the vascular wall has long been recognized as an important regulator of vascular tone (Furchgott & Zawadzki, 1980; Furchgott, 1983; Faraci & Heistad, 1998). Under normal physiological conditions, smooth muscle cell contractility can be directly affected by the endothelium through the production and release of a number of potent vasodilatory and vasocontracting factors, such as NO, prostaglandins, EDHF and endothelin (Mombouli & Vanhoutte, 1997). Evidence would also suggest that the endothelium plays a role in coordinating vascular responses and controlling smooth muscle cell tone via direct electrical coupling with the inner layer of the media (Neild & Crane, 2002).

Since vasomotion has been shown to be associated not only with oscillations in smooth muscle cell calcium, but also oscillations in endothelial cell calcium (Schuster et al., 2001), it is possible that the endothelium may play an important role in regulating rhythmical contractions. Given that the relative contribution of endothelium derived substances has been shown to differ between vascular beds (Hill et al., 2001), it is not surprising that differing effects of the endothelium on vasomotion have also been observed. In the rat iris arteriole, rabbit superior mesenteric artery, rabbit ear artery, rat basilar artery, for example, rhythmical contractions have been shown to occur independently of the endothelium (Omote & Mizusawa, 1994; Chaytor et al., 1997; Hempelmann et al., 1998; Hill et al., 1999). On the other hand, in the hamster aorta, rabbit femoral artery and rat small mesenteric arteries vasomotion has been reported to be endothelium dependent (Jackson, 1988; Gustafsson et al., 1993; Omote & Mizusawa, 1995).

Recently, Peng et al., (2001) have proposed a hypothesis for the mechanism leading to endothelium dependent vasomotion in rat small mesenteric arteries. In this model, vasomotion is dependent on the release of NO from endothelial cells and the subsequent elevation of cGMP in smooth muscle cells. Coordinated contraction is suggested to result
from the intermittent release of calcium from ryanodine stores in the sarcoplasmic reticulum, triggering a cGMP-dependent depolarizing current, which is conducted along the vessel through gap junctions, synchronizing the activation of voltage dependent calcium channels in the smooth muscle. An essential role for the endothelium was confirmed when addition of 8-bromo-cGMP was shown to restore vasomotion in endothelium denuded arteries. On the other hand, much confusion in the literature has arisen due to contradictory observations from studies within this same mesenteric vascular bed (Gustafsson et al., 1994; Peng et al., 2001; Sell et al., 2002; Mauban & Wier, 2004). Indeed, recent studies examining the role of EDHF in agonist induced vasomotion raise the possibility that an endothelial factor other than NO is responsible for the generation of vasomotion within this vascular bed (Dora et al., 2000b; Okazaki et al., 2003; Mauban & Wier, 2004). Thus, the precise role of NO and cGMP in the generation of vasomotion remains somewhat uncertain.

In spite of this confusion, it is likely that rapid synchronization of activity within the vascular wall is critically important in coordinating the contractile and relaxing phases of vasomotion between adjacent cells (Nilsson & Aalkjaer, 2003). Intercellular communication through gap junctions could therefore provide a low resistance pathway for metabolic and electrical cell-to-cell communication in rhythmically active blood vessels. In the present study we have directly investigated the role of the vascular endothelium in spontaneous vasomotion of the juvenile rat basilar artery by correlating changes in smooth muscle cell input resistance, membrane voltage and intracellular calcium in preparations with and without intact endothelium. The contribution of endothelium-derived vasoactive substances was investigated using pharmacological agents. In addition, serial section electron microscopy and immunohistochemistry have been used to investigate the morphological features of cell-to-cell communication within the vascular wall.

5.2 RESULTS

5.2.1 Anatomical characteristics and morphological identification of myoendothelial gap junctions

Arteries had a mean circumference of 294.9 ± 25.5 μm, as determined at the level of the internal elastic laminar (n = 6). The media was typically comprised of 2-3 smooth muscle cell layers (Table 5.1). Serial sections taken through 5 μm long arterial segments were shown to contain both MEGJs and large homocellular endothelial gap junctions (Fig
Figure 5.1

Myoendothelial gap junctions are present in the caudal cerebellar artery. Electron micrographs of the caudal cerebellar artery showing (A) an endothelial cell (EC) projection passing through the internal elastic lamina (IEL) to make contact (indicated by arrow) with an adjacent smooth muscle cell (SMC) (scale: 0.5 μm). Enlargement of myoendothelial contact (inset) shows the characteristic pentalaminar structure indicative of a gap junction (scale: 50 nm). B, Large homocellular contacts could also observed between endothelial cells (indicated by arrow, scale: 1 μm) which had the characteristic pentalaminar structure indicative of a gap junction (inset, scale: 50 nm).

Table 5.1
Characteristics of rat caudal cerebellar artery

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
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<tbody>
<tr>
<td>Circumference (μm)</td>
<td>294.9 ± 25.5</td>
</tr>
<tr>
<td># SMC layers</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td>EC Area (μm²)</td>
<td>388.4 ± 18.6</td>
</tr>
<tr>
<td># MEGJs/EC</td>
<td>3.2 ± 0.9</td>
</tr>
</tbody>
</table>

n = 6; results are mean ± S.E.M. EC, endothelial cell; SMC, smooth muscle cell; MEGJ, myoendothelial gap junction
5.1). All MEGJs were characterized by the presence of pentalaminar membrane structures between the endothelial and smooth muscle cells (Fig. 5.1, inset). The number of MEGJs per endothelial cell was calculated to be 3.2 ± 0.9 MEGJs per endothelial cell based on endothelial cell sizes determined from immunohistochemical staining for Cx40 (Table 5.1). Gap junctions between smooth muscle cells were not examined in the present study.

5.2.2 Connexin specific immunohistochemistry in the juvenile rat basilar artery

Immunoreactivity using antibodies against Cx37 (Fig 5.2A), 40 (Fig 5.2C) and 43 (Fig 5.2E) was detected in the endothelial cell layer in both the basilar and caudal cerebellar arteries. Staining was localized to the endothelial cell perimeters and was typically linear in nature. Cx40 appeared to be the most highly expressed of the three Cx proteins, while Cx37 and Cx43 expression appeared to be less dense. No staining was observed in endothelial cells of either artery using antibodies against Cx45 (Fig 5.2G). In contrast to staining observed in the endothelium, no immunoreactivity was detected in the medial cell layers of either artery with antibodies against Cx40 (Fig 5.2D). Cx43 also appeared to be absent from the smooth muscle cell (Fig 5.2F), although individual cells demonstrating a high density of Cx43 staining could be observed within the media (Fig 5.3). On the other hand, sparse fine punctate staining was observed in the media when arteries were stained with antibodies against Cx37 (Fig 5.2B) and Cx45 (Fig 5.2H). No staining was observed if the primary antibody was not present or if it was pre-absorbed with the immunogenic peptide.

5.2.3 Rhythmical activity in dye identified cells

Electrode recordings from dye identified endothelial (Fig 5.4A) and smooth muscle cells (Fig 5.4B) showed that during spontaneous vasomotion, rhythmical depolarizations could be recorded from both cell types (Fig 5.4C and D). No difference was observed in the resting membrane potential between the two cell types when impalements were made in the same preparation (Fig 5.4C and D; EC: -45 mV, SMC: -45 mV, n = 1). The nuclear marker propidium iodide spread rapidly amongst adjacent endothelial cells (Fig 5.4A), while in smooth muscle cells, dye spread was typically restricted to small groups of 2 to 3 adjacent cells only (Fig 5.4B). The spread of dye between smooth muscle cells was always observed to occur along the length of the vessel, rather than in a radial direction.
Figure 5.2
Figure 5.2
Confocal images showing immunostaining of Cxs in the rat basilar artery. Cy3-conjugated secondary antibodies were used and greyscale confocal images inverted to black on white. Cx37 (A), 40 (C), and 43 (E) were expressed in the endothelium, but not Cx45 (G). Cx37 (B) and 45 (H) were detected in the smooth muscle, but not Cx40 (D) or 43 (F). Vessel axis runs left to right.
Endothelium  Smooth muscle

Cx37

A

Cx40

B

D

Cx43

C

E

Cx45

F

G

H 10 μm
Figure 5.3
Confocal image of smooth muscle-like cells showing immunostaining for Cx43. Cells expressing a high density of Cx43 protein could be detected within the smooth muscle cell layers of the rat basilar artery. Staining was abolished by pre-incubation of the antibody with the immunogenic peptide. Vessel axis runs left to right.
Figure 5.4
Rhythmical activity in dye identified endothelial cells and smooth muscle cells. Propidium iodide filled ECs (A) and SMCs (B). Longitudinal vessel axis runs left to right. Membrane potential oscillations were recorded in both ECs (C) and SMCs (D) and were shown to precede peak contraction.
5.2.4 Role of the vascular endothelium in spontaneous rhythmical vasomotion

Disruption of the endothelium led to irregular and uncoordinated contractions in appearance. Where it was possible to measure them, the frequency of rhythmical contractions appeared to be unchanged (Fig 5.5; intact: 16.1 ± 0.5 min\(^{-1}\), \(n = 53\); denuded: 17.5 ± 0.8 min\(^{-1}\), \(n = 40\)), although the amplitude was significantly reduced (intact: 5.7 ± 0.4 % RVD, \(n = 53\); denuded: 1.8 ± 0.1 % RVD, \(n = 40\); \(P<0.05\); unpaired t-test). Mean resting vessel diameter was not significantly different in the presence or absence of the endothelium (+endothelium: 45.9 ± 2.2 μm, \(n = 53\); -endothelium: 50.7 ± 2.8 μm, \(n = 40\), unpaired t-test). Rhythmical depolarizations (Fig 5.5A) were decreased in amplitude (+endothelium: 6.3 ± 0.7 mV, \(n = 20\); -endothelium: 3.6 ± 0.2 mV, \(n = 20\); \(P<0.05\), unpaired t-test) and increased in frequency (+endothelium: 16.2 ± 1.0 min\(^{-1}\), \(n = 20\); -endothelium: 31.2 ± 1.7 min\(^{-1}\); \(n = 20\); \(P<0.05\), unpaired t-test) and no longer preceded the residual contractions (Fig 5.5A). The smooth muscle cell membrane potential was not significantly different in endothelium denuded preparations (-endothelium: -39.9 ± 0.7 mV, \(n = 20\), compared to those in which the endothelium remained intact (+endothelium: -42.2 ± 0.9 mV, \(n = 20\), unpaired t-test). Measurements of arterial wall calcium (Fig 5.5B) showed that calcium oscillations were smaller and irregular in the absence of the endothelium, although no significant differences were observed in the basal calcium levels between the preparations (Fig 5.5B; + endothelium: 0.53 ± 0.026 F\(_{340/380}\), \(n = 33\); -endothelium: 0.53 ± 0.017 F\(_{340/380}\), \(n = 20\), unpaired t-test). Imaging of smooth muscle cells showed that small calcium oscillations were present in individual cells, but that these now appeared to uncoordinated or asynchronous (Fig 5.5C). Thus, in endothelium intact arteries, individual smooth muscle cells oscillated at the same or nearly identical rates, while in endothelium denuded preparations, oscillations within small groups of 2-3 adjacent cells appeared to be synchronized, while adjacent groups of cells oscillated at different rates (Fig 5.6A). The amplitude of the smooth muscle cell calcium oscillations was reduced in those preparations in which the endothelium had been damaged (Fig 5.6B). In addition, the ratio of the amplitude of the calcium oscillations in individual SMCs, to the amplitude of the calcium oscillations in the arterial wall was significantly increased (Fig 5.6C). When a hyperpolarizing current was applied to short segments of artery, the current-voltage relationship in endothelium denuded preparations was significantly steeper compared to those preparations in which the vascular endothelium remained intact indicating that smooth muscle cell input resistance was increased (Fig 5.7B). It should be emphasized,
Figure 5.5
Influence of the endothelium on rhythmical activity. A, Rhythmical depolarizations, contractions, (B) calcium oscillations in the arterial wall and in (C) individual SMCs, recorded from endothelium intact (+ve endothelium) preparations and endothelium denuded (-ve endothelium) preparations. Increases in \([\text{Ca}^{2+}]_i\) are represented by an upward deflection. Basal smooth muscle cell \([\text{Ca}^{2+}]_i\) levels in endothelium intact preparations and endothelium denuded preparations were 0.64 F_{340/380} and 0.52 F_{340/380}, respectively.
Figure 5.6
Figure 5.6
Comparison of SMC calcium oscillations in endothelium intact and endothelium denuded arteries. A, disruption of the vascular endothelium (▲) caused small groups of adjacent SMCs to oscillate at different frequencies when compared to control preparations with intact endothelium (△) in which adjacent cells oscillated with similar frequencies. Each colour represents 1 animal, comprised of 6 adjacent SMCs. B, removal of the vascular endothelium attenuated the amplitude of the SMC calcium oscillations. C, The ratio of the amplitude of individual SMC calcium oscillations to the amplitude of the calcium oscillations in the arterial wall was significantly increased in endothelium denuded preparations. Values represent mean and S.E.M.* significantly different from control (P<005).
Figure 5.7

I/V relationship and input resistance in endothelium intact and endothelium denuded preparations during spontaneous rhythmical activity. Changes in SMC membrane voltage were recorded during a series of 30 s hyperpolarizing current pulses (0.1-1.0 nA) from preparations with intact endothelium (+ve endothelium) and from those in which the endothelium had been mechanically disrupted (-ve endothelium). Straight lines indicate linear regression analysis. B, SMC input resistance was greater in endothelium denuded preparations than in preparations in which the endothelium remained intact. Values represent mean and S.E.M. * significantly different from control (P<0.05).
however, that the data was obtained from separate preparations in which the endothelium was either intact or had been removed.

5.2.5 Confirmation of successful endothelial removal

In order to assess the effectiveness and selectivity of endothelial cell damage, preparations, both with and without the vascular endothelium, were incubated in the endothelium dependent vasodilator ACh (10 μM). In endothelium intact preparations, ACh hyperpolarized the smooth muscle (Control: -42.2 ± 0.9 mV, ACh: -51.7 ± 1.7 mV; n = 20; P<0.05, paired t-test), decreased basal calcium levels in the arterial wall (ACh: 89.9 ± 0.1 % of control F340/380; n = 33, P<0.05, paired t-test) and caused the vessel to relax (Fig 5.8A; Control: 45.9 ± 2.2 μm, ACh: 50.0 ± 2.2 μm; n = 53; P<0.05, paired t-test). On the other hand, in endothelium denuded preparations, ACh had no apparent effects on smooth muscle cell membrane potential (Control: -39.9 ± 0.8 mV, ACh: -39.0 ± 1.0 mV; n = 20, paired t-test), although basal calcium levels in the arterial wall were increased (ACh: 109.5 ± 0.5 % of control F340/380, n = 20, P<0.05, paired t-test) and a small but significant decrease in vessel diameter was observed (Fig 5.8B; Control: 50.7 ± 2.8 μm, ACh: 49.9 ± 2.8 μm; n = 40; P<0.05, paired t-test). These results confirmed successful removal of the endothelium since vasodilation following the addition of ACh was abolished compared to that observed in endothelium intact preparations (P<0.05, unpaired t-test).

The smooth muscle dependent vasodilator, SNAP (10 μM) was used to assess smooth muscle cell integrity after removal of the endothelium. Only those preparations in which the application of SNAP resulted in vasodilation comparable to the relaxation to SNAP observed in endothelium intact preparations were used in the present study. Thus, in endothelium intact preparations, SNAP had no further effect on membrane potential (SNAP: -52.0 ± 1.3 mV, n = 20, paired t-test), basal calcium levels (SNAP: 91.1 ± 0.3 % of control F340/380, n = 33, paired t-test) or on vessel diameter (Fig 5.8A; SNAP: 50.2 ± 2.2 μm, n = 53, paired t-test) when compared to responses observed in the presence of ACh. In endothelium denuded preparations, SNAP hyperpolarized the smooth muscle cells (SNAP: -51.1 ± 1.2 mV; n = 20; P<0.05, paired t-test), decreased basal calcium levels in the arterial wall (SNAP: 91.0 ± 1.7 % of control F340/380; n = 20; P<0.05, paired t-test) and caused a significant relaxation (Fig 5.8B; SNAP: 53.8 ± 2.8 μm; n = 40; P<0.05, paired t-test).
Figure 5.8

Confirmation of endothelial disruption. A, Shows the vasodilation in response to ACh (10 μM) and SNAP (10 μM) in vessels with intact endothelium (+ve Endothelium). B, In those preparations in which the endothelium was considered to be successfully destroyed (-ve endothelium), application of ACh (10 μM) caused vasonstriction, while SNAP (10 μM) caused the vessel to relax. Bars represent mean and S.E.M. * indicates a significant difference from control (P<0.05).
5.2.6 Effect of nitric oxide inhibition on spontaneous rhythmical activity

To assess the role of nitric oxide, preparations were incubated in the nitric oxide synthase inhibitor, L-NAME (10 μM). L-NAME significantly increased the frequency (Control: 15.4 ± 0.7 min⁻¹, L-NAME: 19.6 ± 0.9 min⁻¹; n = 8; P<0.05, paired t-test) and amplitude of rhythmical depolarizations (Control: 5.3 ± 1.1 mV, L-NAME: 9.4 ± 1.0 mV; n = 4; P<0.05, paired t-test) and contractions (Control: 3.3 ± 0.4 % RVD, L-NAME: 5.8 ± 0.5 % RVD; n = 8; P<0.05, paired t-test) (Fig 5.9A). This effect was also observed on calcium oscillations recorded from both the arterial wall (Fig 5.9B; L-NAME: 153 % of amplitude of control F₃₄₀/₃₈₀ oscillation; n = 4; P<0.05, paired t-test) and in individual smooth muscle cells (Fig 5.9C). L-NAME decreased vessel diameter (Fig 5.9A and B; Control: 46.9 ± 3.9 μm, L-NAME: 44.9 ± 3.5 μm; n = 8; P<0.05, paired t-test), but no apparent effects were observed on membrane potential (Fig 5.9A; Control: -40.5 ± 0.5 mV, L-NAME: -39.5 ± 2.5 mV; n = 4, paired t-test) or on basal calcium levels in the arterial wall (Fig 5.9B; L-NAME: 104 ± 2.9 % of control F₃₄₀/₃₈₀, n = 4, paired t-test). Imaging of individual smooth muscle cells showed that calcium oscillations were increased in frequency and amplitude and appeared to be more synchronized (Fig 5.9C).

5.2.7 Effect of cGMP on spontaneous rhythmical activity in endothelium intact and endothelium denuded preparations

Preliminary experiments in which the second messenger molecule cGMP (dibutyryl cGMP: 30 μM) was added to the superfusing solution for up to 20 min, had no effect on rhythmical activity, resting membrane potential (n = 2), basal calcium levels in the arterial wall (n = 2) or on vessel diameter (n = 4). A higher concentration (300μM) of db-cGMP also had no effect on the frequency of rhythmical contractions (Control: 19.0 ± 1.6 min⁻¹, db-cGMP: 18.3 ± 1.7 min⁻¹; n = 11, paired t-test), depolarizations (Fig 5.10A; n = 7), calcium oscillations in the arterial wall (Fig 5.10B; n = 4) or in individual smooth muscle cells (Fig 5.10C; n = 4). However, db-cGMP (300 μM) reduced the amplitude of the rhythmical depolarizations (Fig 5.10A; Control: 6.4 ± 0.3 mV, db-cGMP: 5.9 ± 0.3 mV; n = 7; P<0.05, paired t-test), contractions (Control: 4.3 ± 0.5 %RVD, db-cGMP: 3.2 ± 0.6 % RVD; n = 11; P<0.05, paired t-test) and calcium oscillations in the arterial wall ( Fig 5.10B; db-cGMP: 98.3 ± 0.4 % of amplitude of control F₃₄₀/₃₈₀ oscillation; n = 4; P<0.05, paired t-test). While no effects were observed on the most negative smooth muscle cell membrane potential (Fig 5.10A; Control: -42.9 ± 0.9 mV, db-cGMP: -42.1 ± 1.0 mV; n =
Figure 5.9

Effect of NOS inhibition on spontaneous activity. The nitric oxide synthase (NOS) inhibitor, L-NAME (10 μM) increased the frequency and amplitude of (A) rhythmical depolarizations and contractions, and (B) [Ca^{2+}]_j oscillations in the arterial wall. C, Imaging showed that [Ca^{2+}]_j oscillations in individual SMCs were more synchronized. Increases in [Ca^{2+}]_j are represented as an upward deflection. The representative control basal calcium levels in the arterial wall was 0.73 F_{340/380}.
Figure 5.10

Effect of db cGMP (300 μM) on spontaneous, rhythmical activity. Incubation in db cGMP had no effect on the most negative membrane potential, nor on the frequency of rhythmical depolarizations, contractions, or calcium oscillations in the arterial wall or in individual SMCs. Rhythmical activity was decreased in amplitude, basal calcium levels in the arterial wall were decreased and the vessel was constricted. Increases in \([\text{Ca}^{2+}]_i\) are represented by an upward deflection. The representative basal calcium level in control is 0.66 $F_{340/380}$. 

### Control vs. db cGMP (300 μM)

<table>
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<tr>
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<th>Control</th>
<th>db cGMP (300 μM)</th>
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<tbody>
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<tr>
<td>Diameter</td>
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<td><strong>C. SMC calcium (n = 6 cells)</strong></td>
<td><img src="image" alt="SMC Calcium" /></td>
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7; P<0.05, paired t-test), basal calcium levels in the arterial wall were decreased (Fig 5.10B; db-cGMP: 99.2 ± 0.7 % of control \( \frac{F_{340/380}}{F_{340/380}} \), Control: 0.63 ± 0.03 \( \frac{F_{340/380}}{F_{340/380}} \); \( n=4 \); P<0.05, paired t-test) and the vessel constricted (Control: 78.2 ± 3.4 μm, db-cGMP: 70.3 ± 2.5 μm; \( n=11 \); P<0.05, paired t-test). No obvious differences were observed regarding the degree of synchronicity of calcium oscillations between individual smooth muscle cells (Fig 5.10C).

In endothelium denuded preparations (Fig 5.11), db-cGMP (300 μM) had no effects on the presence of the irregular contractions, or on the frequency or amplitude of the rhythmical depolarizations (Fig 5.11A), or irregular calcium oscillations in the arterial wall (Fig 5.11B). In addition, no difference was observed in the vessel diameter (Control: 62.7 ± 3.6 μm, db-cGMP: 61.7 ± 4.8 μm; \( n=8 \)), although the smooth muscle cell membrane potential became slightly hyperpolarized (Control: -37.5 ± 1.3 mV, db-cGMP: -38.4 ± 1.3 mV; \( n=4 \); P<0.05, paired t-test) (Fig 5.11A) and the basal calcium levels were decreased. Imaging of individual smooth muscle cells showed that calcium oscillations remained unsynchronized (Fig 5.11C).

5.2.8 Effect of EDHF inhibition on spontaneous rhythmical activity

In a number of different arteries, including cerebral vessels, EDHF mediated responses can be blocked by incubation in the combination of apamin and charybdotoxin, inhibitors of \( \text{SK}_{\text{Ca}} \) and \( \text{BK}_{\text{Ca}}/\text{IK}_{\text{Ca}} \) channels, respectively (Edwards & Weston, 2001; Zygmunt and Hogestatt, 1996; Petersson et al., 1997). In the present study, addition of apamin (0.5 μM) and charybdotoxin (60 nM) to the superfusing solution caused the artery to rapidly constrict (Fig 5.12A and B). In 3 out of the 8 arteries, rhythmical contractions were completely abolished, while in the remaining arteries, rhythmical contractions were irregular and vessel movements appeared uncoordinated. Rhythmical depolarizations were significantly increased in frequency (Fig 5.12A; Control: 21.5 ± 3.5 min\(^{-1} \), apamin + CbTX: 43.5 ± 8.4 min\(^{-1} \); \( n=4 \); P<0.05, paired t-test) and amplitude (Control: 2.7 ± 0.5 mV, apamin + CbTX: 8.8 ± 2.2 mV; \( n=4 \); P<0.05, paired t-test) and the smooth muscle was depolarized (Fig 5.12 A; Control: -44.6 ± 3.5 mV, apamin + CbTX: -27.8 ± 2.2 mV; \( n=4 \); P<0.05, paired t-test). Calcium oscillations in the arterial wall were reduced in amplitude and irregular (Fig 5.12B). Imaging of smooth muscle cells showed that calcium oscillations were unsynchronized (Fig 5.12C). Furthermore, the mean frequency (Fig 5.13A) and the amplitude (Fig 5.13B) of these oscillations was reduced. This asynchronicity was reflected
Figure 5.11

Effect of db cGMP (300 μM) on rhythmical activity in the absence of the vascular endothelium. In endothelium denuded preparations, incubation in db cGMP (300 μM) had no significant effects on rhythmical depolarizations, small contractions, smooth muscle cell membrane potential or vessel diameter. Basal calcium levels and the small irregular calcium oscillations observed in the arterial wall were also not different from control. Imaging showed that calcium oscillations in adjacent SMCs remained unsynchronized. Increases in \([\text{Ca}^{2+}]_i\) are represented by an upward deflection. The basal arterial wall \([\text{Ca}^{2+}]_i\) level in control was 0.44 F_{340/380}. 
Figure 5.12
Figure 5.12

The effect of apamin (0.5 μM) and charybdotoxin (60 nM) in combination, on basilar artery vasomotion. A, Inhibition of SK$_{Ca}$ and BK$_{Ca}$/IK$_{Ca}$ with apamin and charybdotoxin reduced the rhythmical contractions, and caused movements of the vessel to become irregular (trace 10 min in drug). Rhythmical depolarizations were increased in frequency and amplitude and the membrane potential was depolarized. (B) [Ca$^{2+}$]$_i$ oscillations recorded from the arterial wall became irregular (trace 10 min in drug). (C) [Ca$^{2+}$]$_i$ oscillations recorded in individual, adjacent SMCs became asynchronous. Increases in [Ca$^{2+}$]$_i$ are represented as an upward deflection. The representative basal calcium level in control is 0.44 F$_{340/380}$. 
Control  Apamin (0.5 μM)/
Charybdotoxin (60 nM)

A. Membrane potential

-35mV

B. Wall calcium

Diameter

C. SMC Calcium (n = 6 cells)
in the ratio of the amplitude of the $[\text{Ca}^{2+}]_i$ oscillations in individual smooth muscle cells, compared to the amplitude of the $[\text{Ca}^{2+}]_i$ oscillations in the arterial wall, which was significantly increased in the presence of apamin and charbdotoxin (Fig 5.13C; $P<0.05$).

### 5.3 DISCUSSION

The key finding of the present study is that, in the juvenile rat basilar artery, the functional integrity of the endothelium appears to be important for the coordination of calcium fluxes in individual smooth muscle cells. In contrast to previous studies in rat mesenteric arteries, neither endothelium derived NO nor EDHF was responsible for the initiation of vasomotion, however they were both found to provide a modulatory influence. Therefore, we propose that current generated in the smooth muscle passes through MEGJs to the endothelium where it is rapidly conducted along the vessel wall and back to the smooth muscle in order to synchronize rhythmical events.

#### 5.3.1 Cell coupling in the arterial wall

Since vasomotion relies on the coordination, or synchronization of calcium oscillations in individual smooth muscle cells, and gap junctions play a critical role in facilitating transmission of intracellular signals (such as IP$_3$ or current), then cell coupling may be essential to the development of coordinated contraction in rhythmically active vessels (Chaytor et al., 1997; Hill et al., 1999; Bartlett et al., 2000; Sell et al., 2002). Using serial section electron microscopy, we have been able to morphologically identify MEGJs in the juvenile rat basilar artery preparation and provide direct functional evidence for cellular coupling between the endothelial and smooth muscle cell layers, using electrophysiology in which dye identified endothelial and smooth muscle cells from the same artery were shown to have equivalent resting membrane potentials. Furthermore, oscillations in membrane potential, with no discernable differences in the depolarization frequency or amplitude were observed when recording from either cell type. Electrical coupling between the endothelial and smooth muscle cell layers has previously been demonstrated in hamster retractor feed arteries and rat mesenteric arteries, in which the resting membrane potential of either cell type was not significantly different either when cells were quiescent or when displaying rhythmical depolarizations (Emerson & Segal, 2000a; Emerson et al., 2002; Sandow et al., 2002). Studies of calcium dynamics in the rat
Figure 5.13
Figure 5.13
Effect of apamin (0.5 μM) and charybdotoxin (60 nM) in combination on smooth muscle Ca^{2+} oscillations. The effect of EDHF blockade on the frequency (A) and amplitude (B) of [Ca^{2+}]_i oscillations recorded from individual SMCs after the addition of apamin and charbdotoxin. Points represent individual SMCs, whereby 6 adjacent cells were analyzed from 4 different preparations. Frequency and amplitude of SMC [Ca^{2+}]_i oscillations in are expressed as % of the maximum in each preparation. Mean frequency and amplitude for all control preparations was 14.5 ± 1.5 min^{-1} and 0.085 ± 0.025 F_{340/380}, respectively. Straight lines indicate mean value for all data. C, Ratio of the amplitude of individual SMC [Ca^{2+}]_i oscillations to the amplitude of the [Ca^{2+}]_i in the arterial wall is increased in the presence of K_{Ca} channel inhibitors. Values represent mean and S.E.M. * significantly different from control (P<0.05).
Smooth muscle cell

A.

Freq. of \([Ca^{2+}]\) oscillation % of the max. freq.

B.

Amp. of \([Ca^{2+}]\) oscillation % max. amplitude

Control Apamin (0.5 µM) / Charybdotoxin (60 nM)

C.

\[ \Delta SMC / \Delta wall \text{ calcium oscillation amplitude} \]

Control Apamin (0.5 µM) / Charybdotoxin (60 nM)
mesenteric artery have demonstrated calcium fluxes between the smooth muscle and endothelial cell layers during vasomotion, providing additional evidence for myoendothelial cell coupling within this vascular bed (Schuster et al., 2001). In the present study, since the incidence of MEGJs was calculated to be 3 per endothelial cell, and the rat caudal cerebellar artery was shown to have 2 smooth muscle cell layers, this number of MEGJs seems sufficient to effectively couple the two cell layers.

The nuclear marker, propidium iodide, could be observed to spread rapidly amongst endothelial cells and large homocellular gap junctions were detected using serial section electron microscopy. On the other hand, dye only spread amongst small groups of smooth muscle cells (2-4). This is consistent with previous findings in the juvenile rat basilar artery (see chapter 4), that calcium oscillations in individual SMCs appeared to be synchronised within small groups of adjacent cells. Poor coupling between smooth muscle cells has also been described in the hamster feed artery (Emerson & Segal, 2000b). On the other hand, propidium iodide did not appear to pass between the two cell layers. Although transfer of dye between the endothelial and smooth muscle cell layers has been described in the hamster cheek pouch arteriole and in the porcine ciliary artery (Little et al., 1995; Beny, 1999), it has also been noted that electrically coupled cells are not necessarily dye coupled (Pollack, 1976; Beny & Connat, 1992; Beny & Pacicca, 1994; Welsh & Segal, 1998; Beny, 1999). Inconsistencies between electrical coupling and dye coupling can be explained by the selectivity of respective gap junctions to ions versus dye molecules (Little et al., 1995; Veenstra et al., 1995; Brink, 1996). This selectivity is complicated by the fact that connexin(s) subtypes that comprise the MEGJ are unknown. On the other hand, since MEGJs have been reported to show polarity of dye transfer from the endothelium to the smooth muscle only (Little et al., 1995), the observed lack of dye transfer between the cell layers can also be explained by the fact that in the present study, the majority of electrode impalements were made from the smooth muscle.

5.3.2 Connexin distribution in the endothelial and smooth muscle cell layers

All four vascular connexins were expressed in the basilar and caudal cerebellar arteries although these connexin subtypes were differentially distributed throughout the endothelial and smooth muscle cell layers. Thus, Cx37 was shown to be expressed in both the endothelium and smooth muscle layers of the juvenile rat basilar artery, similar to that previously described in the adult rat basilar and caudal arteries and hamster arterioles and
feed arteries (Hill et al., 2002; Rummery et al., 2002a; Sandow et al., 2002), while Cx45 was only found in the smooth muscle as described in rat cerebral arterioles, thoracic aorta and caudal arteries, and in embryonic and adult mice (Alcolea et al., 1999; Kruger et al., 2000; Kumai et al., 2000; Ko et al., 2001; Li & Simard, 2001; Rummery et al., 2002a). On the other hand, in contrast to findings of Li and Simard, (1999) who demonstrated Cx40 expression in the media of the rat basilar artery, we found that Cx40 staining was restricted to the endothelial cell layer only. Since the Cx40 staining reported by Li and Simard, (1999) appears to run parallel to the endothelium, rather than perpendicular to it, it has been suggested that this staining is actually within the endothelium and not in the smooth muscle (Hill et al., 2001).

Expression of Cx43 was only seen in the endothelium, in contrast to Li and Simard, (1999) who have described Cx43 in the smooth muscle only of adult rat basilar arteries. In the present study we have detected a small number of cells located within the smooth muscle cell layers showing intense Cx43 immunoreactivity. These cells appear to resemble interstitial cells of Cajal described in the gastric antum of guinea pigs (Cousins et al., 2003). Cells with anatomical characteristics similar to interstitial cells have also been described in the rabbit portal vein, small guinea pig mesenteric arteries and in human cerebral arteries (Dahl, 1964; Pucovsky et al., 2002; Povstyan et al., 2003). Whether these cells act as pacemaker cells for vasomotion is yet to be determined.

5.3.3 Role of the endothelium in spontaneous vasomotion

Removal of the vascular endothelium led to loss of rhythmical vasomotion and the appearance of smaller and irregular contractions. Calcium oscillations within individual smooth muscle cells were present, but no longer synchronized, suggesting that the endothelium is not responsible for the development of contractions per se. Thus the endothelium is unlikely to be required for the initiation of activity in this vessel. This finding is in agreement with previous studies of vasomotion in the cerebral circulation in which a modulatory influence of the endothelium has been described (Hempelmann et al., 1998) and with previous studies in the rat mesenteric artery suggesting that the endothelium is important for synchronizing contractions (Peng et al., 2001).

Small rhythmical depolarizations could be recorded from the smooth muscle cells of endothelial denuded arteries, consistent with the concept that a functional endothelium is not required to initiate rhythmical activity. This is in direct contrast to findings by
Gustafsson et al., (1993) who demonstrated that rhythmical depolarizations were completely abolished by removal of the endothelium in rat mesenteric arteries. In this artery, Peng et al., (2001) have hypothesized that changes in membrane potential occur subsequent to the activation of a synchronizing cGMP-dependent calcium activated chloride current in the smooth muscle. This current coordinates voltage changes and intracellular calcium release in adjacent smooth muscle cells following basal release of NO from the endothelium. Mauban and Wier, (2004) have also found loss of vasomotion after endothelial cell removal in this vessel, although Schuster et al., (2004) did not.

While rhythmical depolarizations were recorded in the absence of a functional endothelium, they no longer preceded each of the contractions. This phenomenon could be explained by a reduction in coupling in the media leading to individual or small groups of individual smooth muscle cells showing different oscillatory patterns in membrane potential while vessel tone reflects the contributions of all smooth muscle cells. Our assessment of cell coupling, based on the ratio of the amplitude of calcium oscillations in individual smooth muscle cells to oscillations of wall calcium, supported the idea of a decrease in cell coupling. We also show an increase in smooth muscle cell input resistance in the absence of a functional endothelium. This change in input resistance is unlikely to be due to the significant closure of membrane ion channels, since no difference in smooth muscle cell membrane potential was observed between endothelium intact and denuded arteries. Furthermore, following endothelial denudation, calcium oscillations within small groups of 2-3 adjacent cells appeared to be synchronized, while adjacent groups of cells oscillated at different rates. This compared to the near identical frequencies of all cells observed in endothelium intact arteries. Thus, data from the present study could be simply explained if the role of the endothelium was to act as an electrical conduit to rapidly couple groups of smooth muscle cells through MEGJs.

An intriguing finding of the present study was that the asynchronous calcium oscillations were reduced in amplitude, in a manner similar to that described in the rat mesenteric artery following the application of the putative gap junction uncoupler heptanol (Sell et al., 2002). This finding would suggest that by a reduction in cellular coupling between adjacent smooth muscle cells following removal of the endothelium might lower the calcium transient amplitude. On this basis, it might be assumed that calcium is passing through gap junctions to adjacent smooth muscle cells. However this is unlikely as we have previously demonstrated that calcium does not move between smooth muscle cells (see...
chapter 4). Alternatively, the reduction in amplitude of the voltage changes within the smooth muscle would result in a decreased influx of calcium through voltage dependent calcium channels.

It is also possible that subtle damage to the smooth muscle has occurred during the endothelial denudation process. However relaxation to the endothelium independent vasodilator SNAP was chosen as a functional test of smooth muscle cell integrity and only those preparations in which dilation to SNAP was comparable to the relaxation observed in endothelium intact preparations were included for analysis. Furthermore, application of ACh to endothelium denuded preparations was characterized not only by the loss of vasodilation and hyperpolarization, but also by an increase in basal calcium levels in the arterial wall and constriction of the vessel. ACh-induced vasoconstriction has previously been described in the cerebral, coronary, mesenteric and renal circulation under conditions in which the endothelium has either been removed or has been shown to be dysfunctional (Ludmer et al., 1986; Ge et al., 2003; Hegde et al., 1998). The mechanism by which ACh directly contracts vascular smooth muscle is poorly understood, but is thought to occur through calcium release from the sarcoplasmic reticulum following activation of muscarinic receptors located on the smooth muscle (Ge et al., 2003). Those preparations which failed to demonstrate constriction in response to ACh were also discarded, as these preparations were thought to have smooth muscle damage.

5.3.4 Role of NO and cGMP in spontaneous vasomotion

Endothelial derived NO and the subsequent rise in smooth muscle cGMP has been suggested to be essential to the initiation of agonist induced vasomotion in rat mesenteric arteries (Gustafsson, 1993; Peng et al., 2001), although other studies in the same vessel have proposed a more modulatory influence of NO (Huang & Cheung, 1997; Sell et al., 2002; Okazaki et al., 2003; Mauban & Wier, 2004; Schuster et al., 2004). In the present study, incubation in the NOS inhibitor, L-NAME resulted in an increase in the frequency and amplitude of spontaneous depolarizations, contractions, calcium oscillations in the arterial wall and in individual smooth muscle cells. This indicates that spontaneous vasomotion in the juvenile rat basilar artery does not depend on the basal release of NO, but rather, activity of the NO pathway negatively modulates vasomotion. These results are consistent with previous studies of vasomotion in the adult rat basilar artery in vitro, and in cerebral arterioles in vivo, where NOS inhibition was shown to augment or initiate
rhythmical activity (Dirnagl et al., 1993; Hempelmann et al., 1998). Studies of vasomotion in guinea pig lymphatics (von der Weid et al., 1996), hamster skeletal muscle microcirculation (Bertuglia et al., 1994) and rat iris arterioles (see chapter 3) have also shown that endogenous NO production can suppress rhythmical activity.

The hypothesis that a cGMP dependent depolarizing current is responsible for initiating agonist induced vasomotion in the rat mesenteric artery has been supported by recent experimental findings in isolated smooth muscle cells in which a novel cGMP-dependent calcium activated chloride current has been identified (Matchkov et al., 2004a; Piper & Large, 2004). In the present study, however, inhibition of NO failed to significantly alter resting membrane potential. This would suggest that the NO/cGMP pathway does not contribute to a depolarizing membrane current in the juvenile rat basilar artery during spontaneous vasomotion. Moreover, loss of such a current would be expected to desynchronize calcium oscillations in adjacent smooth muscle cells. Thus the present findings are more consistent with a recent study of calcium dynamics in the rat mesenteric artery in which NO production was shown to have a desynchronizing effect on calcium signals in the smooth muscle (Sell et al., 2002) and with another study within the same vascular bed whereby NOS inhibition caused increased vasoconstriction and decreased contraction amplitude of agonist induced vasomotion (Mauban & Wier, 2004).

In the basilar artery of different species, NO is thought to alter vascular responses via the down regulation of L-type voltage dependent calcium channels (Salomone et al., 1997; Porter et al., 1998; Kikkawa et al., 1999; Simard & Li, 2000). Others have reported that NO through cGMP activity can activate calcium or voltage activated potassium channels or decrease calcium release from smooth muscle intracellular stores (Huang & Cheung, 1997; Kitazono et al., 1997; Prakash et al., 1997; Plane et al., 1998; Sobey & Faraci, 1999). Thus in the present study, because incubation in L-NAME not only augmented both the frequency and amplitude of the rhythmical activity, but also synchronized calcium oscillations in neighbouring smooth muscle cells and constricted the vessel, endogenous NO release may decrease calcium influx through voltage dependent calcium channels and reduce synchronicity between adjacent cells. Augmented calcium influx through voltage dependent calcium channels would also account for the increased basal calcium levels in the arterial wall and vasoconstriction observed in the presence of L-NAME. Vasoconstriction in the presence of L-NAME is commonly observed throughout
the cerebral vasculature (Katusic, 1991; Brian & Kennedy, 1993; Dirnagl et al., 1993; Kimura et al., 1994; Salomone et al., 1997; Kikkawa et al., 1999).

In the present study, inhibition of NOS after treatment with L-NAME was unable to mimic the effects of endothelial denudation. Such have previously been documented in the rat basilar artery (Ryman et al., 1993), rat mesenteric artery (Gustafsson, 1993) and in the rat iris arteriole (see chapter 3) after removal of the endothelium. Because products of the NO pathway have been shown to directly regulate gap junction closure (Kwak et al., 1995a; Kwak et al., 1995b) and previous studies in the rat mesenteric artery (Mauban et al., 2001) (Shaw et al., 2004), rat tail artery (Kasai et al., 1997) and rabbit inferior vena cava (Ruehlmann et al., 2000) exhibit dose-dependent, agonist induced increases in smooth muscle cell calcium oscillation frequency after L-NAME, it is possible that the enhanced cellular coupling observed in the present study during NOS inhibition, may increase the strength, amplitude and frequency at which these cells oscillate in an analogous manner. Thus loss of NO may not mimic endothelial denudation if L-NAME were to increase cellular coupling, while at the same time, removal of the endothelium would be expected to decrease cellular coupling. Endothelial derived vasoactive factors other than NO may also influence rhythmical activity in this preparation. Nevertheless, the data obtained in the presence of L-NAME extend our previous observation that the vascular endothelium is an important regulator of rhythmical activity in the juvenile rat basilar artery, through modulation by the NO/cGMP pathway (Dirnagl et al., 1993; Hempelmann et al., 1998). It should also be noted that in the present study, data for endothelium intact and endothelium denuded arteries were obtained from different preparations.

In endothelium intact preparations, incubation in db-cGMP had no significant effects on the frequency of rhythmical depolarizations, contractions, calcium oscillations or on membrane potential, indicating that cGMP activity does not play an important role in regulating vasomotion in the cerebral vascular bed. On the other hand, db-cGMP reduced the amplitude of rhythmical activity and decreased basal calcium levels in the arterial wall, suggesting the negative modulation of vasomotion following release of nitric oxide from the endothelium. The paradoxical effects of cGMP on basal calcium levels and vessel diameter may indicate that cGMP has multiple targets in the vessel wall as this was not seen in the absence of the endothelium may result from increases in cGMP.

Addition of db-cGMP to endothelium denuded preparations failed to resynchronize calcium oscillations in adjacent smooth muscle cells, or restore regular calcium oscillations
and contractions as has been reported for mesenteric arteries (Peng et al., 2001). If indeed cGMP was responsible for activating a synchronizing depolarizing current in the smooth muscle (Peng et al., 2001), then we could predict that db-cGMP would cause the asynchronous calcium oscillations in adjacent cells to become synchronized. However, db-cGMP hyperpolarized the membrane potential and decreased basal calcium levels in the arterial wall. These data suggest that db-cGMP may directly inhibit calcium release from intracellular stores. Similar effects on membrane potential and calcium occur in the juvenile basilar artery following inhibition of intracellular Ca\(^{2+}\)-ATPase or the phospholipase C pathway (see chapter 4), and in cultured bovine aortic smooth muscle cells and isolated guinea pig gastric smooth muscle in which cGMP inhibits IP\(_3\)-sensitive calcium release (Hirata et al., 1990; Murthy et al., 1993).

In summary, neither NO nor cGMP are essential for initiation of vasomotion in the cerebral vasculature. Instead, the NO/cGMP signaling cascade can be categorized as modulatory, since rhythmical activity continues following pharmacological manipulation of these factors.

### 5.3.5 Role of EDHF in spontaneous vasomotion

In rat small mesenteric arteries, the SK\(_{Ca}\) and IK\(_{Ca/BK}\) channel inhibitors, apamin and charybdotoxin, have been reported to abolish rhythmical contractions, suggesting that EDHF production is essential to the development of agonist induced vasomotion in this vascular bed (Dora et al., 2000b; Okazaki et al., 2003; Mauban & Wier, 2004). In the present study, however, we have been able to record small, irregular contractions in the presence of these inhibitors and therefore, we suggest a modulatory, but not essential role for EDHF in spontaneous vasomotion. We can attribute the loss of rhythmical activity in some preparations to the increased vasoconstriction associated with inhibiting K\(_{Ca}\) channels in this vascular bed. In addition, we found that application of apamin and charybdotoxin caused calcium oscillations in adjacent smooth muscle cells to become asynchronous. Mauban and Wier (2004) also demonstrated the appearance of asynchronous calcium waves following inhibition of EDHF with charybdotoxin and apamin in the rat mesenteric artery. Since production of EDHF in the endothelium would be expected to hyperpolarize the SMCs, the loss of such an influence would be expected to activate more VDCCs. Indeed block of SK\(_{Ca}\) and BK\(_{Ca}\)/IK\(_{Ca}\) leads to depolarization, constriction and increase in the amplitude and frequency of the rhythmical depolarizations, consistent with the
activation of these channels. Paradoxically, it can be predicted that this would lead to synchronization of smooth calcium transients and augmentation of vasomotion. However, in the rabbit inferior vena cava, increases in agonist concentration switch synchronized calcium oscillations and coordinated rhythmical contraction to asynchronous calcium oscillations and tonic constriction (Ruehlmann et al., 2000).

In vivo studies indicate that the production of EDHF is stimulus dependent (Parkington et al., 2002). In the present study, EDHF does not appear to be tonically released during vasomotion since no change in membrane potential was observed after removal of the endothelium. Therefore, apamin and charybdotoxin may have direct effects in the smooth muscle cells where IK\text{Ca} channels provide negative feedback for vasomotion (see chapter 4). Thus results are not consistent with a role for endogenous EDHF, but rather an inhibitory effect on IK\text{Ca} in smooth muscle cells leading to increased vasoconstriction. Nevertheless, EDHF could provide an important negative modulation of vasomotion through reduction of voltage dependent calcium channel activity and reduced synchronization of smooth muscle cell calcium oscillations.

5.3.6 Proposed model of cellular coupling in rat basilar artery vasomotion

In conclusion, the endothelium of the juvenile rat basilar artery does not appear to be responsible for the initiation of vasomotion through the production of vasoactive factors such as NO or EDHF. Instead, the NO and EDHF signaling pathways may provide an endogenous mechanism through which vasomotion can be negatively modulated. Thus, we propose that calcium oscillations are generated within the smooth muscle, but that these cells are poorly coupled. The principle activity of the endothelium then, appears to be its ability to facilitate the electrotonic spread of voltage along the vascular wall in order to coordinate calcium fluxes through voltage dependent calcium channels within adjacent smooth muscle cells (Fig 5.14). Transfer of electrical current passes from the smooth muscle, through MEGJs, along the endothelial cells and back into the smooth muscle cells to synchronize rhythmical activity and vasomotion takes place. Serial section electron microscopy confirming the presence of MEGJs, and immunohistochemistry revealing extensive connexin distribution throughout the vascular wall are consistent with this proposition. EDHF may reduce membrane potential and significantly attenuate vasomotion by reducing calcium influx through voltage dependent calcium channels, while NO may
Figure 5.14
Figure 5.14
Proposed role of the endothelium in spontaneous vasomotion in the rat basilar artery. Calcium oscillations are generated solely within the SMCs (1), but that these cells are not particularly well coupled (2). Transfer of electrical current then passes through MEGJs (3), along the endothelial cells (4) and back into the smooth muscle cells to synchronise rhythmical activity (5) through the activation of voltage dependent calcium channels (VDCC) and calcium induced calcium release from ryanodine receptors (Ry) and vasomotion takes place (6). The endothelium derived vasoactive factors NO and EDHF provide an endogenous mechanism through which vasomotion can be negatively modulated by reducing voltage dependent calcium channel activity (7).
reduce calcium release from intracellular stores or directly reduce voltage dependent calcium channel activity.
6.1 INTRODUCTION

Intercellular communication via gap junctions between and within the endothelial and smooth muscle cell layers of the arterial wall has been suggested to be critical to synchronizing cellular responses and coordinating vasomotion (Peng et al., 2001; Matchkov et al., 2004b). Several studies have investigated the role of gap junctions in vasomotion using compounds thought to interfere with cellular coupling. Accordingly, incubation in the long chain alcohols, octanol and heptanol, were shown to inhibit rhythmical contractions and desynchronize calcium oscillations in the hamster aorta and rat small mesenteric arteries, respectively (Jackson et al., 1991; Sell et al., 2002), while in the rat iris arteriole and rat pulmonary artery, vasomotion was rapidly abolished by the licorice derivate, 18α-glycyrrhetinic acid (Hill et al., 1999; Bonnet et al., 2001). However, many of these putative uncouplers have been reported to exert non-specific effects on cellular targets other than gap junctions (Spray & Burt, 1990; Chaytor et al., 1997; Hashitani & Suzuki, 1997; Coleman et al., 2001, 2002). An alternative and apparently more selective approach by Chaytor et al., (1997), has made use of short peptides which mimic connexin sequences to study the role of gap junctions in agonist induced vasomotion in the rabbit mesenteric artery.

Connexin mimetic peptides are designed to imitate the extracellular loops of connexins and are thought to disrupt gap junctional coupling by interfering with the docking of connexins between adjacent cells (Chaytor et al., 1999; Kwak & Jongsma, 1999; Evans & Boitano, 2001; Li & Simard, 2001; Dhein, 2002). Since vasomotion relies on the synchronization of cellular events, the reported specificity of these mimetic peptides makes them ideal candidates with which to study gap junctional coupling in this phenomenon. Furthermore, since these peptides have been designed to locate to amino acid sequences against particular connexin subtypes, the potential exists for connexin selective activity during vasomotion to be directly examined. On the other hand, as many of the
details regarding the precise mechanism of action remain unclear (Chaytor et al., 1997; Evans & Boitano, 2001; Sandow, 2004) and similarities in the peptide sequences amongst the different connexins suggests that there may be overlap in their activity, an investigation into the specificity of these peptides is warranted.

In chapter 5, we found that the two cell layers of the juvenile basilar artery were connected by MEGJs and that Cx37, 40 and 43 were found within the endothelium while Cx37 and Cx45 were expressed to a lesser extent in the media. Moreover, we were able to demonstrate a critical role for myoendothelial coupling in synchronizing the cellular events that underlie vasomotion in this artery. Thus, the particular aims of this chapter were to use connexin mimetic peptides against two extracellular regions involved in docking Gap 27 and Gap 26, to directly examine the role of specific connexin subtypes during spontaneous vasomotion in the juvenile rat basilar artery. Additional peptides to another region involved in docking (Kwak & Jongsma, 1999; Li & Simard, 2001) were also tested in order to assess the specificity of the effects seen.

6.2 RESULTS

6.2.1 Cx mimetic peptides homologous to the extracellular Gap 27 sequence

6.2.1.1 Effect of \(^{37,43}\)Gap 27.

In arteries with intact endothelium, incubation in the Cx-mimetic peptide homologous to the extracellular Gap 27 sequence of connexins 37 and 43 (\(^{37,43}\)Gap 27; 100 \(\mu\)M; \(n = 12\)) abolished the rhythmical depolarizations, contractions (Fig 6.1A), calcium oscillations in the arterial wall (Fig 6.1B) and in individual smooth muscle cells (Fig 6.1C) within 10 min of the peptide entering the bath (\(P<0.05\), paired t-test). Smooth muscle cells were hyperpolarized (Fig 6.1A; Control: \(-42.8 \pm 2.3 \text{ mV}\), \(^{37,43}\)Gap 27: \(-54.3 \pm 2.8 \text{ mV}\); \(n = 4\); \(P<0.05\), paired t-test), basal calcium levels were decreased (Fig 6.1B; \(^{37,43}\)Gap 27: 99.0 \(\pm 4.2 \% F_{340/380}\); \(n = 8\); \(P<0.05\), paired t-test) and the vessel relaxed (Fig 6.1A and B; Control: \(37.6 \pm 4.6 \text{ pm}\), \(^{37,43}\)Gap 27: 41.8 \(\pm 4.8 \text{ pm}\); \(n = 12\); \(P<0.05\), paired t-test).

To further explore the functional effects of the gap junction peptide, \(^{37,43}\)Gap 27, rhythmical activity was also studied in a number of preparations from which the endothelium had been removed (Fig 6.2). In contrast to endothelium intact preparations, small rhythmical depolarizations and asynchronous contractions could be recorded in the presence of \(^{37,43}\)Gap 27 (Fig 6.2A). The rhythmical depolarizations were increased in
The effect of $^{37,43}$Gap 27 (100 μM) on rhythmical activity. $^{37,43}$Gap 27, targeting Cx37 and 43, hyperpolarized SMCs and abolished (A) rhythmical depolarizations, contractions and (B) calcium oscillations in the arterial wall. Basal calcium levels were decreased and the vessel relaxed. C, calcium oscillations in individual SMCs were also abolished. Increases in [Ca$^{2+}$]$_i$ are represented as an upward deflection. The representative control basal SMC [Ca$^{2+}$]$_i$ was 0.64 F$_{340/380}$. 
frequency (Control: 19.4 ± 4.4 min⁻¹, 37,43Gap 27: 25.7 ± 3.9 min⁻¹; n = 4; P<0.05, paired t-test) but reduced in amplitude (Control: 4.4 ± 0.3 mV; 37,43Gap 27: 3.4 ± 0.2 mV; n = 4; P<0.05, paired t-test). Furthermore, no significant effects were observed on membrane potential (Fig 6.2A; Control: -38.7 ± 1.8 mV, 37,43Gap 27: -37.7 ± 1.6 mV; n = 4), basal calcium levels (Fig 6.2; 37,43Gap 27: 105.5 ± 3.3 % F₃₄₀/₃₈₀; n = 4) or vessel diameter (Control: 58.3 ± 5.8 μm, 37,43Gap 27: 58.6 ± 5.8 μm). On the other hand, and in a manner similar to that observed in endothelium intact preparations, 37,43Gap 27 abolished calcium oscillations in the arterial wall and in individual SMCs (Fig 6.2C; n = 4; P<0.05, paired t-test).

6.2.1.2 Effect of ⁴⁰Gap 27.

To assess the role of Cx40 in rat basilar artery spontaneous vasomotion, preparations were incubated in the Cx-mimetic peptide homologous to the extracellular Gap 27 sequence of connexin 40. Within 5 min of the peptide entering the recording chamber, ⁴⁰Gap 27 (100 μM) caused a significant reduction in the frequency (Control: 17.3 ± 2.6 min⁻¹, ⁴⁰Gap 27: 9.4 ± 3.2 min⁻¹; n = 8; P<0.05, paired t-test) and amplitude of rhythmical contractions (Control: 4.9 ± 0.8 % RVD, ⁴⁰Gap 27: 1.5 ± 0.3 % RVD; n = 8; P<0.05, paired t-test) resulting in the appearance of uncoordinated movements (Fig 6.3A and B). Electrophysiological measurements revealed the presence of small rhythmical depolarizations (Fig 6.3A; P<0.05, paired t-test) and smooth muscle cells appeared to settle at their most negative membrane potentials (Control: -38.7 ± 1.9 mV, ⁴⁰Gap 27: -39.4 ± 1.3 mV, n = 4). Measurements of arterial wall [Ca²⁺]j revealed the presence of small irregular calcium oscillations and basal calcium levels were unaltered (Fig 6.3B; ⁴⁰Gap 27: 111.1 ± 2.2 % F₃₄₀/₃₈₀, n = 4). Vessel tone showed a trend to be decreased, as evidenced by a small but insignificant increase in diameter (Control: 31.9 ± 3.5 μm, ⁴⁰Gap 27: 32.7 ± 4.3 μm; n = 8). Imaging of SMCs showed that small calcium oscillations were present in individual cells, but that these were unsynchronized (Fig 6.3C). Further analysis showed that the mean frequency (Fig 6.4A) and the amplitude (Fig 6.4B) of these oscillations was significantly reduced (P<0.05, unpaired t-test) and the ratio of the amplitude of [Ca²⁺]j oscillations in individual SMCs, to the amplitude of the [Ca²⁺]j oscillations in the arterial wall was increased (Fig 6.4C; P<0.05, unpaired t-test).

In those preparations from which the endothelium had successfully been removed, incubation in ⁴⁰Gap 27 (100 μM) caused the irregular contractions to regain rhythmicity (Fig 6.5A and B). Contractions (Control: 15.7 ± 1.5 min⁻¹, ⁴⁰Gap 27: 18.8 ± 2.2 min⁻¹; n = 8,
Figure 6.2

Effect of $^{37,43}\text{Gap 27 (100 \, \mu M)}$ on rhythmical activity in endothelium denuded preparations. Representative traces show that in the presence of the Cx mimetic peptide, $^{37,43}\text{Gap 27}$, targeting Cx37 and 43, small rhythmical depolarizations could be recorded in those preparations from which the vascular endothelium had been disrupted. A, No effect was observed on the SMC membrane potential or on vessel diameter. B, Irregular calcium oscillations in the arterial wall and C, asynchronous calcium oscillations in individual SMCs were abolished (C). Increases in $[\text{Ca}^{2+}]_i$ are represented by an upward deflection and the representative basal calcium level in control was 0.49 $F_{340/380}$. 
Figure 6.3
Effect of the gap junction peptide, $^{40}$Gap 27 (100 μM) on rhythmical activity. The peptide homologous to the extracellular loop of Cx40, $^{40}$Gap 27 abolished the large rhythmical depolarizations and coordinated contractions. B, calcium oscillations in the arterial wall were also abolished. C, Calcium oscillations recorded in individual, adjacent SMCs were reduced in amplitude and became asynchronous. Increases in $[Ca^{2+}]_i$ are represented by an upward deflection. The representative control basal calcium level in the arterial wall was 0.48 F$_{340/380}$. 
Figure 6.4
Effect of $^{40}$Gap 27 (100 µM) on rhythmical $[Ca^{2+}]_i$ oscillations. The frequency (A) and amplitude (B) of calcium oscillations recorded from adjacent smooth muscle cells before and after the addition of $^{40}$Gap 27. Points represent individual SMCs, whereby 6 adjacent cells were analyzed from 4 different preparations and the cell with the frequency or greatest amplitude designated as 100% in each preparation. The absolute frequency and amplitude of SMC $[Ca^{2+}]_i$ oscillations in control was $17.0 \pm 2.5\ min^{-1}$ and $0.43 \pm 0.003\ F_{340/380}$. Straight lines indicate mean values. C, The ratio of the amplitude of individual SMC calcium oscillations to the amplitude of the calcium oscillations in the wall is increased in the presence of $^{40}$Gap 27. Values represent mean and S.E.M. * significantly different from control (P<0.05).
P<0.05, paired t-test) and rhythmical depolarizations (Control: 24.7 ± 1.5 min⁻¹, 40Gap 27: 25.8 ± 1.5; n = 4, P<0.05, paired t-test) were increased in frequency (Fig 6.5A). No significant effects were observed on membrane potential (Control: -43.0 ± 1.1 mV, 40Gap 27: -40.5 ± 1.9 mV; n = 4, paired t-test) or on vessel diameter (Fig 6.5A; Control: 69.8 ± 11.1 μm, 40Gap 27: 70.5 ± 10.7 μm; n = 8, paired t-test). Calcium oscillations in the arterial wall mimicked the depolarizations in that the presence of 40Gap 27, calcium oscillations returned to regular cyclic patterns (Fig 6.5B) and were increased in frequency (Control: 15.7 ± 1.5 min⁻¹, 40Gap 27: 18.8 ± 2.2 min⁻¹; n = 4; P<0.05, paired t-test). No significant effects were observed on basal calcium levels in the arterial wall (40Gap 27: 104 ± 3.3 % F₃₄₀/₃₈₀; n = 4, paired t-test). Calcium oscillations in adjacent smooth muscle cells were increased in frequency (Fig 6.6A; n = 4; P<0.05, unpaired t-test) reduced in amplitude (Fig 6.6B; n = 4; P<0.05, unpaired t-test) and apparently synchronized as evidenced by a decrease in the ratio of the amplitude of calcium oscillations in adjacent smooth muscle cells, to the amplitude of the calcium oscillations in the arterial wall (Fig 6.6C). When hyperpolarizing current pulses (0.1-1.0 nA) were applied to short arterial segments, the current voltage relationship was significantly steeper in the presence of 40Gap 27 indicating significant increase in smooth muscle cell input resistance (Fig 6.7B).

6.2.1.3 Effect of 45Gap 27.

The role of Cx45 was investigated using the synthetic mimetic peptide 45Gap 27 (100 μM) homologous to the Cx45 sequence in the Gap 27 extracellular region. Incubation in 45Gap 27 abolished rhythmical depolarizations (Fig 6.8A), contractions and (Fig 6.8B) calcium oscillations in the arterial wall (Control: 18.5 ± 2.3 min⁻¹, 45Gap 27: 2.1 ± 0.8 min⁻¹; n = 8; P<0.05, paired t-test). Furthermore, 45Gap 27 substantially hyperpolarized the membrane potential (Fig 6.8A; Control: -40.0 ± 0.7 mV, 45Gap 27: -51.5 ± 2.1 mV; n = 4; P<0.05, paired t-test) and dilated the vessel (Control: 65.4 ± 2.4 μm, 45Gap 27: 70.5 ± 2.9 μm; n = 8; P<0.05, paired t-test).

6.2.2 Cx mimetic peptides homologous to the extracellular Gap 26 sequence

6.2.2.1 Effect of 37,40Gap 26.

Incubation in the synthetic mimetic peptide 37,40Gap 26 (100 μM) abolished rhythmical depolarizations, contractions (Fig 6.9A) and oscillations in the arterial wall (Fig 6.9B; n = 11; P<0.05, paired t-test). In addition 37,40Gap 26 (100 μM) hyperpolarized (Fig 6.9A; Control: -41.0 ± 1.7 mV, 37,40Gap 26: -47.6 ± 3.1 mV; n = 7; P<0.05, paired t-test)
Figure 6.5

The effect of $^{40}$Gap 27 (100 µM), targeting Cx40, on spontaneous rhythmic activity in the absence of the vascular endothelium. A, In endothelium denuded preparations, incubation in $^{40}$Gap 27 had no significant effects on smooth muscle cell membrane potential or vessel diameter. B, Irregular calcium oscillations in the arterial wall and contractions became more regular and increased in frequency. C, Imaging showed that the unsynchronized calcium oscillations recorded from adjacent smooth muscle cells appeared to more coordinated. Increases in $[\text{Ca}^{2+}]_i$ are represented as upward deflection. The representative control SMC basal calcium was $0.54 F_{340/380}$. 
Figure 6.6
Figure 6.6

Effect of $^{40}$Gap27 (100 μM) on SMC Ca$^{2+}$ oscillations in endothelium denuded arteries. A, Disruption of the vascular endothelium caused small groups of adjacent SMCs to oscillate at different frequencies (□), while addition of the Cx mimetic peptide $^{40}$Gap 27 (▲) to these preparations caused adjacent SMCs to oscillate at similar frequencies. B, Effect of $^{40}$Gap27 on the SMC [Ca$^{2+}$]$_i$ oscillation amplitude in endothelium denuded arteries. Mean frequency and amplitude of SMC [Ca$^{2+}$]$_i$ in control endothelium denuded preparations was $11.2 \pm 0.8$ min$^{-1}$ and $0.041 \pm 0.002$ F$_{340/380}$. Points represent values for individual SMCs whereby 6 adjacent cell were analyzed from 4 different preparations and the cell with the frequency or greatest amplitude designated as 100% in each preparation. C, The ratio of the amplitude of individual SMC [Ca$^{2+}$]$_i$ oscillations to the amplitude of the [Ca$^{2+}$]$_i$ is decreased in the presence of the peptide. Values represent mean and S.E.M * significantly different from control (P<0.05).
A. Smooth muscle cell

- Freq. of $[Ca^{2+}]$ oscillation % of the max. freq.

B. Amp. of $[Ca^{2+}]$ oscillation % of max. amplitude

C. SMC/wall calcium oscillation amplitude

- EC
- EC + $^{40}$Gap27 (100 µM)
Figure 6.7

The effect of $^{40}$Gap 27 (100 μM), targeting Cx40, on smooth muscle cell input resistance in the absence of the vascular endothelium. A, Smooth muscle cell membrane potential (mV) was recorded during 30 s hyperpolarizing current pulse steps (0.1, -1.0 nA) in control Krebs' solution (-ve endothelium) and in the presence of $^{40}$Gap 27. Straight lines indicate linear regression curves. B, In endothelium denuded preparations, $^{40}$Gap 27 significantly increased the SMC input resistance. Values represent mean and S.E.M. * significantly different from control (P<0.05).
### Control

<table>
<thead>
<tr>
<th>A. Membrane potential</th>
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<tr>
<td>45Gap 27 (100 μM)</td>
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</table>

**Diameter**

- 44 mV
- 48
- 66 μm
- 60

10 s

<table>
<thead>
<tr>
<th>B. Wall calcium</th>
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<tr>
<td>0.1 340/380</td>
</tr>
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**Diameter**

- 85 μm
- 75

10 s

<table>
<thead>
<tr>
<th>C. SMC calcium (n=6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 340/380</td>
</tr>
</tbody>
</table>

10 s

**Figure 6.8**

Effect of the Cx mimetic peptide targeting Cx45 on spontaneous rhythmical activity. A, Incubation in the peptide 45Gap 27 (100 μM) abolished rhythmical depolarizations, hyperpolarized and relaxed the SMCs. B, Calcium oscillations in the arterial wall were inhibited and the basal calcium levels were decreased. C, In some preparations, small synchronized calcium oscillations could be recorded from individual SMCs. Increases in [Ca^{2+}]_i are represented as an upward deflection. The basal calcium level in the arterial wall in the the control representative trace is 0.61 F_{340/380}. 
and relaxed the smooth muscle cells (Control: 44.3 ± 3.9 μm, 37,40 Gap 26: 46.5 ± 4.5 μm; n = 11; P<0.05, paired t-test). No effects were observed on basal calcium levels in the arterial wall (Fig 6.9B; 37,40 Gap 26: 108.4 ± 3.6 % F340/380; n = 4; P<0.05, paired t-test). Imaging of individual SMCs showed that [Ca^{2+}]_i oscillations were also abolished (Fig 6.9C). The effects of 37,40 Gap 26 were observed within 8 min of the peptide entering the bath.

6.2.2.2 Effect of 43 Gap 26.

The connexin mimetic peptide 43 Gap 26 (100 μM; n = 11) abolished rhythmical depolarizations and contractions (Fig 6.10A; P<0.05, paired t-test), calcium oscillations in the arterial wall (Fig 6.10B; P<0.05, paired t-test) and in individual smooth muscle cells (Fig 6.10C). The membrane potential showed a trend to be more hyperpolarized (Fig 6.10A; Control: -41.3 ± 2.3 mV, 43 Gap 26: -46.5 ± 3.9 mV; n = 6, paired t-test), basal calcium levels in the arterial wall were decreased (Fig 6.10B; 43 Gap 26: 101.0 ± 2.0 % F340/380; n = 5; P<0.05, paired t-test) and the vessel diameter significantly increased (Fig 6.10A and B; Control: 39.9 ± 6.7 μm, 43 Gap 26: 42.9 ± 7.0 μm; n = 11; P<0.05, paired t-test). The effects of 43 Gap 26 on rhythmical activities were observed within 10 min of entering the bath.

6.2.3 Cx mimetic peptides homologous to additional extracellular sequences

6.2.3.1 Effect of Gap 37.

In the majority of preparations, Gap 37 (100 μM), homologous for amino acid sequences 181-194 of Cx37, caused a significant reduction in the frequency (Control: 19.1 ± 1.9 min⁻¹, Gap 37: 6.8 ± 2.3 min⁻¹; n = 4; P<0.05, paired t-test) and amplitude (Control: 5.2 ± 1.6 % RVD, Gap 37: 1.3 ± 0.4 % RVD; n = 4; P<0.05, paired t-test) of rhythmical contractions (Fig 6.11A and B). Interestingly, spontaneous depolarizations were increased in frequency, but decreased in amplitude and the SMCs appeared to settle at their most negative membrane potentials (Fig 6.11A; Control: -41.0 ± 1.2 mV, Gap 37: -40.2 ± 2.6 mV; n = 4). Calcium oscillations in the arterial wall were abolished (Fig 6.11B; n = 4; P<0.05, paired t-test) and basal calcium levels in the arterial wall were decreased (Gap 37: 97.8 ± 1.5 % F340/380; n = 4; P<0.04, paired t-test). Imaging of individual SMCs showed that calcium oscillations appeared to remain synchronized but were significantly reduced in both amplitude and frequency (Fig 6.11C). The effects of the Gap 37 on rhythmical activity were observed within 10 min of entering the bath.
Figure 6.9
Effect of \(37,40\text{Gap 26} (100 \mu \text{M})\) on spontaneous, rhythmical activity. Incubation in \(37,40\text{Gap 26}\), the mimetic peptide with amino acid sequences homologous with the extracellular loops of Cxs37 and 40, abolished rhythmical depolarizations and contractions, hyperpolarized and relaxed SMCs (A). Calcium oscillations in the arterial wall were abolished and basal calcium levels were decreased. (B). Imaging showed that calcium oscillations in individual SMCs were also abolished (C). Increases in \([\text{Ca}^{2+}]_i\) are represented by an upward deflection. The representative control basal calcium was 0.42 F\(_{340/380}\).
Figure 6.10
Effect $^{43}$Gap 26 (100 μM) on rhythmical activity. Incubation in $^{43}$Gap 26, targeting Cx43, reduced the amplitude of rhythmical depolarizations and contractions. $[\text{Ca}^{2+}]$ oscillations in the arterial wall were abolished and were decreased in individual SMCs. SMCs were hyperpolarized (A), basal calcium levels were decreased (B) and the vessel relaxed (C). Increases in $[\text{Ca}^{2+}]$ are represented as upward deflection. The representative control SMC basal calcium was 0.51 $F_{340/380}$. 
Figure 6.11

Effect on Gap 37 (100 μM) on spontaneous, rhythmical activity. A, Gap 37, generated to specific amino acid sequences against Cx37, reduced the amplitude of rhythmical depolarizations and (B) contractions. Calcium oscillations in the arterial wall were abolished. C, Small [Ca^{2+}]_i oscillations were recorded in individual, adjacent SMCs. Increases in [Ca^{2+}]_i are represented by an upward deflection. The representative control basal calcium level in the arterial wall was 0.68 F_{340/380}.
6.2.3.2 Effect of Gap 43.

The effect of the gap junction peptide, gap 43 (100 μM), generated to specific amino acid sequences 181-194 of Cx43 was observed within 10 min of the peptide entering the bath. Thus, Gap 43 abolished the rhythmical depolarizations (Fig 6.12A; P<0.05), contractions and calcium oscillations in the arterial wall (Fig 6.12B; P<0.05, paired t-test) and in individual smooth muscle cells (Fig 6.12C; P<0.05, paired t-test). Smooth muscle cells showed a trend to be more hyperpolarized (Fig 6.12A; Control: -39.0 ± 0.9 mV, Gap 43: -44.9 ± 5.7; n = 4; paired t-test), basal calcium levels in the arterial wall were significantly decreased (Fig 6.12B; Gap 43: 89.8 ± 8.9 % F340/380; n = 4; P<0.05, paired t-test) and the vessel relaxed (Control: 70.6 ± 6.3 μm, Gap 43: 79.7 ± 5.8 μm; n = 8; P<0.05, paired t-test).

6.2.3.3 Effect of Gap 37 and 43 in combination

Incubation in the Cx-mimetic peptide combination Gap 37 / Gap 43 (100 μM; n = 8) abolished the rhythmical depolarizations, contractions (Fig 6.13A) and calcium oscillations in the arterial wall (Fig 6.13B) and in individual smooth muscle cells (Fig 6.13C) within 10 min of the peptides entering the bath (P<0.05, paired t-test). Smooth muscle cells were hyperpolarized (Fig 6.13A; Control: -39.2 mV ±1.1 mV, Gap 37/Gap 43: -48.5 ± 3.1 mV; n = 4; P<0.05, paired t-test), basal calcium levels were decreased (Fig 6.13B; Gap 37/ Gap 43: 98.1 ± 2.1 % F340/380, n = 4; P<0.05, paired t-test) and the vessel relaxed (Control: 70.0 ± 5.1 μm, Gap 37/ Gap 43: 79.7 ± 5.7 μm; n = 4; P<0.05, paired t-test).

6.2.3.4 Effect of Gap 40.

Incubation in the mimetic peptide, Gap 40 (100 μM), homologous for amino acid sequences of 171-191 Cx40, appeared to mimic the majority of effects observed in the presence of the peptide, Gap 27 (Fig 6.3) or endothelial removal (Fig 5.5). Thus, Gap 40 caused a decrease in both the frequency (Control: 18.0 ± 1.8 min⁻¹, Gap 40: 9.7 ± 1.8 min⁻¹; n = 8; P<0.05, paired t-test) and amplitude (Control: 4.1 ± 0.8 % RVD, Gap 40: 1.6 ± 0.3 % RVD; n = 8; P<0.05, paired t-test) of rhythmical contractions (Fig 6.14A and B). Furthermore, contractions appeared to be irregular and uncoordinated. The large depolarizations were significantly reduced in amplitude (P<0.05, paired t-test) and no longer preceded contractions (Fig 6.14A). Smooth muscle cells appeared to settle at their most negative potentials (Control: -44.9 ± 1.3 mV; Gap 40: -45.8 ± 1.8 mV). Measurements of arterial wall calcium revealed the presence of small irregular calcium oscillations,
Figure 6.12
Effect of Gap 43 (100 µM) on spontaneous rhythmical activity. Incubation in the mimetic peptide against Cx43, Gap 43, abolished (A) rhythmical depolarizations and contractions, (B) calcium oscillations in the arterial wall and (C) in individual SMCs. B, Basal calcium levels in the arterial wall were decreased and the vessel relaxed. Increases in \([Ca^{2+}]_i\) are represented as an upward deflection, whereby the basal calcium level in the representative control trace was 0.67 \(F_{340/380}\).
Figure 6.13
Figure 6.13
The effects of the Cx mimetic peptides Gap 37 (100 μM) and Gap 43 (100 μM) in combination, on spontaneous rhythmical activity. Incubation in the combined peptides, Gap 37 and Gap 43 hyperpolarized SMCs and abolished spontaneous depolarizations and contractions (A), inhibited calcium oscillations in the arterial wall (B) and in individual SMCs (C). Increases in [Ca^{2+}]_{i} are represented by an upward deflection. In control, the representative basal calcium level in the arterial wall was 0.53 F_{340/380}.
Control Gap 37/Gap 43 (100 μM)

A. Membrane potential

-35 mV

Diameter

70 μm

B. Wall calcium

0.1

340/380

Diameter

74 μm

C. SMC calcium (n=6 cells)

0.1

340/380

10 s
Figure 6.14
Effect of disrupting Cx 40 on spontaneous, rhythmical activity. The synthetic mimetic peptide, Gap 40 (100 μM), reduced large rhythmical depolarizations, coordinated contraction (A) and calcium oscillations in the arterial wall (B). \([\text{Ca}^{2+}]_i\) oscillations recorded in individual, adjacent SMCs were reduced in amplitude and became asynchronous (C). Increases in \([\text{Ca}^{2+}]_i\) are represented by an upward deflection. The representative basal calcium level in control is 0.64 F_{340/380}. 
however no effects were observed on basal calcium levels (Fig 6.14B; Gap 40: 110.4 ± 9.6 % F$_{340/380}$). Gap 40 caused a significant vasodilatation (Control: 70.7 ± 6.8 μm, Gap 40: 79.3 ± 6.4 μm; n = 8; P<0.05, paired t-test) and imaging of individual smooth muscle cells showed that small calcium oscillations were present in individual cells, but that these were unsynchronized (Fig 6.14C). The frequency (Fig 6.15A; P<0.05, unpaired t-test) and amplitude (Fig 6.15B; P<0.05, unpaired t-test) of these unsynchronized oscillations was reduced. Furthermore, the ratio of the amplitude of [Ca$^{2+}$], oscillations in individual SMCs, to the amplitude of the [Ca$^{2+}$], oscillations in the arterial wall was significantly increased (Fig 6.15C). Effects were observed with 10 min of the peptide entering the recording chamber.

**6.2.3.5 Effect of Gap 45.**

In three out of 8 preparations, Gap 45 (100 μM), homologous for amino acid sequences 203-216 of Cx45, completely abolished the spontaneous activity recorded in the juvenile rat basilar artery (Fig 6.16A; P<0.05, paired t-test). In the remaining 5 preparations, Gap 45 caused a substantial reduction in the frequency (Fig 6.16B; Control: 17.9 ± 0.3 min$^{-1}$, Gap 45: 5.7 ± 1.7 min$^{-1}$; n = 5; P<0.05, paired t-test) and amplitude (Control: 4.2 ± 0.7 % RVD, Gap 45: 1.0 ± 0.4 % RVD; n = 5; P<0.05, paired t-test) of rhythmical contractions, depolarizations (Control: 5.5 ± 1.0 mV; Gap 45: 1.1 ± 0.8 mV, n = 2) and calcium oscillations (Gap 45: 25.3 % amplitude of control F$_{340/380}$ oscillation, n = 3) recorded in the arterial wall. In both cases, Gap 45 hyperpolarized (Fig 6.16A; Control: -39.3 ± 2.8 mV; -48.3 ± 2.9 mV; n = 4; P<0.05, paired t-test) and relaxed the smooth muscle (Fig 6.16A and B; Control: 61.6 ± 5.1 μm, Gap 45: -48.3 ± 2.9 mV; n = 8; P<0.05, paired t-test). Basal calcium levels in the arterial wall were not significantly altered (Fig 6.16B; Gap 45: 101.4 ± 1.3 % F$_{340/380}$; n = 4) and in those preparations from which activity could still be recorded, imaging revealed the presence of small calcium oscillations in adjacent smooth muscle cells (Fig 6.16C).

**6.3 DISCUSSION**

Vasomotion has been abolished by the addition of a variety of different Cx mimetic peptides. While some of these effects may confirm the involvement of cellular coupling and specific Cx subtypes in this phenomenon, other observations suggest that there may be non-specific actions of these peptides.
Figure 6.15
Figure 6.15

Effect of Gap 40 (100 μM) on rhythmical \([Ca^{2+}]_i\) oscillations. Gap 40 reduced the frequency and amplitude of calcium oscillations recorded from adjacent SMCs. Points represent individual SMCs, whereby 6 adjacent cells were analyzed from 4 different preparations and the cell with the frequency or greatest amplitude designated as 100% in each preparation. The absolute frequency and amplitude of SMC \([Ca^{2+}]_i\) oscillations in control was 17.9 ± 1.9 min\(^{-1}\) and 0.06 ± 0.003 F\(_{340/380}\). Straight lines indicate mean values. * significantly different from control.

C. The ratio of the amplitude of individual SMC calcium oscillations to the amplitude of the calcium oscillations in the arterial wall was significantly increased in the presence of Gap 40. Values represent mean and S.E.M. * significantly different from control.
A. Smooth muscle cell

B. Amp. of [Ca^{2+}] oscillation % of max. amplitude

C. SMC wall calcium oscillation amplitude

Control | Gap 40 (100 μM)

* indicates statistical significance
Figure 6.16

Effect on Gap 45 (100 µM) on spontaneous, rhythmical activity. A, Gap 45, generated to specific amino acid sequences against Cx45, abolished rhythmical depolarizations, hyperpolarized and relaxed the SMCs. Gap 45 typically abolished rhythmical contractions, however in some preparations small, irregular contractions and calcium oscillations in the arterial wall could be recorded. Under these conditions, imaging revealed small calcium oscillations in adjacent SMCs. Increases in [Ca²⁺]ᵢ are represented by an upward deflection. The representative control basal calcium level in the arterial wall is 0.61 F₃₄₀/₃₈₀.
6.3.1 $^{37,43}$Gap 27 and Gap 37 and 43 in combination abolish vasomotion

Incubation in the Cx mimetic peptide $^{37,43}$Gap 27, incorporating the SRPTEK amino acid sequence which is involved in Cx docking through the second extracellular loop of Cx37 and 43, or the combined peptides Gap 37 and Gap 43, incorporating the sequences PCPH also located in the second extracellular loop of Cx37 and 43 respectively, abolished vasomotion. This suggests that functional cellular communication involving Cx37 and 43 is essential to the development of this phenomenon in the juvenile rat basilar artery. Such observations are consistent with the findings of Chaytor et al., (1997), who showed that $^{37,43}$Gap 27 reversibly inhibited agonist induced vasomotion in the rabbit mesenteric artery. The specificity of $^{37,43}$Gap27 has previously been assessed in rabbit arteries, where a control peptide with sequence homology to the intracellular loop of Cx43 was shown to be inactive (Chaytor et al., 1998) and in confluent COS fibroblasts where $^{37,43}$Gap 27 was shown to inhibit intracellular dye transfer in cells expressing Cx43 (Chaytor et al., 1999).

Surprisingly, $^{37,43}$Gap 27 abolished calcium oscillations in individual smooth muscle cells and hyperpolarized and relaxed the arteries. While $^{37,43}$Gap 27 has been demonstrated to inhibit the propagation of calcium waves in cultured tracheal epithelial cells (Boitano & Evans, 2000) calcium waves have not been seen in the basilar artery suggesting that neither calcium nor IP$_3$ pass through gap junctions. On the other hand, the effects of $^{37,43}$Gap 27 were largely abolished by removal of the endothelium, indicating that the primary site of action was at MEGJs or endothelial gap junctions. Consistent with these observations, we have demonstrated expression of both Cx37 and 43 in the endothelial cell layer of the rat basilar artery (see chapter 5). Thus it would be expected that the effect of $^{37,43}$Gap 27 would be mimicked by removal of the endothelium. However this was not the case as endothelial removal desynchronized calcium oscillations in individual smooth muscle cells without affecting basal tone. $^{37,43}$Gap 27 may therefore be acting in a non-specific manner, for example, to promote the release of an endothelium derived vasodilatory factor or to induce endothelial hyperpolarization. Such an action would be inconsistent with previous studies that gap peptides have no effect on NO release (Chaytor et al., 1998; Sandow et al., 2003a; Karagiannis et al., 2004), while they block EDHF activity in a variety of vascular beds (Chaytor et al., 2001; Taylor et al., 2001; Berman et al., 2002; Griffith et al., 2002; Sandow et al., 2002; Sandow et al., 2003b; Sandow et al., 2004). While there appears to be no clear cut explanation for the actions of $^{37,43}$Gap 27 in abolishing calcium oscillations and decreasing vessel tone, the same effects were seen with the combination of Gap 37 and Gap
43 which are targeted to different sequences in the extracellular loops. It is interesting that the gap junction inhibitor heptanol also hyperpolarized membrane potential, decreased basal calcium levels and reduced tone of rhythmically active rat small mesenteric arteries (Matchkov et al., 2004b). These effects were attributed to non-specific activation of potassium channels as well as direct inhibition of calcium channels (Matchkov et al., 2004b). In the present study, there was a further effect of 37,43Gap 27 directly in the smooth muscle since calcium oscillations in smooth muscle cells were decreased in endothelium denuded preparations. While this is consistent with the detection of Cx37 protein in the media, non-specific effects on intracellular calcium stores cannot be ruled out.

6.3.2 Role of Cx43 in spontaneous vasomotion

In order to further investigate the role of Cx43 in intercellular communication during vasomotion, we have compared the inhibitory action of the connexin mimetic peptides 43Gap 26 and Gap 43, containing the SHVR and PCPH amino acid sequences important for docking of the first and second extracellular loops of Cx43, respectively (Chaytor et al., 1997; Kwak & Jongsma, 1999). 43Gap 26 has previously been reported to inhibit agonist induced vasomotion in the rat mesenteric artery (Chaytor et al., 1997) and has been shown to selectively inhibit dye transfer between HeLa cells transfected with Cx43 (Berman et al., 2002). In the present study, both peptides designed against Cx43 abolished rhythmical depolarizations, calcium oscillations and contractions. When taken together with the data obtained in 37,43Gap 27, these results indicate a critical role of Cx43 in the development of coordinated rhythmic contractions of blood vessels. It is interesting that studies of arteries from Cx43 heterozygote deficient mice have correlated decreased Cx43 expression with a decrease in the incidence of rhythmical activity (Slovut et al., 2004).

Like 37,43Gap 27, 43Gap 26 and Gap 43 both decreased basal calcium levels in the arterial wall, decreased vessel tone and hyperpolarized the smooth muscle. This is in direct contrast to findings in rabbit arteries and veins where the effects of 43Gap26 and 37,43Gap 27 were shown to be independent of the regulation of vessel tone (Chaytor et al., 1998; Taylor et al., 1998; Griffith & Taylor, 1999). The effects of 43Gap 26 or Gap 43 are unlikely to be due to the loss of signaling molecules moving between smooth muscle cells through gap junctions, since immunohistochemistry has revealed that Cx43 is absent from the media of the juvenile rat basilar artery. On the other hand, we have been able to identify a number of
cells demonstrating high expression of Cx43 located within the smooth muscle layers, with anatomical characteristics similar to interstitial cells of Cajal (Cousins et al., 2003). Recently, interstitial like cells have been isolated from the rabbit portal vein where they are suggested to act as specific pacemakers to drive rhythmical contractions in this vessel (Povstyan et al., 2003). It is therefore possible that peptides designed to target the extracellular loops of Cx43 can disrupt the cellular function of these cells, which in turn could account for the loss of rhythmical activity in the presence of $^{43}$Gap 26, Gap 43 and $^{37,43}$Gap 27.

6.3.3 Cx40 in the endothelium synchronizes spontaneous rhythmical activity

In the present study the gap peptides, $^{40}$Gap 27, containing the SRPTEK amino acid motif, and Gap 40, containing the PCPH amino acid sequence present in the second extracellular loop of Cx40 were used to investigate the role of Cx40. Both $^{40}$Gap 27 and Gap 40 had similar effects in that they significantly reduced the large rhythmical depolarizations and calcium oscillations in the arterial wall and produced small and irregular contractions and unsynchronized calcium oscillations in individual smooth muscle cells. These findings are similar to the effects of endothelial removal and also to the spontaneous and irregular vasomotion previously reported in cremaster muscle arterioles in Cx40-deficient mice (de Wit et al., 2000). Since we found strong expression of Cx40 only in the endothelium, we suggest that Cx40 located in the endothelium is essential for coordination of cells in the smooth muscle layer. This data supports our hypothesis that current generated in the smooth muscle passes through MEGJs, to the endothelium where it is rapidly conducted along the vessel wall, and back to the smooth muscle in order to synchronize rhythmical events.

Surprisingly, in the absence of endothelium, $^{40}$Gap 27 caused the irregular activity to regain rhythmicity and the small uncoordinated calcium oscillations in individual smooth muscle cells to become synchronized. These data suggest that $^{40}$Gap 27 increased cell coupling within the media of the juvenile rat basilar artery. Our assessment of cell coupling based on the ratio of amplitude of individual smooth muscle cells to wall calcium also supported the idea of an increase in cell coupling. Paradoxically, smooth muscle cell input resistance was increased consistent with a decrease in cell coupling. As we did not find any evidence for Cx40 in the media and the gap peptides would be expected to decrease coupling, the synchronizing effects of $^{40}$Gap 27 may suggest a non-selective action.
6.3.4 Role of Cx37 in spontaneous vasomotion

In the present study we have developed a peptide containing the amino acid sequence PCPH involved in docking of the second extracellular loop of Cx37, as the Gap 27 and Gap 26 amino acid sequences are not selective for Cx37. In contrast to the mimetic peptides used to inhibit gap junctional communication involving Cx43, we have been able to record small rhythmical depolarizations and reduced calcium oscillations in individual smooth muscle cells in the presence of Gap 37. Since the effects of Gap 37 were similar to those of Gap 27 or endothelial denudation we suggest that the effects of Gap 37 are mediated through Cx37 in the endothelium. The results of the present study appear to correlate with the observations of Lagaud et al., (2002) in cerebral vessels of the rat, in which inhibitors of gap junctions were shown to increase the number of smooth muscle cells exhibiting oscillations in membrane potential. The reason for this anomaly is unclear.

6.3.5 Role of Cx45 in spontaneous vasomotion

Previous studies using Gap 45 have demonstrated functional Cx45 gap junction channels in isolated rat basilar artery smooth muscle cells (Li & Simard, 2001) and our immunohistochemical studies have shown Cx45 expression in the media (see chapter 5). Our finding that mimetic peptides Gap27 or Gap 45, corresponding to different amino acids in the second extracellular loop of Cx45, abolished rhythmical activity in the juvenile rat basilar artery would further support a role for working Cx45 channels in rat cerebral vessels. However, the peptides to Cx45 also hyperpolarized and relaxed the arteries and decreased calcium oscillations in individual smooth muscle cells. These effects suggest non-selective actions of peptides on intracellular IP3 stores.

6.3.6 Conclusions

In conclusion, we have shown that short synthetic peptides, designed to target specific amino acid sequences in the extracellular loops of the vascular connexins can rapidly and reversibly inhibit rhythmical activity in the basilar artery. However we found additional effects on intracellular calcium oscillations in smooth muscle cells, membrane potential and vessel tone apparently unrelated to effects on gap junctions. While these latter effects suggest that the peptides have non-specific actions at intracellular calcium stores, results obtained with peptides against different extracellular sequences of the same connexin were remarkably consistent. Additional studies are therefore required to
determine whether there may be a functional interaction between the conductance of gap
ejunctions and the activity of intracellular calcium stores.
CHAPTER 7

GENERAL DISCUSSION

In order to maintain homeostasis, the circulatory system must ensure an adequate supply of oxygen, nutrients and other essential substances to bodily organs and the removal of potentially harmful waste products through alterations in regional blood flow. In this regard, blood flow is determined by the contractile activity, or vascular tone of the smooth muscles cells that lie within the vessel wall (Rossitti et al., 1995). Contractile events in vascular smooth muscle depend on an increase in [Ca$^{2+}$]_i and activation of the contractile apparatus. Since calcium influx through voltage dependent calcium channels, following changes in membrane potential, and calcium release from intracellular stores, following activation of intracellular second messenger pathways, are the major trigger source of calcium, mechanisms underlying smooth muscle contraction have been interpreted as voltage dependent, voltage independent or relying on both voltage dependent and independent events (Hirst & Edwards, 1989; Jackson, 2000). Not surprisingly, rhythmical contractions or vasomotion have also been described to result from three different mechanisms, one based entirely on voltage dependent events (Fujii et al., 1990a; Omote & Mizusawa, 1995), one involving entirely voltage independent intracellular mechanisms (Hill et al., 1999) and the other relying on both voltage dependent and independent events (Gustafsson & Nilsson, 1993). A growing body of literature would also suggest that in many vascular beds, such events can be influenced by endothelial derived vasoactive factors (see chapter 1). At present however, the precise connections between these components in the generation of vasomotion is not clear. Nevertheless, it is evident that functional cellular communication, via gap junctions is necessary for the integration, modulation and coordination of these different Ca$^{2+}$ signals in order for vasomotion to occur (Christ et al., 1996). Thus it becomes increasing clear that the mechanisms underlying vasomotion between different vascular beds may indeed be heterogeneous. Although it can be postulated that such heterogeneity may be due to variations in the distribution and interaction of membrane receptors, ion channels and the production/ and or diffusion of second messengers, it remains essential to find common underlying principles in the generation of this phenomenon so that potential therapeutic targets and treatments of disorders of blood flow can be found.
7.1 A comparative study of vasomotion

This thesis has examined the mechanisms underlying different models of vasomotion, one relying on voltage independent mechanisms and the other on voltage dependent events in two apparently diverse vascular beds, arterioles of the iris and the basilar artery and its major branches. Results obtained in the present study would suggest that variations must exist in the expression of different ion channels and intracellular signaling mechanisms between these vessels however similarities also exist.

A major similarity in the mechanisms underlying spontaneous vasomotion in the rat iris arteriole and basilar artery preparations is the dependence of vasomotion on the release of Ca$^{2+}$ from the IP$_3$ sensitive intracellular store. In both vascular beds, inhibition of the PLC pathway with U73122 was shown to abolish rhythmical activity, hyperpolarize the membrane potential and increase vessel diameter. The importance of intracellular Ca$^{2+}$ release was confirmed using the IP$_3$ intracellular Ca$^{2+}$ chelator BAPTA-AM and the Ca$^{2+}$-ATPase inhibitor, thapsigargin. A role for IP$_3$ sensitive Ca$^{2+}$ release during agonist induced vasomotion has also been described in the mesenteric vascular bed (Lamont & Wier, 2004).

Another major similarity between the iris arteriole and basilar artery preparations is that Ca$^{2+}$ released from the IP$_3$ sensitive intracellular Ca$^{2+}$ store is responsible for the activation of a depolarizing current. In both chapter 3 and chapter 4, the identity of this channel was suggested to be a Cl$_{Ca}$ channel. The presence of this channel in the rat basilar artery was confirmed using the putative Cl$_{Ca}$ channel inhibitor niflumic acid (Hogg et al., 1994b; Criddle et al., 1997). On the other hand, another Cl$_{Ca}$ channel inhibitor, DIDS was shown to have additional effects on intracellular Ca$^{2+}$ stores within the smooth muscle of both vessels. A recent study of vasomotion in the rat mesenteric artery has reported that membrane depolarization occurs following the activation of a cGMP dependent Cl$_{Ca}$, as a consequence of NO released from the endothelium. In the present study however, it is unlikely that this channel is present in the rat iris arteriole, since vasomotion occurred independently of the endothelium. Given that NO had an inhibitory effect on rhythmical activity in the rat basilar artery, a similar conclusion regarding the lack of involvement of this channel in the development of vasomotion was reached. In both vessels then, NO played a negative modulatory role only. These results were consistent with previous studies of vasomotion in the cerebral circulation and in arterioles of the hamster (Dirnagl et al., 1993; Bertuglia et al., 1994; Hempelmann et al., 1998). Much confusion in the literature
still exists regarding the role of NO in vasomotion in vessels of the systemic circulation (see chapter 1).

In contrast to the foregoing discussion, major differences must exist in the receptor/ion channel expression between the iris arteriole and basilar artery preparations. The most negative membrane potential recorded in the iris arteriole was significantly more hyperpolarized than that of the basilar artery and the form and frequency of rhythmical contractions were also quite dissimilar. Perhaps the most fundamental difference between the two models of vasomotion is the involvement or lack of, VDCCs in the generation of rhythmical contractions. In this regard, the iris arteriole appears to be unique. In the basilar artery, like in the mesenteric artery, the simultaneous activation of the VDCCs and influx of extracellular Ca\(^{2+}\) is critical to the development of coordinated contractions (see chapter 4, Peng et al., 2001). One striking difference between vessels of the systemic and cerebral circulation however, is the effect of VDCC channel inhibition in the maintenance of vascular tone. Thus mesenteric arteries have been reported to relax in the presence of nifedipine (Gustafsson & Nilsson, 1993), while in the basilar artery nifedipine had no effect on vascular tone (see chapter 4). It can be postulated that these differences may be due to the relative distribution of the VDCCs between the two arteries, whereby in the mesenteric artery, calcium that has entered the cell is more readily accessible to the contractile apparatus and thus makes a greater contribution to the maintenance of vascular tone.

The involvement of the ry sensitive intracellular stores in vasomotion also varied substantially between the different arterial preparations. In the rat iris arteriole, ryanodine had no effect on spontaneous contractions (see chapter 3) while in the basilar artery Ca\(^{2+}\) released from the ry sensitive intracellular store was involved in the negative feedback loop of the oscillatory cycle by activating a hyperpolarizing current. In direct contrast, Ca\(^{2+}\) released from the ry sensitive intracellular store in the mesenteric artery has been reported to be involved in the activation of a depolarizing current responsible for initiating vasomotion in these vessels (Peng et al., 2001).

Another difference in the mechanisms underlying vasomotion in the iris arteriole and rat basilar artery relates to the role of additional intracellular pathways. In the iris arteriole, PKC or metabolites of the lipoxygenase arm of the arachidonic acid signaling pathway have been shown to be essential to the development of rhythmical contractions. In contrast, no significant effects were observed on rhythmical activity in the basilar artery following incubation in the PLA\(_2\) inhibitor, AACOCF\(_3\) (see chapter 4) or in the mesenteric
artery following inhibition of metabolites of this pathway (Okazaki et al., 2003; Mauban & Wier, 2004). In a similar fashion, PKC activity was shown to have a modulatory effect on vasomotion in the basilar artery, rather than an essential role as described for the iris arteriole.

Studies of vasomotion in endothelium denuded irideal arterioles and basilar artery preparations would suggest two very different roles for this cellular layer between the two preparations. Thus in the iris arterioles vasomotion was endothelium independent, while in the basilar artery vasomotion was endothelium dependent. It was suggested that the principle activity of the endothelium in the latter artery was to facilitate the spread of current along the vascular wall in order that Ca\(^{2+}\) fluxes through VDCCs occurred simultaneously in adjacent SMCs (see chapter 5). At present the role of the endothelium in vasomotion in vessels of the systemic circulation appears somewhat ambiguous (see chapter 1 and chapter 5).

In conclusion, it is evident that while some similarities exist in the mechanisms underlying vasomotion in different vascular beds, a vast number of differences exist to account for the disparity of voltage dependency between the iris arteriole and basilar artery preparations. These variations may be due to the differential expression and localization of different ion channels and receptors involved in intracellular signaling pathways.

7.2 Vasomotion in the basilar artery: a working model

Recently, Peng et al., (2001) proposed a model to explain agonist induced vasomotion in the rat mesenteric artery. In this vascular bed, calcium released from intracellular stores occurs in a cyclical manner, activating an endothelium dependent depolarizing inward current across the smooth muscle plasma membrane, generating an oscillating membrane potential. Thus vasomotion is thought to occur when a sufficient number of smooth muscle cells are activated and depolarization spreads via gap junctions to synchronize calcium influx through VDCCs across the vascular wall (Peng et al., 2001). The synchronized activation of membrane ion channels is thought to further entrain the release of calcium from intracellular stores and coupling of the oscillating smooth muscle cells is strengthened (Suzuki & Hirst, 1999; Van Helden et al., 2000; Peng et al., 2001). Such a model then, relies on unsynchronized [Ca\(^{2+}\)]\(_i\) waves to provide a pacemaking mechanism for the initiation of vasomotion in this vessel (Peng et al., 2001).
In chapter 4, a similar model was proposed to explain the presence of spontaneous vasomotion in the rat basilar artery preparation, whereby the oscillations in membrane potential were suggested to be a consequence of cyclical release of calcium from the IP$_3$ sensitive intracellular calcium store and the activation of calcium dependent chloride channels in the SMC membrane. However, this model is not entirely supported by data obtained in this thesis, since it cannot explain the observation that the L-type VDCC inhibitor nifedipine abolished rhythmical depolarizations. If the membrane potential oscillations in individual smooth muscle cells were indeed the consequence of cyclical release from the IP$_3$ sensitive intracellular calcium store and the subsequent activation of a depolarizing current in the cell membrane, it should be possible to record rhythmical depolarizations from individual SMCs following inhibition of VDCCs. To the best of this author’s knowledge, there is no evidence in the literature examining the effect of VDCC inhibition on membrane potential changes in the rat mesenteric artery during vasomotion.

An alternative model instead relies on the cyclical interaction between membrane ion channels and the Ry sensitive intracellular store. In this model, Ca$^{2+}$ from the IP$_3$ sensitive intracellular store is able to activate a depolarizing current which is not cyclical. This would account for the quiescent membrane potential observed in the presence of nifedipine. Rhythmical depolarizations are subsequently generated following the influx of extracellular calcium through VDCCs, activation and release of calcium from the Ry sensitive intracellular Ca$^{2+}$ store. The resulting activation of IK$_{\text{Ca}}$ channels provides an inhibitory hypolarizing feedback loop which closes the VDCCs in preparation for the cycle to begin again. By definition then, spontaneous vasomotion in the juvenile rat basilar artery constitutes a model of a voltage dependent, membrane bound oscillating system (Berridge & Galione, 1988; Gustafsson & Nilsson, 1993).

The oscillating system that underlies vasomotion in the cerebral vascular bed however, remains complex. In chapter 4, it was demonstrated that Ca$^{2+}$ released from the IP$_3$ sensitive intracellular Ca$^{2+}$ store appears not only a necessary component for the generation of vasomotion, but also for the maintenance of vascular tone. Therefore, it would seem likely that vasomotion in the cerebral vascular bed is a consequence of membrane potential oscillations superimposed on a basal level of vascular tone. By implication this finding suggests that the basal [Ca$^{2+}$]$_i$ levels and membrane potential must be primed in order for rhythmical contractions to occur. A recent study in the rat mesenteric artery has also demonstrated that basal [Ca$^{2+}$]$_i$ within smooth muscle cells must reach a
threshold level in order for oscillations to occur (Schuster et al., 2004). Since we were able to detect the presence of Ca\(^{2+}\) waves in the presence of nifedipine, but not following exposure to ryanodine (see chapter 4), data would suggest that Ca\(^{2+}\) released from the Ry sensitive intracellular store must also play an important role in augmenting this phenomenon. Based on the above discussion then, it is apparent that dynamic interaction between the membrane voltage oscillations and [Ca\(^{2+}\)]\(_i\) events controlled by the intracellular stores are necessary for the generation of vasomotion in this artery. Thus according to the data presented in this thesis, we can propose the following hypothetical model examining the ionic and intracellular events underlying spontaneous vasomotion in the juvenile rat basilar artery (Fig 7.1).

In this model, constitutive activity of the PLC pathway leads to the production of IP\(_3\) and release of Ca\(^{2+}\) from the IP\(_3\) sensitive intracellular store following activation of IP\(_3\)R channels within the smooth muscle. Release of Ca\(^{2+}\) from this store contributes to the generation of a basal level of vascular tone and formation of Ca\(^{2+}\) waves. Given that results demonstrate that IP\(_3\)R1 is the most abundantly expressed receptor subtype within the smooth muscle of this artery (see Appendix), it is likely that calcium release from this store occurs following the activation of IP\(_3\)R1. Ca\(^{2+}\) released from the IP\(_3\) intracellular store results in CICR from the Ry sensitive intracellular store, and formation of the Ca wave. Since recent mRNA studies in the juvenile rat basilar artery have demonstrated that RyR3 is the most abundantly expressed receptor subtype in this artery (personal communication, Dr H. Grayson), it can be hypothesised that RyR3 is critically involved in this regenerative process. Ca\(^{2+}\) released from the IP\(_3\) sensitive intracellular store must also activate a Cl\(_{Ca}\) channel which depolarizes the membrane potential and opens VDCCs. Ca\(^{2+}\) influx through the VDCCs further activates the IP\(_3\) sensitive intracellular store, amplifying the Ca\(^{2+}\) wave. On the other hand, calcium that has entered through VDCCs must also activate RyRs, providing synchronized Ca\(^{2+}\) for the activation of IK\(_{Ca}\) channels and subsequent membrane hyperpolarization. It is possible that these Ry sensitive intracellular stores may be functionally distinct from those contributing to the calcium wave. Since mRNA for RyR2 was also detected in this artery, different receptor isotypes may mediate these two events. Interestingly, recent immunohistochemical studies in the juvenile rat basilar artery, examining the distribution of IK\(_{Ca}\) channels within the vascular wall have demonstrated that IK\(_{Ca}\) channels are located within the smooth muscle and also at the medial side of the internal elastic lamina coincident with the holes through which endothelial cells project to
Figure 7.1
Figure 7.1

Calcium waves and integration of ionic mechanisms leading to vasomotion in the rat basilar artery. 1) Constitutive activity of phospholipase C (PLC) leads to the production of IP₃ and release of calcium (Ca²⁺) from the IP₃ sensitive intracellular Ca²⁺ store following activation of IP₃R1 channels within the SMCs. 2) Ca²⁺ induced Ca²⁺ release (CICR) from both IP₃R1 and ryanodine receptor channels (RyR) results in the formation of a Ca²⁺ wave along the cell. Ca²⁺ waves in adjacent cells are asynchronous. 3) Ca²⁺ from the IP₃ store then activates a Ca²⁺ dependent chloride channel (ClCa) which depolarizes the membrane potential and opens voltage dependent calcium channels (VDCC) causing extracellular Ca²⁺ influx into the cell. 4) The Ca²⁺ that has entered the cell through the VDCCs further activates the IP₃ sensitive intracellular Ca²⁺ store increasing the amplitude of the Ca²⁺ wave. 5) Ca²⁺ that has entered through VDCCs can also activate RyR2, causing Ca²⁺ release from the ryanodine sensitive store and 6) the subsequent activation of intermediate conductance calcium activated potassium (IKCa) channel causing the membrane to hyperpolarise. Activation of the VDCCs and RyR2 channels are essential to the membrane potential oscillation and electrical current passes through MEGJs to the endothelium. 7) Ca²⁺ released from intracellular Ca²⁺ stores in the SMCs may also pass through MEGJs to cause the additional activation of endothelial cell IKCa channels located near the MEGJ lying within the internal elastic lamina (IEL). 8) Membrane voltage oscillations are conducted along the endothelium. 9) Current then passes back into the SMCs to synchronize VDCC activity and CICR from RyR2s. Ca²⁺ signaling and membrane potential oscillations within the SMCs are entrained and vasomotion takes place. Closure of the VDCCs following SMC hyperpolarization ensures down regulation of the cycle in preparation for it to begin again.
form MEGJs with the inner layers of smooth muscle (personal communication, Ms K. Belfrage). Since the endothelium was shown to be essential in coordinating rhythmical events in adjacent smooth muscle cells, it is possible that Ca\textsuperscript{2+} released from intracellular stores in the SMCs may also pass through MEGJs, as has previously been described during vasomotion in the rat mesenteric artery (Schuster et al., 2001), to cause the additional activation of IK\textsubscript{Ca} channels located on endothelial projections. This phenomenon would augment conduction of electrical activity along the endothelium and amplify the electrical signal that passes back into the SMCs to synchronize VDCC activity and CICR from RyR2s. This proposal is consistent with experimental data obtained in chapter 5, in which removal of the endothelium was shown to reduce the amplitude of the membrane potential oscillations. Finally, Ca\textsuperscript{2+} signaling and membrane potential oscillations within the SMCs are entrained and vasomotion takes place. Closure of the VDCCs following SMC hyperpolarization ensures down regulation of the cycle in preparation for it to begin again.

Results from this thesis have also demonstrated that Ca\textsuperscript{2+} waves, initiated at one end of the cell and propagating to the other, were consistently recorded from the smooth muscle during spontaneous vasomotion (see chapter 4). This finding is contrary to the non-wavelike Ca\textsuperscript{2+} flashes described to occur during agonist induced vasomotion in the rat mesenteric artery (Peng et al., 2001; Sell et al., 2002; Lamboley et al., 2003; Mauban & Wier, 2004). In order to reconcile such differences, it can be suggested that in the juvenile rat basilar artery, receptor/ion channel distribution may be highly localized to specific regions of the SMC. This would allow both rapid voltage dependent and slower intracellular events to occur simultaneously. However, we did not find such a selective distribution of IP\textsubscript{3}R1 in vascular SMCs (see Appendix). On the other hand, due to the overall shape and cellular dimensions of vascular SMCs such a proposal may not be necessary. In general, SMCs have been described to be spindle shaped with tapered ends (Rhodin, 1967). This morphological characteristic would reduce the distance required for Ca\textsuperscript{2+} released from intracellular stores to reach membrane bound ion channels which are located at the ends of cells compared to those located near the middle. CICR events would therefore be expected to be initiated at the ends of the cells rather than in the middle.

7.3 Methodological limitations

As with any body of scientific work, it is important to make some remarks regarding the potential limitations of the techniques used in the collection of data, in order
to ensure that caution is employed when interpreting variations in results between different studies and appropriate conclusions are reached.

While the importance of performing *in vivo* experiments, or at the very least using preparations which are perfused and pressurized cannot be underestimated, the appearance, form and cessation of spontaneous contractions and the gradual increase in vascular tone observed in both the iris arteriole and basilar artery preparations used in this study were similar to those previously described in isolated pressurized arterioles (Duling *et al.*, 1981). Furthermore, the resting membrane potential recorded in the basilar artery was comparable to that observed in pressurized cerebral arteries (Knot & Nelson, 1998). Thus although caution must be exercised when extrapolating between models of vasomotion studied under different experimental protocols, it would seem likely that the cellular and ionic events reported to occur in these blood vessels closely mimics those events occurring under more physiologically relevant conditions.

Because of the technical difficulties involved in simultaneously measuring changes in membrane potential, \([Ca^{2+}]_i\) and mechanical activity, electrophysiological data were collected from different preparations than those used to measure \([Ca^{2+}]_i\) changes. However, for both sets of measurements, simultaneous changes in vessel diameter were recorded and these mechanical responses showed similar changes to pharmacological inhibitors, suggesting that the differences in methods did not account for the results. Future work to develop a standard technique whereby simultaneous electrophysiological, \([Ca^{2+}]_i\) and mechanical measurements are made is warranted.

In the present study, we were unable to detect the presence of Ca\(^{2+}\) sparks. Traditionally, calcium sparks are formed when a cluster of RyR channels are activated following Ca\(^{2+}\) influx through VDCCs, resulting in a highly localized increase in \([Ca^{2+}]_i\) (Nelson *et al.*, 1995). In smooth muscle tissues, these events have been shown to activate K\(_{Ca}\) channels in the cell membrane to elicit a transient hyperpolarizing current (Benham & Bolton, 1986). Ca\(^{2+}\) sparks appear abruptly, reaching peak amplitudes within 10 ms, and dissipating in another 20 ms (Fill & Copello, 2002; Wang *et al.*, 2004). Since in the present study changes in SMC \([Ca^{2+}]_i\) were imaged approximately every 250 ms, it is not surprising that we were unable to detect their presence. High speed, high resolution confocal microscopy needs to be employed to resolve this matter.

7.4 Implications for future research
The studies described in this thesis contribute to a better understanding of the events underlying the rhythmical contractions or vasomotion observed in two distinct vascular beds of the juvenile wistar rat, the iris arteriole and the basilar artery. However, some additional experiments as outlined below may assist in confirming the models proposed.

Iris arteriole:

Characterization of LOX metabolites

In order to determine exactly which metabolite is involved in initiating calcium release from the IP$_3$ sensitive intracellular calcium store, a more detailed study of the lipoxygenase arm of the arachidonic acid pathway is required.

Role of nifedipine insensitive VDCCs

Experiments should be performed using the nifedipine insensitive VDCC antagonist, mibefradil, in order to rule out a role of these channels in the oscillatory voltage changes.

Identity of the depolarizing current

Since the putative Cl$_{Ca}$ channel inhibitor DIDS was shown to affect [Ca$^{2+}$]$_i$ oscillations another Cl$_{Ca}$ channel inhibitor, such as niflumic acid, should be tested. It would also be of interest to examine the role of SOCs/ROCs in the development of the rhythmical depolarizations since calcium oscillations are coincident with these voltage changes and activity of these channels may be an alternate source of the depolarizing current.

Basilar artery:

Voltage dependence of intracellular stores/vasomotion

Since production of IP$_3$ has been suggested to be voltage dependent, it would be of interest to examine the effects of changing the membrane potential under current clamp conditions on rhythmical activity and vascular tone. Alternatively, the PLC inhibitor U73122 could be used to hyperpolarize the membrane potential and inhibit Ca$^{2+}$ release from the IP$_3$ intracellular store. Under current clamp conditions, the effects of a depolarizing current in the reestablishment of rhythmical activity and vascular tone could then be examined. Since nifedipine was shown to have no effect on vascular tone, the nifedipine insensitive VDCC channel inhibitor, mibefradil could also be used in order to rule out a
role for these channels in membrane depolarization and vasomotion. Likewise, the role of SOCs/ROCs should also be investigated.

Location of KCa channels

Responses attributed to EDHF are thought to arise following the activation of IKCa and SKCa channels located in the endothelium. Since vasomotion in the basilar artery is negatively modulated by EDHF and membrane potential oscillations are critically dependent on the activation of IKCa, the effects of the IKCa and SKCa channel inhibitors, charybdotoxin and apamin, on membrane potential should be examined in endothelium denuded preparations. This would enable us to further elucidate the role of the endothelium during rhythmic activity by determining the location of the KCa channels.

Cx mimetic peptides

Since Cx mimetic peptides were shown to have additional effects on mechanisms unrelated to gap junction channel inhibition, control experiments using peptides targeting amino acid sequences in the intracellular loops of connexins should be performed. Results in Chapter 6 suggest that effects of Gap 37 on rhythmical activity are mediated through Cx37 located in the endothelium. Control experiments examining the effects of Gap 37 on SMC input resistance in endothelium denuded arteries could be performed to rule out additional effects of this peptide in the smooth muscle. Effects of the Cx mimetic peptides 43Gap 26 and Gap 45 on cellular coupling should also be studied in endothelium denuded preparations. This would enable us to determine the specific effects of these peptides on Cxs located within the smooth muscle layer.
APPENDIX
DIFFERENTIAL EXPRESSION OF INOSITOL 1,4,5-TRISPHOSPHATE RECEPTOR SUBTYPES BETWEEN THE SMOOTH MUSCLE AND ENDOTHELIAL CELL LAYERS OF RAT ARTERIES

A.1 INTRODUCTION

Changes in intracellular calcium concentration ([Ca$^{2+}$]) control many fundamental cellular responses including transcriptional activation, cell differentiation and proliferation, hormone secretion, apoptosis and muscle contraction (Berridge et al., 2000). In blood vessels, contractile responses within smooth muscle cells can be mediated by influx of extracellular calcium through voltage dependent calcium channels, calcium-induced calcium release from ryanodine receptors or the release of calcium from inositol 1,4,5-trisphosphate (IP$_3$) receptor (IP$_3$R) stores located on the endoplasmic reticulum (ER) or sarcoplasmic reticulum (SR) (Hill et al., 2001; Bultynck et al., 2003). The latter occurs following activation of phospholipase C and the formation of IP$_3$ which binds to the IP$_3$R (Bultynck et al., 2003). Conversely, in endothelial cells, the increase in [Ca$^{2+}$] following activation of the IP$_3$ pathway leads to synthesis of nitric oxide and other vasodilatory factors that mediate relaxation of the surrounding smooth muscle (Hill et al., 2001; Ding, 2003).

There are 3 molecular subtypes of the IP$_3$R (Thrower et al., 2001; Bultynck et al., 2003) which have been shown to exhibit different binding affinities for IP$_3$ in the low nM range of calcium concentrations (IP$_3$R2 > IP$_3$R1 > IP$_3$R3) (Newton et al., 1994; Wojcikiewicz & Luo, 1998). Furthermore, IP$_3$R1 has been shown to exhibit [Ca$^{2+}$]-dependent inhibition whereas IP$_3$R2 and IP$_3$R3 do not (Hagar, 1998; Ramos-Franco et al., 2000). It has therefore been suggested that differences in the distribution and diversity of IP$_3$R subtypes, according to tissue, cell type or subcellular location as well as the ability to form heterotetrameric channel complexes, are responsible for different patterns of calcium release (Wojcikiewicz, 1995; Oberdorf et al., 1999; Hayashi et al., 2000; Leite et al., 2003; Morel et al., 2003; Inoue et al., 2004) ultimately resulting in different physiological outcomes.
To the best of our knowledge, the expression and distribution of IP$_3$R subtypes have not been examined in the cellular layers of intact blood vessels, including those from which calcium oscillations are commonly recorded, such as the mesenteric and basilar arteries (Peng et al., 2001; see chapters 3 and 4). A clear understanding of the expression and distribution of receptor subtypes within and between the vascular cell layers may enable IP$_3$R subtypes to be specifically assigned to particular responses enabling therapeutic interventions in cases where these responses are perturbed. Therefore, in the present study we have examined the mRNA expression and protein distribution for the IP$_3$R subtypes in the aorta, basilar and mesenteric arteries of the rat.

A.2 METHODS

All experiments were performed according to the guidelines approved by the Animal Experimentation Ethics Committee of the Australian National University.

A.2.1 Messenger RNA extraction and reverse transcription

Male Wistar rats aged from 14-17 days (juvenile) and from 9-13 weeks (adult) postnatal were anaesthetized with ether and decapitated. The thoracic aorta, basilar and mesenteric arteries were removed, immersed in RNAlater (Ambion) and extraneous material was carefully removed in cold phosphate-buffered saline (PBS). For every sample (n=3), basilar arteries were pooled from 12 juveniles or from 5 adults, mesenteric arteries were pooled from 5 juveniles or from 3 adults, and thoracic aorta was removed from 2 juveniles or 1 adult. Basilar artery preparations included major and minor side branches. Mesenteric artery preparations contained only primary and secondary branches supplying the upper ileum, but not the superior mesenteric artery. Tissues were snap frozen in liquid nitrogen, homogenized and processed using the RNeasy Mini Kit (Qiagen), including 30 min incubation with DNase, according to the manufacturer's instructions. Messenger RNA was reverse transcribed to cDNA (42°C 1h, 50°C 1h, 90°C 10 min) using oligo dT primers (500 ng 1$^{-1}$, Invitrogen) and Superscript II (200 U 1$^{-1}$, Invitrogen). For each sample, reactions from which either reverse transcriptase or RNA was omitted were run in parallel to control for contaminating DNA.
A.2.2 Quantitative PCR

Quantitative PCR was used to determine the absolute copy numbers of IP$_3$R1, IP$_3$R2, and IP$_3$R3 in mRNA extracted from the thoracic aorta, basilar and mesenteric arteries of Wistar rats. The copy numbers were obtained using standard curves constructed by amplification of known quantities of plasmid DNA containing a cloned PCR product insert corresponding to each IP$_3$R subtype. Specific primers were designed that were complementary to the published sequences for each subtype of the IP$_3$R as follows: IP$_3$R1 forward 5'-gagatgagctggctgaggttcaa-3' and reverse 5'-tgttgcctcttccagaagtgcga-3', IP$_3$R2 forward 5'-caaccagaccttgagagcttgac-3' and reverse 5'-ttgcccagaggttgtatgcactc-3', IP$_3$R3 forward 5'-agacccgctgctactatagaa-3' and reverse 5'-gtcaggaactggcagatggcaggt-3'. Each 25 µl PCR reaction mixture was prepared using the SYBR Green Core Reagents kit (Applied Biosystems) and contained 1 µl of each primer (final concentration 800 nM), 2 µl of 12.5 mM dNTPs, 3 µl of 25 mM MgCl$_2$, 0.125 µl of AmpliTaq Gold (5U µl$^{-1}$), 2.5 µl of 10 x SYBR Green buffer, 10.375 µl H$_2$O and 5 µl of template containing either 10 ng of cDNA or diluted plasmid DNA containing the relevant cloned PCR product. All samples were diluted in water containing tRNA (2 ng µl$^{-1}$). All reactions were performed in duplicate using the ABI Prism 7700 Sequence Detection System (Applied Biosystems) at 95°C for 10 min, and 40 cycles of 20 sec at 95°C, 20 sec at 65°C, 45 sec at 72°C. Every experiment included duplicate control reactions containing the corresponding untranscribed RNA sample, and water and the expression of all of the genes was determined in every sample. The integrity of each PCR reaction was checked using dissociation curve analysis after every reaction. The identity of each PCR product was confirmed by direct sequencing using the ABI 3730 Capillary Genetic Analyser at the Biomolecular Resource Facility, John Curtin School of Medical Research, ANU.

For each gene, the absolute mRNA concentration was determined by comparing the fluorescent signal at threshold (equal to 10 x S.D. of baseline fluorescence) to that generated by a standard curve using serial dilutions (from $\sim 10^5$ to 46 copies) of a known concentration of purified plasmid DNA containing the relevant cloned PCR product. The concentration of each IP$_3$R mRNA was normalized by comparison with 18S rRNA concentration, which was determined by using primers complementary to a region of the 5' end of the molecule and a standard curve using serial dilutions of plasmid DNA containing the cloned fragment of the 18S rRNA gene (Rummery et al., 2002a). Results were analyzed
for statistical significance (P<0.05) using one-way analysis of variance (ANOVA) and Student's t-test.

A.2.3 Immunohistochemistry

The presence of protein corresponding to each of the IP_3R subtypes was confirmed by staining wholemount preparations with rabbit antibodies that specifically recognize the 18 amino acid residues of the carboxy terminus of either IP_3R1 or IP_3R2 (Wojcikiewicz, 1995) or with a mouse monoclonal antibody that recognizes an epitope corresponding to residues 22-230 at the N terminal region of IP_3R3 (Oberdorf et al., 1999) (BD Transduction Labs). Wholemount preparations of the thoracic aorta, mesenteric artery and basilar artery were prepared from male Wistar rats aged 14-17 days and 10 weeks postnatal. Animals were anaesthetized with an intraperitoneal injection of 44 mg kg⁻¹ ketamine and 8 mg kg⁻¹ rompun and perfused via the right atrium at a pressure of 60 mmHg with saline (0.9 % NaCl) containing 0.1% NaNO₃, 5U ml⁻¹ heparin and 0.1% bovine serum albumin (BSA) followed by 2% paraformaldehyde in 0.1 M L⁻¹ sodium phosphate buffer for 10 min. Thoracic aorta, basilar, and mesenteric arteries were removed into PBS and pinned flat on Sylgard resin (Dow Corning). Vessels from adult rats and the thoracic aorta from juveniles was first cut open longitudinally before pinning.

Arteries were pre-incubated (1h, room temperature=RT) in 2% BSA and 0.2% Triton X-100 (Tx) in PBS, and then incubated for 2h at 37°C (or 37°C for 1h, then overnight at RT) with the primary IP_3R antibodies (1:50 rabbit anti- IP_3R1 and 1:10 rabbit anti- IP_3R2; 1:50 mouse anti- IP_3R3, BD Transduction Laboratories) prepared in 2% BSA and 0.2% Tx in PBS. After washing with PBS, the tissues were incubated (1h, RT) with Cy3-conjugated goat anti-rabbit IgG or Cy3-conjugated donkey anti-mouse IgG (1:100; Jackson Immunoresearch Laboratories) in 0.01% Tx in PBS. Tissues were washed with PBS and mounted in buffered glycerol. As a test of specificity, some samples were incubated in antibodies that had been pre-incubated in the presence of the appropriate immunizing peptide (10 μg ml⁻¹, 1h, RT).

For co-localization studies of the IP_3R with cellular components, basilar arteries from juvenile rats were used. IP_3R staining was detected with FITC-conjugated swine anti-rabbit immunoglobulins (1:40; DAKO) or FITC-conjugated goat anti-mouse IgG (1:40; Jackson Immunoresearch Laboratories), and actin was detected with phalloidin-Texas Red (1:40, 1h, RT; Molecular Probes) or nuclei were detected with propidium iodide (0.01%, 5
min, RT; Molecular Probes).

For co-localization studies of the IP$_3$R with cell membranes of endothelial cells, sheep anti-connexin40 (anti-Cx40) was used to highlight cellular perimeters. Preparations were pre-incubated (1h, RT) in 2% BSA and 0.2% Tx in PBS, and then placed in sheep anti-Cx40 (1:100, 2h, 37°C). The tissue was washed in PBS and incubated (1h, RT) in Cy3-conjugated donkey anti-sheep IgG (1:100; Jackson Immunoresearch Laboratories). Following further washes in PBS, preparations were incubated (1h, RT) in IP$_3$R antibodies, washed and detected with appropriate secondary antibodies conjugated with FITC as described above.

Preparations were examined using a confocal laser-scanning microscope (TCS 4D, Leica Instruments) with excitation from an argon/krypton laser and fitted with filters appropriate for the detection of Cy3 and FITC fluorescence. Optical sections were collected at 1 μm intervals (x 100 objective, 1.4 NA, pinhole: 21/255) in the z axis and digitized images were obtained with line averaging. Each series of images was recombined to create a single image incorporating all smooth muscle or endothelial cell labeling (ScanWare, Leica). Images were then converted to greyscale and inverted for clarity (Photoshop 6.0, Adobe). For co-localization studies, single frames were combined for the two fluorophores. Care was taken to maintain similar gain settings for comparisons of the same antibody staining between preparations. For each preparation, images were collected from at least 3 different fields and each artery was sampled from at least 3 different animals.

A.3 RESULTS

A.3.1 Differential mRNA expression for each IP$_3$R subtype

The DNA sequences of each PCR product matched the corresponding IP$_3$R subtype to which complementary primers were designed. To correct for variations in the overall efficiency of the procedure, the copy numbers for each subtype were normalized to the number of copies of 18S rRNA and were expressed as copies/10$^6$ copies of 18S rRNA (Fig.A.1A and B). These data show that within all arteries IP$_3$R1 was the predominantly expressed subtype (P<0.05). In juvenile rats (Fig. A.1A), IP$_3$R1 and IP$_3$R3 were expressed at a lower level in the thoracic aorta than in the mesenteric (P<0.001) or basilar arteries (P<0.05) while IP$_3$R2 was more highly expressed in the mesenteric artery (P<0.05). This contrasts with adult rats (Fig. A.1B) where IP$_3$R2 and IP$_3$R3 were expressed at a similar
Figure A.1

Expression of IP$_3$R subtype mRNA in the basilar and mesenteric arteries and thoracic aorta from juvenile and adult rats. Copy numbers for each IP$_3$R (mean ± SEM) are expressed per 10$^6$ copies of 18S rRNA (normalized copy number) for (A) 14-17 day, and (B) 9-13 week postnatal rats. Within each artery, the relative contribution of each IP$_3$R subtype to overall IP3R expression was calculated from normalized copy numbers (mean ± SEM) for (C) 14-17 day and, (D) 9-13 week post-natal rats. ThA: thoracic aorta; BA: basilar artery; MA: mesenteric artery. * indicates significant difference between arteries.
level in all 3 arteries but the expression of IP3R1 in the mesenteric artery was about half that of the thoracic aorta and the basilar artery (P<0.05). Compared with the juvenile, there was a substantial (>7-fold) increase in expression of IP3R1 in the adult thoracic aorta (P<0.001) which did not occur in the other arteries, and a substantial reduction in the expression of IP3R2 in the mesenteric artery of the adult (P<0.05) and in the expression of IP3R3 in the mesenteric artery and basilar arteries of the adult.

To clarify whether the differences in IP3R mRNA expression between each age group represented an alteration to the pattern of overall IP3R expression within each artery, the relative contribution to overall expression that was made by each subtype was calculated. IP3R1 comprised a greater proportion of overall IP3R expression in each of the arteries of adult rats than in juvenile rats (P<0.05, Fig. A.1C and D). This was accompanied by a reduction in the contribution of IP3R2 in the thoracic aorta (P<0.01) and IP3R3 in the basilar artery (P<0.05) compared to the juvenile rats. Within the juvenile rats, IP3R2 was proportionately lower in the basilar artery than the mesenteric artery (P<0.05) and thoracic aorta (P<0.001).

A.3.2 Differential protein expression for each IP3R subtype

A.3.2.1 Distribution of IP3R1.

IP3R1 was highly expressed in the smooth muscle and in the endothelium in each of the 3 arteries in both juvenile and adult rats. In the endothelial cells of all arteries, IP3R1 staining appeared as elongated structures comprised of distinct punctate regions, although this staining did not appear to be localized to the perimeter of individual cells (Fig. A.2A-A.7A). Staining within the smooth muscle cells was also arranged in a linear fashion. (Fig. A.2B-A.7B). Images obtained at the vessel edge from small branching arteries of the basilar (Inset: Fig. A.2B) and mesenteric arteries (Inset: Fig. A.4B) showed IP3R1 staining in close proximity to the smooth muscle cell membrane. In these smaller vessels, it was also possible to see that central regions of the cells were devoid of staining (Fig. A.2B, A.4B, insets). A clear demarcation was seen between the two cell layers with regard to the orientation of the immunostaining, being in line with the vessel axis in the endothelium and perpendicular to the vessel axis in the smooth muscle (Fig. A.2A,B-A.7A,B).

A.3.2.2 Distribution of IP3R2.

In juvenile and adult rats IP3R2 was almost entirely restricted to the endothelium of each of the 3 arteries (Fig. A.2-A.7C), except for some weak punctate staining observed in
Figure A.2
Confocal images showing immunostaining of IP$_3$R in the basilar artery from animals aged 14-17 days postnatal. Cy3-conjugated secondary antibodies were used and greyscale confocal images inverted to black on white. All three IP$_3$R subtypes were observed in the endothelium (A, C, E; IP$_3$R1-3, respectively). IP$_3$R1 (B) and weak expression of IP$_3$R2 (D) were detected in the smooth muscle, however IP$_3$R3 appeared to be absent from the media (F). Inset panel B: IP$_3$R1 at the smooth muscle cell periphery in a small branch of the artery (scale bar: 10 µm). Arrow indicates region devoid of staining. Vessel axis runs left to right.
the media of the basilar artery of both juvenile and adult rats (Fig. A.2D and A.3D). In comparison with the adult, the endothelial cell staining was noticeably stronger in each of the arteries of juvenile animals (Fig. A.2C, A.4C and A.6C, compare A.3C, 5C and 7C). In addition, the pattern of staining appeared to change during development, from a predominantly linear form parallel to the endothelial cell and vessel axis, to a predominantly punctate form.

In adult arteries the staining did not lie close to the cell membrane since there appeared to be areas devoid of staining between adjacent cells (Fig. A.3C, A.5C and A.7C). In the juvenile however, the relationship of IP$_3$R2 with the endothelial cell membrane, was difficult to discern, particularly in the basilar and mesenteric arteries, while in the thoracic aorta clear spaces existed between the individual cells (Fig. A.6C).

**A.3.2.3 Distribution of IP$_3$R3.**

In arteries of the juvenile rats IP$_3$R3 was limited to the endothelium (Fig. A.2E, A.4E and A.6E) in the 3 arteries tested. The staining pattern was generally similar to that for IP$_3$R2, but punctate staining was more prevalent in the thoracic aorta and mesenteric arteries than in the basilar artery (Fig. A.4E and A.6E). Areas devoid of staining could be clearly seen between adjacent cells, particularly in the basilar artery (Fig. A.2E) and thoracic aorta (Fig. A.6E). In adult animals the endothelial cell staining was less intense and more punctate, especially in the thoracic aorta and mesenteric arteries (Fig. A.3E, A.5E and A.7E). Areas devoid of staining could be clearly seen between adjacent cells. Some sparse punctate staining of the media was apparent in the basilar and mesenteric arteries of adult rats (Fig. A.3F and A.5F), but not in the juvenile vessels.

**A.3.3 Antibody specificity**

The specificity of the rabbit antibodies to IP$_3$R1 (Fig. A.8A and C) and IP$_3$R2 (Fig. A.8E and G) was confirmed by peptide block, which completely abolished the staining in both the endothelial (Fig. A.8B and F) and smooth muscle cell (Fig. A.8D and H) layers in the adult rat basilar artery. It was not possible to test the specificity of the monoclonal antibody in the same manner because the immunizing peptide is not commercially available. However, the latter antibody is reported to be specific for IP$_3$R3 (Oberdorf et al., 1999) and recognizes a single band of ~300 kDa in Western blots of HeLa cell lysates (BD Transduction Laboratories).
Figure A.3
Figure A.3
Confocal images showing immunostaining of IP$_3$R in the basilar artery from animals aged 10 weeks postnatal. Cy3-conjugated secondary antibodies were used and greyscale confocal images inverted to black on white. IP$_3$R1 is strongly stained in the endothelium (A) and smooth muscle (B). In addition, IP$_3$R2 and IP$_3$R3 are present in the endothelium (C, E) and are also weakly detected in the smooth muscle. Vessel axis runs left to right.
Figure A.4
Figure A.4
Confocal images showing immunostaining of IP₃R in the mesenteric artery from rats aged 14-17 days postnatal. Cy3-conjugated secondary antibodies were used and greyscale confocal images inverted to black on white. IP₃R1 is present in the endothelium (A) and smooth muscle (B) while IP₃R2 and IP₃R3 are present in the endothelium (C, E) but not the smooth muscle (D, F). Inset: IP₃R1 was present close to the periphery of smooth muscle cells in a small branch of the artery (scale bar: 8 μm). Arrow indicates region devoid of staining. Vessel axis runs left to right.
Endothelium

IP₃R1

IP₃R2

IP₃R3

Smooth Muscle

A

B

C

D

E

F

25 μm
Figure A.5
Figure A.5

IP$_3$R immunostaining of the mesenteric artery of 10 weeks postnatal rats. Cy3-conjugated secondary antibodies were used and greyscale confocal images inverted to black on white. IP$_3$R1 and IP$_3$R3 are present in the endothelium (A, E) and smooth muscle media (B, F), while IP$_3$R2 is present in the endothelium (C) but not smooth muscle. Vessel axis runs left to right.
Figure A.6
Confocal images showing immunostaining of IP$_3$R in the thoracic aorta of rats aged 14-17 days postnatal. Cy3-conjugated secondary antibodies were used and greyscale confocal images inverted to black on white. IP$_3$R1 is present in the endothelium (A) and smooth muscle (B) while IP$_3$R2 and IP$_3$R3 are present in the endothelium (C, E) but not the smooth muscle (D, F). Vessel axis runs left to right.
Endothelium  •  Smooth Muscle

IP$_3$R1

IP$_3$R2

IP$_3$R3

25 µm
Figure A.7
Figure A.7

IP$_3$R immunostaining of the thoracic aorta of 10 weeks postnatal rats. Cy3-conjugated secondary antibodies were used and greyscale confocal images inverted to black on white. IP$_3$R1 is present in the endothelium (A) and smooth muscle media (B) and IP$_3$R2 and IP$_3$R3 are present in the endothelium (C, E) but not the smooth muscle. Vessel axis runs left to right.
**Figure A.8**

Confocal images from the 10 week postnatal rat basilar artery, showing that staining of IP$_3$R1 (A, C) and IP$_3$R2 (E,G) in the endothelium (EC) and smooth muscle media (SMC) was completely abolished when either IP$_3$R antibody was preincubated with the appropriate immunizing peptide (IP$_3$R1: B and D; IP$_3$R2: F and H). Cy3-conjugated secondary antibodies were used and greyscale confocal images inverted to black on white. Vessel axis runs left to right.
A.3.4 Subcellular localization of receptor subtypes in basilar arteries

Since the staining pattern for each subtype appeared to be similar amongst the 3 arteries, we chose to investigate the subcellular distribution of the receptors by comparing subtype distribution with known cellular elements within the juvenile basilar artery. This artery was also the subject of our physiological studies into vasomotion (chapters 4 and 5). We chose to use the smaller diameter vessels since the staining was similar amongst the different sized branches but the smaller vessels had fewer layers of smooth muscle cells. In larger vessels the staining of the smooth muscle cells (eg with IP$_3$R1) tended to obscure the endothelial cells.

A.3.4.1 IP$_3$R subtypes in the endothelium.

Antibodies against Cx40 were used as a marker to delineate the endothelial cell periphery (Fig. A.9B, E and H). No evidence could be found for co-localization of any IP$_3$R subtypes with Cx40 and therefore it appeared that in the endothelium, at least for the majority of IP$_3$Rs, there was no close association with the cell membrane (Fig. A.9C, F, I). Due to the small size of the vessels studied, some staining of smooth muscle with IP$_3$R1 could be seen, particularly at the edges of the vessels, at right angles to the majority found in the endothelium.

In preparations in which actin filaments were stained with Texas Red-conjugated phalloidin (Fig. A.10B, E and H), a close association was found between the majority of the staining for each IP$_3$R subtype (Fig. A.10A, D and G) and the actin filaments. In these preparations, staining for each of the subtypes, especially IP$_3$R2 and IP$_3$R3, appeared to be intimately associated with the F-actin fibres (Fig. A.10C, F and I). Smooth muscle cell staining could be seen at the edges of the vessels (Fig. A.10A, B and C).

In preparations stained with the nuclear marker, propidium iodide (Fig. A.11B, E and H), all receptor subtypes (Fig. A.11A, D and G), in particular IP$_3$R1 and IP$_3$R2, appeared to lie near the edge of the nucleus. There was little evidence for intranuclear staining with any of the IP$_3$R subtypes (Fig. A.11C, F and I), although the optical section thickness of 1 μm was greater than the depth of the endothelial cell nuclei in similarly dilated basilar arteries, as determined from electron microscopy (T.Brackenbury, personal communication). While the concentration of propidium iodide used in this study was kept to a minimum, some nuclear staining could be seen in the FITC channel (Fig. A.11A, D and G). This nuclear staining was not due to the IP$_3$ receptors as it was not seen in the absence of propidium iodide (Fig. A.2-7). Some staining of smooth muscle cell nuclei,
Figure A.9 Confocal images of small branches of intact basilar arteries which were immunostained with antibodies against each IP3R subtype (FITC, green; panels A, D, G) and with connexin40 (Cy3, red; panels B, E, H). IP3Rs did not appear to be colocalised with the plasma membrane immunostained for connexin40 (overlay; panels C, F, I). Endothelial cells run parallel to the longitudinal axis of the vessel wall (left to right). Some perpendicular staining can be seen in the smooth muscle in panels A-C.
Figure A.10 Confocal images of small branches of basilar arteries which were immunostained with antibodies against each IP$_3$R subtype (FITC, green; panels A, D, G) and with phalloidin texas-red (red; panels B, E, H). IP$_3$Rs showed a close association with phalloidin-labelled actin filaments (overlay; panels C, F, I). Endothelial cells run parallel to the longitudinal axis of the vessel wall (left to right). Some perpendicular staining can be seen in the smooth muscle in panels A-C.
Figure A.11 Confocal images of small branches of basilar arteries were immunostained with antibodies against each IP$_3$R subtype (FITC, green; panels A, D, G) and with the nuclear marker, propidium iodide (red; panels B, E, H). IP$_3$R1 (Panel C) and IP$_3$R2 (Panel F), and to a lesser extent IP$_3$R3 (Panel I), were found along the periphery of the nuclei in the endothelium. Endothelial cells run parallel to the longitudinal axis of the vessel wall (left to right). Some staining of smooth muscle nuclei can be seen in panels A-C and G-I.
aligned top to bottom in the panels, can also be seen in Fig. A.11A-C and G-I.

A.3.4.2 IP$_3$R subtypes in the smooth muscle.

In the smooth muscle cells, IP$_3$R1 appeared to be distributed throughout the cytosol, interwoven with the actin filaments (red) in a manner similar to that observed in the endothelial cells (Fig. A.12A and C). The central region of the cell was however essentially devoid of IP$_3$R1 staining (Fig. A.12A). This area corresponded well with the nucleus and prominent IP$_3$R1 staining was observed around its perimeter (Fig. A.12A and C). This was confirmed when cells were stained with the nuclear marker, propidium iodide (Fig. A.12B and D). IP$_3$R1 staining could also be seen extending linearly along the length of the cell (Fig. A.12B and D). This location of staining was consistent with an association with cell membranes (Fig A.12B, arrows) and the longitudinally arranged actin filaments (Fig. A.12B, D, compare A.12A, C). In Figure 1A.12D, some endothelial cell nuclei can be seen oriented left to right in the vessel.

A.4 DISCUSSION

The present study has demonstrated that all three IP$_3$R subtypes are expressed in the thoracic aorta, basilar and mesenteric arteries but that the distribution of subtypes consistently varied between the endothelial and smooth muscle layers of the vascular wall, as well as between vessels from juvenile and adult rats. Co-localization studies in the juvenile rat basilar artery demonstrated an association between all three IP$_3$R subtypes and actin filaments. While no association was observed between any of the subtypes and the endothelial cell membrane, IP$_3$R1 staining could be observed near the cell membrane in the smooth muscle. IP$_3$R1 and IP$_3$R2 were also shown to be localised near the nuclear membrane in both cell types. The differential distribution of the IP$_3$R subtypes amongst the cell layers suggests subtype specificity may be responsible for the different physiological responses observed throughout the vascular network.

A.4.1 IP$_3$R subtypes and mRNA expression

In the present study, IP$_3$R1 mRNA was shown to be the predominant subtype in each of the arteries examined. Since mRNA was extracted from intact vessels, containing multiple layers of smooth muscle but only a single layer of endothelium, expression in smooth muscle would be expected to predominate. Accordingly, mRNA expression
Figure A.12  Confocal images of small branches from the basilar artery showing the distribution of IP$_3$R1 (FITC, green) in relation to actin filaments (A, C), stained with phalloidin-Texas Red (red) or the cell nuclei (B, D), stained with propidium iodide (red). IP$_3$R1 staining could be observed extending linearly, close to the region of the cell membrane (B, arrows). Endothelial cells are oriented left to right and smooth muscle cells are oriented top to bottom. Each image was obtained from a different vessel.
appeared to correlate well with protein expression, in that IP3R1 was observed in the both smooth muscle and endothelial cell layers of all arteries in both age groups, while IP3R2 and IP3R3 were almost entirely restricted to the endothelium. Our work is consistent with previous studies of the aorta of adult rats (Tasker et al., 2000; Tovey et al., 2000) in which mRNA for IP3R1 predominates over IP3R2 and IP3R3.

The proportional contribution of IP3R1 mRNA expression was increased in adult compared to juvenile animals. It is possible that the increase in the number of smooth muscle cell layers in the arterial wall of adult compared to juvenile rats (Blackburn et al., 1997) may account for the greater proportional contribution of IP3R1 mRNA in the adult vessels. In this regard, each of the vessels from juvenile rats IP3R2 and IP3R3 were shown to provide a greater contribution to the total mRNA than they did in vessels from adult rats. This was correlated with an increased protein expression of these subtypes in the endothelium of the juvenile vessels. On the other hand, in the smooth muscle of the basilar and mesenteric arteries, IP3R3 protein which was absent in juvenile vessels, was weakly expressed in adult vessels. Developmental changes in IP3R subtypes have previously been described in smooth muscle cells of neonatal rat aorta and portal vein with IP3R3 showing higher and IP3R1 lower protein expression in neonatal than in adult vessels (Tasker et al., 1999). In the current study, however, we did not find any evidence for protein expression of IP3R3 in the smooth muscle of the aorta at either age. The reason for this discrepancy may be related to variation in the ages of young rats or to residual endothelium in the aorta and portal vein samples.

A.4.2 IP3R subtypes are differentially distributed amongst the vascular cell layers

In the present study, IP3R1 was the predominant subtype expressed in the smooth muscle of all three arteries. Weak expression of IP3R3 was observed in the smooth muscle of the basilar and mesenteric arteries from adult rats and weak expression of IP3R2 could be observed in the basilar artery from both age groups. On the other hand, neither IP3R2 nor IP3R3 were found in the aorta. Since IP3R1 and IP3R3 have been previously identified in renal vascular smooth muscle (Monkawa et al., 1998), it is possible that differences may exist in subtype expression between elastic and muscular arteries. It is interesting then, that the basilar artery of adult rats, in vivo (Fujii et al., 1990b) and of juvenile rats in vitro (see chapter 4) have been described to undergo spontaneous rhythmical contractions or vasomotion.
A.4.3 IP3R subtype expression and vasomotion

During vasomotion, rhythmical oscillations in smooth muscle cell \([Ca^{2+}]_i\) precede changes in vessel diameter. Recent studies have shown that calcium oscillations generated in cultured rat portal vein myocytes and rat adrenal chromaffin cells appear to be dependent on IP3R2 or an interaction between IP3R1 and IP3R2 (Morel et al., 2003; Inoue et al., 2004). Thus, while IP3R1 appears to be an ideal candidate for establishing calcium oscillations, since it shows both calcium dependent activation and inactivation (Thrower et al., 2001) and high levels of expression in the muscle, interaction with another IP3R subtype, such as IP3R2, may be necessary to initiate the cycle. Given that vasomotion can be recorded in large elastic arteries during hypertension but not under control conditions (Shimamura et al., 1999), it will be of interest to determine whether expression of IP3R2 is upregulated in the smooth muscle of these vessels.

A.4.4 Subcellular localization of receptor subtypes in juvenile basilar arteries

A.4.4.1 Endothelial cell layer.

In the endothelial cell layer of the basilar artery, none of the three receptor subtypes appeared to be closely associated with the cell membrane, as delineated with antibodies against Cx40. On the other hand, all three subtypes, especially IP3R2 and IP3R3, appeared to be located along the actin filaments. The association of actin and IP3R suggests that calcium could be released close to the actin filaments and may be associated with structural changes following activation of the cells. Association of IP3R1 with actin filaments has been described previously in cultured bovine endothelial cells (Fujimoto et al., 1995) and of IP3R2 and IP3R3 with actin filaments in passaged smooth muscle cells (Sugiyama et al., 2000). This is the first description of such an association in an intact blood vessel. IP3R1 and IP3R2 also appeared to be localised along endothelial cell nuclei, but were not detected within the nucleus, in contrast to previous findings in cultured endothelial cells (Leite et al., 2003). However, it should be noted that the optical section thickness of 1 µm was greater than the depth of the endothelial cell nuclei in similarly dilated basilar arteries, as determined from electron microscopy (0.67 ± 0.04 µm, \(n = 4\) animals).

A.4.4.2 Smooth Muscle cell layer.

In the smooth muscle cells of the basilar artery, IP3R1 staining was detected close to both the nuclear and plasma membranes. IP3R1 was also observed throughout the cytoplasm intermingled with the actin filaments. This observation suggests that an
extensive SR network spreads from the nuclear region, throughout the cell, and comes into close association with the plasma membrane. Localisation of IP3R close to the cell membrane may account for the selective activation of calcium-dependent chloride channels which are proposed to provide the depolarisation necessary for the initiation of vasomotion (see chapter 4). Intriguingly, previous studies have suggested that IP3R may be physically coupled to store-operated channels in the cell membrane which may open in response to depletion of intracellular stores, resulting in calcium influx from external sources (Kiselyov et al., 1998; Boulay et al., 1999; Bishara et al., 2002; Rosado et al., 2002). On the other hand, localisation of IP3R subtypes to the nuclear membrane may provide a mechanism for the independent regulation of calcium dependent cellular processes in the nuclear compartment as has been described for HepG2 cells (Leite et al., 2003).

In a manner similar to that observed in endothelial cells of the basilar artery, IP3R1 distribution in the smooth muscle appeared to be closely associated with actin filaments. The association between phospholipase C- coupled calcium signalling pathways and actin filament reorganisation is well established (Lassing & Lindberg, 1985). Actin polymerisation modulates calcium release from IP3 and ry sensitive calcium stores (Wang et al., 2002). The intimate arrangement of different IP3 subtypes and the cytoskeleton also extends the possibilities for spatial interaction with a variety of G protein-coupled receptors in signalling microdomains (Delmas et al., 2002), plasmalemmal PIP2 and PLC (Lupu et al., 1998), and a range of intracellular target proteins that exert their effects through calcium-regulated interactions (Mandinova et al., 1998; Schell et al., 2001). It is possible that the activation of MLCK after calcium release in smooth muscle and subsequent events leading to contraction may occur as a consequence of such a relationship.

A.4.5 Concluding remarks

In conclusion, we have shown that IP3R2 and IP3R3 are predominantly distributed throughout the vascular endothelium of intact thoracic aorta, basilar and mesenteric arteries, and as such are likely targets to provide the calcium necessary for the production of important vasodilatory factors such as NO, prostaglandins and EDHF. IP3R1 being the major subtype expressed in the smooth muscle is likely to mediate the action of vasoconstrictors, such as phenylephrine and endothelin, which activate receptors coupled to the phospholipase C pathway. We also speculate that expression of both IP3R1 and IP3R2 in basilar artery smooth muscle from juvenile rats may be essential for the generation of
spontaneous vasomotion recorded in this preparation. The development of subtype selective antagonists to the IP$_3$R$_s$ will enable functional studies to test these predicted roles for the 3 subtypes in vasomotor responses.


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155


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169


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