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The Role of Intracellular Calcium Stores in the Myogenic Response of Rat Middle Cerebral Arteries

by

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Abstract

The myogenic response is the ability of resistance arteries to constrict in response to blood pressure increases. As a key autoregulatory mechanism, this ability limits fluctuations in blood flow even when large blood pressure changes occur. The aim of my research is to examine the role of intracellular calcium stores in the myogenic response of rat middle cerebral arteries. I have addressed this aim by using pharmacological approaches to test two hypotheses:

1. smooth muscle sarcoplasmic reticulum (SR) provides an internal calcium source for myogenic response

2. the endothelium can modulate myogenic tone, and disruption of the endothelial endoplasmic reticulum will disturb myogenic reactivity

My data demonstrate that in rat middle cerebral arteries, disruption of the smooth muscle cell SR results in loss of myogenic response. Also, although the endothelium does not actively regulate the myogenic response, disruption of endothelial cell calcium regulation can lead to myogenic tone loss.

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Abbreviations

2-APB	2-aminoethoxydiphenyl borate
ANOVA	analysis of variance
ATP	adenosine triphosphate
BK_{Ca}	large conductance calcium-activated potassium channel
CaM kinase	calcium/calmodulin-dependent protein kinase
cAMP	cyclic adenosine monophosphate
CICR	calcium- -induced calcium release
COX	cyclooxygenase
CPA	cyclopiazonic acid
DAG	diacylglycerol
DMSO	dimethyl sulfoxide
ECM	extracellular matrix
EDH	endothelium-dependent hyperpolarization
EDTA	ethylenediamine tetra-acetic acid
EETs	epoxyeicosatrienoic acids
EGTA	ethylene glycol tetra-acetic acid
FKBP	FK506-bindin protein
GPCR	G-protein coupled receptors
IbTX	iberiotoxin
ID	internal diameter
IK_{Ca}	intermediate conductance calcium-activated potassium channel
IP_3	inositol 1,4,5-trisphosphate
IP_3R	IP_3 receptor
K_V	voltage-sensitive potassium channel
L-NAME	N^G-Nitro-L-Arginine Methyl Ester
MLC	myosin light chain
MLCK	MLC kinase

MLCP	MLC phosphatase
NO	nitric oxide
NOS	NO synthase
NSCC	non-selective cation channel
OD	outer diameter
PE	phenylepherine
PKA	protein kinase A
PLB	phospholamban
PLC	phospholipase C
RyR	ryanodine receptor
SERCA	sarco/endoplasmic reticulum calcium-ATPase
SK_{Ca}	small conductance calcium-activated potassium channel
SR	sarcoplasmic reticulum
STOC	spontaneous transient outward current
TRP	transient receptor potential
TRPC	canonical transient receptor potential
TRPM	melastatin transient receptor potential
TRPV	vanilloid transient receptor potential
VOCC	voltage-operated calcium channel

Chapter 1: Introduction

1.1 Cerebral circulation

The cerebral circulation is an elaborate network of blood vessels which ensures sufficient blood flow for the delivery of nutrients to the brain and removal of metabolic waste. In humans and in rats, the principal arterial inflow for the cerebral circulation is through four arteries; two internal carotids and two vertebrals, with the carotids quantitatively more significant (Prado *et al.,* 2008). The vertebral arteries originate from the sub-clavian arteries which supply the shoulders, lateral chest and arms and unite to form the basilar artery at the base of the brain (Waxman, 2011). The vertebral artery system supplies the brain stem, cerebellum, occipital lobe and parts of the thalamus (Waxman, 2011). The internal carotid arteries branch off the common carotid artery which runs vertically up the neck. The two internal carotids fuse with the basilar artery to form the Circle of Willis which rests just below the hypothalamus. Off the Circle of Willis, three pairs of large vessels supply the left and right hemispheres of the cortex; the anterior, posterior and middle cerebral arteries (Figure 1.1A).

The importance of a constant supply of blood to the brain is demonstrated by situations in which the blood flow through these major cerebral blood vessels is disrupted. For example, in orthostatic hypotension, there is a temporary reduction in cerebral blood flow due to a sudden postural change such as standing up or stretching. As a result, an individual may

experience light-headedness and possibly syncope, thus illustrating that even a temporary fluctuation in cerebral blood flow can lead to significant physiological symptoms. Furthermore, many cerebrovascular diseases can also be attributed to an interruption of cerebral blood flow. One of the commonest and most devastating cerebrovascular disorders is ischemic stroke. It accounts for 80% of all strokes and is the third leading cause of death in Canada (Statistics Canada). This condition is fundamentally due to a sudden disruption of blood flow to the brain. Hence, it is clear that a constant supply of blood into the brain is critical for the survival and normal functioning of the human body.

1.2 Resistance arteries

In order to study the mechanisms responsible for the regulation of blood flow through the cerebral network, we must first examine the physiology of the vessels involved. Cerebral arteries belong to a group of arteries called resistance vessels. These arteries are small arteries and arterioles with diameter less than 500 μm and are major contributors to regulation of peripheral vascular resistance, which in turn determines blood pressure (Mulvany and Aalkjaer, 1990). Resistance arteries can be found in mesenteric, hepatic, renal, cerebral and skeletal circulations, and consist of two cell types; smooth muscle cells making up the wall and a single layer of endothelial cells lining the lumen (Figure 1.2). Smooth muscle cells are not exclusive for resistance arteries but can also be found in stomach, intestines, bladder, airways, and uterus (Webb, 2003). In the

case of blood vessels, contraction of the vascular smooth muscles cells leads to vasoconstriction. The degree of constriction is modulated by the endothelial cell layer via the release of diffusible factors and by direct electrical coupling between with the smooth muscle cells.

1.3 Regulation of resistance artery diameter

Maintenance of constant blood flow throughout the brain in physiological conditions involves a number of extrinsic and intrinsic processes. Extrinsic pathways are defined as processes which involve factors originating away from the blood vessels, e.g. circulating hormones (e.g. estrogen which relaxes blood vessels), and neurotransmitters (e.g. noradrenaline which constricts blood vessels). In contrast, intrinsic mechanisms originate from the blood vessel itself. The most important intrinsic mechanism in the autoregulation of resistance arteries is the myogenic response which is defined as the ability of vessels to constrict when intraluminal pressure increases and to dilate when pressure decreases (Schubert *et al.,* 2008).

1.4 The myogenic response: History

Myogenic response, also known as the "Bayliss effect", was first coined by Bayliss in 1902 to describe the intrinsic property of vascular smooth muscle to respond to changes in intravascular pressure (Bayliss, 1902). Truly intrinsic to the smooth muscle cells, it occurs in arteries and arterioles denuded of endothelium

and in sympathetically denervated animals (Busija and Heistad, 1984; Fog, 1937; Folkow, 1949; 1952) but can be modulated by factors released from endothelium or nerves (Anschütz and Schubert, 2005; Meininger and Faber, 1991). In general, the myogenic response in small arteries can be categorized into two phenomena: myogenic tone (i.e. tone at a constant level of pressure) and myogenic reactivity (i.e. alteration of tone in response to a change in pressure; Davis and Hill, 1999). Collectively, the two are referred to as the "myogenic response".

1.5 The function of the myogenic response

Although most prominent in the cerebral circulation, which is the focus of my thesis, the myogenic response has been described in several vascular beds including the mesenteric, skeletal muscle, uterine, renal and coronary circulations (Davis and Hill., 1999; Schubert and Mulvany *et al.,* 1999; Hill *et al.,* 2001; Hill *et al.,* 2006; Hemmings *et al.,* 2005). Within these vascular beds, the myogenic response is most pronounced in the arterioles, but is occasionally demonstrated in arteries, venules, veins, and lymphatics (Johnson, 1981). In longitudinal comparisons made among arterioles of a given vascular network, Davis (1993) found that the strength of the myogenic response consistently increased inversely to vessel size. The exception to this is the cerebral circulation in which all vessels demonstrate a comparable level of myogenic reactivity (Osol, 1995). The ability of resistance arteries to respond to changes in intravascular pressure is crucial for several cardiovascular functions: 1) it allows the setting of

peripheral resistance and thus blood pressure, 2) it permits resistance arteries to constrict to maintain constant flow in the face of increases in pressure and 3) it allows control of capillary hydrostatic pressure during variations in systemic arterial pressure (Johnson, 1986; Cipolla, 2009; Cole and Welsh, 2011). These processes in turn protect "smaller downstream arterioles and capillaries from damage in the face of changing perfusion pressures" and "maintain tissue perfusion during periods of decreased blood pressure" (Kontos *et al.,* 1978; Mellander, 1989). In particular for the cerebral circulation, functions two and three are the most critical as they allow the maintenance of a constant blood flow to the brain and prevents injury to brain tissue during periods of systemic blood pressure fluctuations.

1.6 Pathways underlying development of the myogenic response

Since the first description over a hundred years ago, our knowledge of myogenic response has vastly expanded due to "increased availability of exteriorised tissue preparations in combination with video microscopy, and later the advent of isolated, and pressurized arteriole preparations" (Hill *et al.,* 2006). Nonetheless, our knowledge of the exact signalling mechanisms underlying the myogenic response remains incomplete. Currently, the established mechanism is that increases in intraluminal pressure causes a graded depolarization of the smooth muscle cell membrane potential via an as yet unidentified transducer (see below); in rat isolated skeletal muscle arterioles increasing intraluminal

pressure from 0 to 150 mmHg resulted in progressive membrane depolarization from -55 to -30 mV (Kotecha and Hill, 2005). This depolarization leads to the opening of voltage-operated calcium channels (VOCC) and the influx of extracellular calcium which interacts with the contractile machinery in the smooth muscle cells to elicit contraction. The calcium dependence of the myogenic response can further be finely regulated by protein kinase C and Rho kinase, which can alter the calcium sensitivity of the contractile machinery. The mechanism of the myogenic response is summarized in Figure 1.3.

1.7 The stimulus and the sensor

A number of candidates have been proposed to play a role in the sensing of changes in blood pressure which then lead to depolarization of the smooth muscle cell membrane potential and development of myogenic response. Depending on the sensing mechanism proposed, the type of stimuli detected has also been variably defined. Nonetheless, the general consensus is that it is a change in arterial wall tension which is the primary stimulus triggering the myogenic response. Several studies have shown that alterations in wall tension, but not arterial diameter, correlate with changes in smooth muscle cell intracellular calcium levels and the activation of the contractile machinery (Johnson, 1989; Hui *et al.,* 1995; Hill *et al.,* 2000). For example, in the study by Hui *et al.* (1995), the correlation between wall tension and myosin light chain

(MLC) phosphorylation was very strong but no correlation was found between wall diameter and phosphorylation.

Regarding how changes in wall tension are detected, three mechanisms have been proposed: 1) stretch-activated cation channels, 2) mechanosensitive proteins within the plasma membrane and 3) the complex interaction between the extracellular matrix, integrins and the cytoskeleton. Of these, the role of non-selective cation channels, specifically those encoded by the superfamily of transient receptor potential (TRP) channels has received most attention. These channels carry an inward current during the course of the myogenic response and thus could provide a direct link between a mechanical signal and membrane depolarization (Brayden *et al.,* 2008).

1.7.1 Mechanosensitive TRP channels

TRP channels, first identified in Drosophila, are non-selective cation channels gated by temperature, light, pressure and chemical stimuli. Categorized into six subfamilies, 28 distinct genes are involved in the coding of this superfamily. TRP channels proteins are expressed as six-transmembrane-domain polypeptide subunits, four subunits coming together to form a homomeric or heteromeric cation-permeable pore (Clapham, 2003 - Figure 1.4A & 1.4B). In vascular smooth muscle cells, canonical (TRPC), vanilloid (TRPV) and melastatin (TRPM) subclasses are robustly expressed (Gonzalez and Trebak *et al.,* 2010; Yin and Kuebler*,* 2010). In particular, TRPC6 and TRPM4 are regarded as

the most likely candidates responsible for the mechanosensor in myogenic response because of their demonstrated sensitivity to mechanical stimuli.

1.7.1.1 TRPC6

According to Welsh *et al.* (2002), down-regulation of TRPC6 expression using antisense oligodeoxynucleotides attenuates muscle membrane depolarization and vasoconstriction induced by elevation of intraluminal pressure in intact cerebral arteries. This proposal is supported by the observation that pressure stimuli (negative pipette pressure) activated TRPC6 channels in expression systems (HEK293) and that activation of these channels was inhibited by reported toxins of mechanosensitive ion channels (GsMTx-4)(Spassova *et al.,* 2006; Suchya *et al.,* 2000). Interestingly, TRPC6-deficient mice show elevated mean arterial blood pressure, exaggerated agonist responsiveness and myogenic response of isolated arteries, opposite of what we would expect if TRPC6 is responsible for triggering the myogenic response (Dietrich *et al.,* 2005). However, these observations may be attributed to the over-compensatory expression of TRPC3 in these animals (Dietrich *et al.,* 2005).

1.7.1.2 TRPM4

The alternate candidate for a mechanosensing channel is the TRPM4 channel. Several studies indicate that these channels contribute to stretch-induced myogenic depolarization of cerebral artery myocytes. In the presence of

TRPM4 antisense oligonucleotides, expression of TRPM4 was attenuated and smooth muscle depolarization and constriction in response to elevated intravascular pressure were greatly reduced both *in vitro* and *in vivo* (Brayden *et al.,* 2008; Earley *et al.,* 2004). Furthermore, cerebral arterial "myogenic tone and cerebral blood flow autoregulation also decreased after exposing cerebral arteries to TRPM4 antisense oligonucleotides *in vivo*" in the rat (Reading and Brayden*,* 2007). Together, these results indicate that TRPM4 may also play an important role in the translation of mechanical stimuli to a cellular signal in resistance arteries.

1.7.2 Mechanosensitive proteins in the plasma membrane

Several mechanosensitive plasma membrane proteins have also been proposed to contribute to the mechanical sensing component of the myogenic response. One such example is G protein coupled receptors (GPCRs). It has been shown that mechanical stimuli can activate $G_{q/11}$-GPCRs (in the absence of chemical agonists) which then activate TRPC channels via a G-protein and phospholipase C (PLC)-coupled mechanism (Mederos *et al.,* 2008; Spassova *et al.,* 2006). Consequently, the role of TRPC channels in mechanosensing may be as downstream targets following activation of G proteins rather than as actual sensors. However, in a recent study by Anfinogenova *et al.* (2011), activation of $G_{q/11}$-GPCRs did not augment the mechanosensitivity of a TRPC-like current.

Thus, the potential interaction between TRPC and GPCRs in detecting changes in intravascular pressure remains to be defined.

1.7.3 Integrins, extracellular matrix and cytoskeleton complexes

In addition to mechanical sensing ion channels and membrane proteins described above, integrins have also been considered as an important link between changes in intravascular pressure and vasoconstriction. Integrins are a class of membrane-spanning glycoproteins composed of α,β-heterodimers that link the extracellular matrix (ECM) with the cytoskeleton (Davis *et al.,* 2001). In a study by Wang *et al.* (1993), integrins were found to transduce mechanical forces across cell membrane to the cytoskeleton. Furthermore, mechanical stimulation of these molecules led to a rapid increase in intracellular free calcium response in osteogenic cell line (Pommerenke *et al.,* 1996). Though these results were not obtained from arteries, they set the ground for integrins having the potential to be a mechanosenor for the myogenic response. Subsequent studies further suggested the possibility that these molecules may have a role in myogenic response. For example, Muller *et al.* (1997) found that the inhibition of integrin binding to the ECM inhibited shear stress-induced vasodilation and Mogford *et al.* (1996) found that myogenic tone was disrupted when integrin function was disrupted in rat cremaster resistance arterioles. The strongest supporting evidences later came from Waitkus *et al.* (2003) who observed a significant loss in myogenic tone during integrin blockage and from Wu *et al.* (1998) who found

that integrin ligands are linked to the activity of L-type VOCCs, an established key component of myogenic tone. These data place integrins as strong alternate candidates to TRP channels as a mechanosensor for the myogenic response but further work is required to define how integrins may be linked to membrane depolarization.

1.8 Which ion channels are responsible for the membrane depolarization underlying the myogenic response?

Once changes in wall tension/stretch are detected, the mechanical stimuli are converted into electrical signal in the form of smooth muscle cell membrane depolarization. Despite many years of study, the ionic conductances responsible for the depolarization remain to be defined. As described above, mechanosensitive TRP channels provide an inward cation current and thus may contribute but another prime candidate Cl^- channels. It is established that depolarization increases the open probability of VOCCs but it has also been suggested that calcium entry through these channels could contribute to further changes in membrane potential. Additionally, as depolarization also increases the open probability of large conductance calcium-activated (BK_{Ca}) and voltage-sensitive (K_v) potassium channels which have been proposed to act as a voltage- and calcium-dependent brake to limit further depolarization and thus calcium entry and constriction.

I will now provide more detail on the evidence that the ionic conductance plays a role in mediating or modulating the depolarization associated with the myogenic response.

1.8.1 Chloride channels

The reversal potential of Cl^- (-30 to -20 mV) in smooth muscle cells is substantially more positive than the normal smooth muscle cells resting membrane potential, which in myogenically active arteries at physiological pressures is between -60 to -40 mV (Davis and Hill, 1999). Thus, Cl^- channels are potential candidates to contribute to the depolarization of the cell membrane (Atkin, 1991). Two key studies support this proposal. Inhibitors of Cl^- channels led to concentration-dependent hyperpolarization and dilation of myogenically active posterior cerebral arteries (Nelson *et al*., 1997), and Cl^- channels were found to be sensitive to mechanical stimuli (Yamazaki *et al.,* 1998).

However, later studies suggested that the Cl^- inhibitors used by Nelson *et al*. were non-specific and are also inhibitors of VOCCs and non-selective cation channels (Welsh *et al.,* 2000; Doughty *et al.,* 1998). Nonetheless, direct measurement of Cl^- fluxes revealed pressure-dependent efflux of Cl^- during myogenic tone development, further supporting the idea that Cl^- channels may have a role in the initial membrane depolarization in response to increases in intravascular pressure (Doughty *et al.,* 2001).

1.8.2 VOCCs as a contributor to membrane depolarization

Limited evidence has also showed that VOCC may contribute to the initial depolarization required for activation of myogenic response. Langton *et al*. (1993) and McCarron *et al*. (1997) showed that VOCC currents in isolated smooth muscle cells were substantially increased by membrane stretch evoked by applying positive pressure to the patch electrode. However, Knot and Nelson (1995) found that stretch-induced depolarization persisted in the presence of Ca^{2+} channel inhibition, hence suggesting the two are separate processes.

1.8.3 Potassium channels

On the other end of the spectrum, BK_{Ca} and K_v channels are believed to provide a negative feedback mechanism to limit smooth muscle cell depolarization and contraction during the myogenic response. Depolarization causes an increase in the open probability of K_v channels and in rabbit cerebral and rat mesenteric arteries, inhibitors of K_v channels augmented myogenic tone (Knot and Nelson, 1995; Plane *et al.*, 2005).

Smooth muscle cell membrane depolarization and the subsequent increase in calcium influx through VOCCs during the myogenic response also increase the open probability of BK_{Ca} channels. In 1992, Brayden and Nelson found that BK_{Ca} channel inhibition by charybdotoxin led to depolarization and constriction of myogenically active cerebral arteries indicating that these

channels may modulate myogenic tone. Since then, numerous studies have been published in order to further address the role of these channels in the myogenic response. BK_{Ca} channels were stretch-sensitive and were activated by calcium in mesenteric (Dopico *et al.,* 1994) and coronary artery smooth muscle cells (Piao *et al.,* 2003; Wu and Davis, 2001). Furthermore, according to a group in Vermont, inhibition of BK_{Ca} channels by the selective inhibitor iberiotoxin (IbTX) caused membrane depolarization, elevated arterial wall calcium and caused a tonic constriction of pressurized cerebral arteries thus strongly suggesting that BK_{Ca} channels may provide a negative mechanism to limit the myogenic response (Knot *et al.,* 1998; Nelson *et al.,* 1995; Brayden *et al.,* 1992).

1.9 The role of VOCCs in the myogenic response

Although the mechanism underlying the depolarization in response to changes in intravascular pressure is unclear, it is well established that following depolarization of cell membrane, the open probability of VOCCs is increased. In vascular smooth muscle, there are two types of VOCCs expressed, T-type and L-type. Evidence showing T-type channel involvement in vascular function has been accumulating but its role in the myogenic response has yet to be fully defined (Kuo *et al.,* 2011; VanBavel *et al.,* 2002*)*. A high-voltage-activated, T-type-antagonist-sensitive calcium current was found to comprise 20% of current in smooth muscle cells of the main cerebral arteries and approximately 45% of current in smooth muscle cells from their branches, suggesting that there may be

a T-type splice variant involved (Kuo *et al.,* 2010). Nonetheless, of the two, L-type VOCCs are still thought to be more important in arterial smooth muscle (Bean *et al.,* 1986; Benhan *et al.,* 1987; Ganitkevich and Isenberg, 1990; Nelson *et al.*, 1990).

L-type VOCCs are highly sensitive to changes in the membrane potential and changes of 20 to 35 mV can increase open probability by 15 fold (Nelson *et al.,* 1990). This permits the large influx of calcium needed for myogenic vasoconstriction. In smooth muscle cells, CaV1.2 channels are expressed and are known to open as the membrane potential depolarizes beyond about -30 mV (Lipscombe *et al.,* 2004). Functional evidence to support the role of L-type VOCCs in the myogenic response have come from *in vitro* studies showing that in basilar, cerebral, skeletal, and mesenteric arteries, L-type VOCC blocker (dihydropyridines) greatly attenuate and in some cases completely abolish the myogenic response (Asano *et al.,* 1993; Harder, 1984; Hill and Meininger, 1994; Wesselman *et al.*, 1996). Conversely, openers of VOCCs enhance the myogenic response in mesenteric arteries (Wesselman *et al.,* 1996). Additionally, in isolated vascular smooth muscle cells, pressure- and stretch-induced calcium currents are attenuated by dihydropyridines (Davis and Sikes, 1990).

All of these studies collectively demonstrate the importance of VOCC function in myogenic vasoconstriction. Reverse mode sodium-calcium exchange has also been suggested to possibly contribute to calcium influx during this

process but evidence remains limited and VOCC continue to be regarded as the primary channel for calcium influx into smooth muscle cells in response to increases in intravascular pressure (Raina *et al.,* 2008; Potocnik and Hill*,* 2001).

1.10 Activation of contractile machinery

Following the opening of VOCCs, intracellular calcium increases to allow activation of the contractile machinery in smooth muscle cells. If extracellular calcium is removed, a reduction in intracellular calcium will follow and myogenic tone will be lost. The interaction of calcium and the contractile machinery in the myogenic response is the same as described for agonist-induced contraction (Barrett *et al*., 2011). Cytoplasmic calcium binds to calmodulin and the resulting complex activates the calmodulin-dependent myosin light chain kinase (MLCK). MLCK then phosphorylates the myosin light chain (MLC_{20}) on serine at position 19 which enable the molecular interaction of myosin with actin. This cross-bridging with actin leads to contraction. Biochemical analysis has demonstrated "arteriolar myogenic tone is dependent on calcium-calmodulin activation of myosin light-chain kinase with subsequent phosphorylation of the 20-kDa myosin regulatory light chain" (Zou *et al.,* 1995*,* 2000). This process is reversed by the myosin light chain phosphatase (MLCP) which removes high-energy phosphate from the light chain myosin and hence, reducing the cross-bridging between myosin and actin (Figure 1.5).

1.11 Calcium sensitization

Although the interaction between cytoplasmic calcium and contractile machinery in myogenic response is quite clear, an additional mechanism which may modulate this process is sensitization of the contractile proteins to calcium. This proposal is largely based on studies by D'Angelo *et al.* (1997) and Osol *et al.* (2002) in which they showed that the amplitude of the myogenic response is not always directly dependent on the level of steady-state calcium. Based on this finding, other groups then demonstrated that myogenic tone can occur independently of changes in intracellular calcium levels and can be modulated by modifying the balance between MLCK and MLCP activity through Rho-Kinase (Lagaud *et al.,* 2002; Gokina *et al.*, 2005; Somlyo and Somlyo*,* 2003). This is analogous to the mechanism whereby agonists can invoke contraction of smooth muscle cells with very little or no rise in cytoplasmic calcium.

1.12 Intracellular calcium stores in vascular smooth muscle

As described above, the role of extracellular calcium in the myogenic response is well defined: membrane depolarization increases the open probability of VOCCs, calcium enters the cell through these channels and interacts with contractile machinery to elicit vasoconstriction. However, vascular smooth muscle cells contain well-defined intracellular calcium stores which play a key role in determining intracellular calcium levels via release and sequestration (Nishimura *et al.,* 1989). Intracellular calcium stores play a key role

in modulating vasoconstriction evoked by agonists (Shima *et al.,* 1992) but their role in the myogenic response of arteriolar smooth muscle remains poorly defined.

1.12.1 Intracellular stores: an historical perspective

The sarcoplasmic reticulum (SR) is a specialized type of endoplasmic reticulum found in many excitable cells including smooth and striated muscle cells. In vascular smooth muscle cells, the SR is the primary storage and release site of intracellular calcium (Laporte *et al.,* 2004).

The first detailed description of the endoplasmic reticulum was made by Emilio Veratti in 1902 in which he described it as a "true reticular apparatus constituted of filaments" (Mazzarello *et al.*, 2003). For the next 50 years, it was drowned out by other cytological discoveries which flooded the biological literature in the first decades of the twentieth century (Mazzarello *et al.*, 2003) and it was not until 1954 that the endoplasmic reticulum was rediscovered with the use of the electron microscope and new research into its function was stimulated (Palade *et al.*, 1954). It took several more decades before the role of the endoplasmic reticulum as an intracellular calcium store was defined. It was also then that the term SR was coined to describe the endoplasmic reticulum found in muscle cells. The role of the SR as a calcium store in smooth muscle was most elegantly demonstrated by the works of Gabella *et al.* (1971), Somlyo *et al.* (1971) and Bond *et al.* (1984). In their studies, they identified that calcium

is stored in the SR, visualized the calcium and linked the cycling of calcium to and from the SR to smooth muscle contraction. In the time that followed, much of the work in the field examined the role of the SR in excitation-contraction coupling; based on the assumption that SR in smooth muscle cells are similar that of striated muscle. It has only been recent years that alternate functions of the SR in smooth muscle cells such as localized activation of ion channels (Nelson *et al.,* 1990, 1995, 1997) have been explored.

1.12.2 Structure of the SR

Most of what we know of the structure of SR is based on studies using electron-microscopy. Within smooth muscle cells, this organelle can be seen as sheets of reticulum running along the longitudinal axis of the cell (Devine *et al.,* 1972; Kuo *et al.,* 2003; Sweeney *et al.,* 2006). Occupying 1.5 to 7.5% of the total cell volume, these folds can be distributed close to the cellular periphery (peripheral/superficial SR) or span deep into the cell (central/deep SR) (Devine *et al.,* 1972), this differential localization being linked to distinct functions. "The peripheral SR has been associated with calcium homeostasis, local calcium release, and interactions with plasma membrane ion channels and hence excitability, whereas the central SR has been suggested to be more directly involved in contraction by supplying calcium to the myofilaments" (Wray and Burdyga *et al.,* 2010). This view is supported by observations that SR are found predominately at the periphery in vas deferens smooth muscle cells, a phasic

muscle, but deeper in a central location in tonic aortic smooth muscle cells (Nixon *et al.,* 1994).

Findings on the general shape and structure of SR from electron microscopy have been further reinforced by newer confocal imaging techniques. The same layered tubular structure and the orientation relative to the nucleus have been shown in imaging studies on aortic, mesenteric artery and portal vein smooth muscle cells (Tribe *et al.,* 1994; Golovoa and Blaustein, 1997; Gordienko *et al.,* 2001) and the correlation between function and location relative to the nucleus has been supported by the use of confocal imaging studies. The continuity of SR to the nucleus and its close association to mitochondria has also been revealed by improved imaging techniques (Shmygol and Wray, 2004; Gordienko *et al,* 2001).

1.12.3 Calcium storage in the SR

As an intracellular calcium store, attempts have been made to investigate in the calcium content of the SR. However, even with improvements in imaging and calcium detection techniques, there remains large variation in the reported estimates of calcium concentration within intracellular stores. This is largely due to the calcium detection methods and the types of cell under investigation (Laporte *et al.,* 2004). For example, aequorin (a lower affinity florescent calcium indicator) can be used to measure free calcium in the SR, but electron probe X-ray microanalysis gives a measure of total calcium (Laporte *et al.,* 2004).

Depending on the methods used for measurement, the estimated range of calcium in these stores is between 1 μM to 5 mM (Kendall *et al.,* 1992; Montero *et al.,* 1995; Meldolesi and Pozzan *et al.,* 1998). For comparison, the concentration of calcium in the cytoplasm of unstimulated cells is between 50 and 100 nM (Foskett *et al.,* 2007). More recently, the availability of high affinity calcium indicators such as fluro-3 or fura-2 has allowed a more accurate quantification of the calcium level in the SR. However, these indicators also have their limitations as they are prone to compartmentalize in various other intracellular organelles as well (Takahashi *et al.,* 1999). Hence, better detection methods are still needed to accurately determine the calcium content of the SR.

1.12.3.1 Calcium storage proteins

Upon entry into the SR, the majority of calcium becomes loosely bound to SR storage proteins. Such binding prevents precipitation of insoluble calcium phosphate during cation accumulation, allows rapid mobilization and calcium release when release mechanisms are triggered, and maintains free calcium at a low concentration to prevent negative feedback on uptake mechanism (Pozzan *et al.,* 1994). In the SR, the two most abundant forms of storage proteins are the calsequestrin and calreticulin. Both of these can bind to calcium at very high capacity (25-50 mol/mol) but relatively low affinity (1-4 mM) (Lytton *et al.,* 1992; Pozzan *et al.,* 1994). This low affinity binding permits rapid release when release channels are activated. Furthermore, to facilitate rapid calcium release, binding

proteins are generally localized close to calcium release channels; Villa *et al.* (1993) observed calsequestrin was predominantly located in the peripheral portion of SR which happens to be rich in inositol trisphosphate channels (IP_3R).

1.12.4 Calcium uptake into the SR

Although the SR has a very efficient mechanism to maintain calcium levels within the SR lumen, it also requires effective mechanisms to release and take up calcium. The role of filling these stores is fulfilled by the sarco/endoplasmic reticulum calcium-ATPase (SERCA). SERCA belongs to the P family of cation transport ATPases and is responsible for transporting calcium into the SR at the expense of ATP (Verkhratsky, 2004). All transporters of this family form a phosphorylated enzyme intermediate by covalent interaction of the ATP terminal phosphate with an aspartyl residue at the catalytic site, setting them apart from the F and V families of cation transport proteins (Moller *et al.*, 2010). Specifically for SERCA, it can transport two calcium ions for every one ATP hydrolyzed, distinguishing it from the plasma membrane calcium-ATPases which transport one calcium ion for every one ATP hydrolyzed (Inesi, 1987; Grover and Samson, 1986).

1.12.4.1 SERCA: an historical view

Evidence for the existence of calcium pumps within the SR membrane first appeared in the early 1960s when SR fractions were shown to accumulate

calcium at the expense of ATP hydrolysis (Ebashi and Lipmann, 1962). Though the protein within this fraction was not identified nor coined as SERCA at the time, the properties of this protein were fairly well established by this group and others (Ebashi and Lipmann, 1962; Hasselbach and Makinose *et al.*, 1961). It took about a decade for the protein in the SR fraction to be purified and 40 years later for the crystal structure of this protein to be resolved (MacLennan, 1970; Toyoshima *et al.*, 2000). Despite the relatively recent identification of the crystal structure, the general organization of SERCA in the membrane had been inferred from morphological data well before information on the molecular structure became available. The 3D crystal structure of SERCA however, allows the settling of some of the more controversial aspects of the structure, including controversy over the location of the ATP-binding sites (Toyoshima *et al.,* 2000).

1.12.4.2 SERCA structure

SERCA is a 110-kDa protein that consists of a single polypeptide formed into four major domains: 1) transmembrane (M) domain, 2) actuator (A) domain, 3) phosphorylation (P) domain, and 4) the nucleotide binding (N) domain (Figure 1.6A & 1.6B).

The M domain, which forms the pore through which calcium passes, comprises 10 α-helixes of varying inclination and length (Toyoshima *et al.,* 2000). Of the 10 helixes, referred to as M1-M10, calcium binding site 1 lies between M6 and M5 and calcium binding site 2 is located between M6 and M4 (Wuytack *et*

al., 2002). According to Toyoshima *et al.* (2000), the two calcium binding sites are 5.7Å apart. It should also be noted that the α-helixes may undergo rotation or winding/unwinding during calcium transport and to accommodate the calcium binding sites (Wuytack *et al.,* 2002). The P domain is connected to the M domain through M4 and M5 helixes. The P domain is "built up of two parts of the primary sequence, separated by a long stretch that forms the N domain" (Toyoshima *et al*., 2000). Functionally, the P domain houses the target which ATP phosphorylates to allow the transport of calcium into the SR. Of note is the amino acid ASP 351 which has been identified as the amino acid that becomes transiently phosphorylated during the catalytic cycle (Moller *et al.,* 2010). Mutations in ASP 351 or neighbouring amino acids can destabilize the phosphorylated intermediate, disrupt the catalytic cycle, and hence prevent calcium transport (Andersen, 1995; Clausen *et al.*, 2001). Resting on the upper cytoplasmic part of the SERCA complex is the N domain which is shaped like a top-hat and linked to the P domain (Moller *et al.,* 2010). Functionally, it provides the binding pocket for the nucleotide moiety of ATP. Fitting into the groove toward the lower end of the N domain, the triphosphate part of ATP can reach and interact with ASP 351 residue of the P domain by swivelling of the whole N domain (Sorensen *et al.,* 2004; Xu *et al.,* 2002). This permits the phosphorylation of ASP 351 which is needed for the catalytic cycle. Finally, the A domain is the smallest of all the domains (Toyoshima *et al.,* 2000). Connected to the M domain by long loops, it has the potential to move substantially during the catalytic cycle

(Wuytack *et al*., 2002). As a result, it has been proposed that the "rotary movements that the A domain performs" may facilitate the phosphorylation and dephosphorylation or release of calcium to SR lumen during the catalytic cycle (Wuytack *et al.,* 2002; Xu *et al.,* 2002). To summarize, each of these SR domains has a specific role and together they work cooperatively as the pump moves through the catalytic cycle.

1.12.4.3 The SERCA catalytic cycle

The term catalytic cycle is in reference to the conformational changes in SERCA during calcium uptake into the SR (Figure 1.7). In general, two conformational states are used to describe the SERCA enzyme as it transports calcium from the cytoplasm into the SR: E1 and E2. E1 refers to the high calcium affinity (K_D 10^{-7} M) conformation which can be phosphorylated by ATP to form a high-energy intermediate, 2calcium-E1-P_i. In the E1 state, the calcium binding site is just accessible to the cytoplasm side while access through the luminal side stays closed. Once ATP binds, autophosphorylation occurs at ASP351 of the P domain and ADP is released. The energy released from the high energy phosphate bond allows a major conformation change thus forming a 2calcium-E2-P_i intermediate. In this state, the calcium binding site changes to a low affinity state (K_D 10^{-3} M) and access to the binding site is switched to the luminal side. Consequently, calcium leaves and enters into the SR. Shortly after, the phosphate comes off of the N domain, and the enzyme becomes a low-energy

intermediate, E2. Two or three protons are counter-transported as the enzyme converts back to the E1 condition and the cycle starts again.

1.12.4.4 SERCA isoforms

There are three isoforms of SERCA: SERCA1, SERCA2 and SERCA3 encoded by the genes *ATP2A1*, *ATP2A2* and *ATP2A3* respectively. Various alternative splicing gives rise to two subtypes of SERCA1 (neonatal vs adult), two subtypes of SERCA2 and 5 subtypes of SERCA3. In vascular smooth muscle, which is the interest of my research, SERCA2b is the predominant isoform (greater than 70%) with the remainder composed of SERCA2a and SERCA3 (Eggermont *et al.,* 1990; Amrani *et al.,* 1995). SERCA1is found in high amounts in the SR of fast-twitch skeletal-muscle fibers (Wray and Burdyga*,* 2010). In contrast, SERCA3 is much more widespread, being expressed in epithelial and endothelial cells, platelets, many endocrine glands, cerebellum, pancreas, and spleen (Wray *et al*., 2010). Other than the cell types these variants are expressed in, the major difference between these types and subtypes are the structural differences in the carboxyl terminal. These structural changes, whilst minor, may lead to differences in calcium sensitivity (Brandl *et al.,* 1987; Lytton *et al.,* 1992). For example, Verboomen *et al.* (1992) found that in COS cells, SERCA2b showed a two-fold higher apparent affinity for calcium than SERCA2a. For the most part though, the 3D structure and the function in calcium uptake in these variants are very similar.

1.12.4.5 SERCA regulation

Depending on the variant of SERCA, its primary regulator may be different. In smooth muscle cells, SERCA2b is predominantly expressed and is regulated by phospholamban (PLB), a small 52 amino acid membrane-spanning protein which reversibly binds to SERCA to inhibit its activity. Young *et al.* (2001) suggested that binding of PLB to SERCA may resist the large domain movement of SERCA during the catalytic cycle, thus limit its turnover rate. They further proposed that during such inhibition, each molecule of PLB can interact with two molecules of SERCA and reduce its calcium affinity (Wray and Burdyga*,* 2010). While PLB regulates SERCA, PLB activity is regulated by two kinases: cAMP-dependent protein kinase A (PKA) and calcium/calmodulin-dependent protein kinase II (CaM kinase II; Tada *et al.,* 1982). When PLB is phosphorylated by these kinases, its charge changes and that greatly reduces its inhibitory effect on SERCA (Wray and Burdyga*,* 2010). Hence, SERCA activity increases. As a side note, a homolog of PLB, sarcolipin, is also an inhibitor of SERCA. However, this is mostly found in skeletal and cardiac muscle and there is very little data on its role in smooth muscle (Wuytack *et al.,* 2002).

1.12.5 Calcium release channels

In order for calcium in the SR to be of use to the cell, it must be released into the cytoplasm. There are two families of channels which facilitate the release of calcium from the SR; ryanodine receptor (RyR) channels and IP_3R

channels. These two channels have substantial similarities in structure. Nonetheless, when arranged into their three dimensional conformation, noticeable differences between the two can be detected (Jiang *et al.,* 2002).

1.12.5.1 RyR channels: structure and mechanism

The RyR is a high-conductance ion channel responsible for the release of calcium from the SR in response to increases in calcium in the cytoplasm (calcium-induced calcium release; CICR; Meissner*,* 1994). With a molecular weight of approximately 2.3 MDa, these channels are homotetramers composed of subunits weighing about 565kDa each (Kimlicka and Van Petegem*,* 2011). Structurally, these channels can be described as a "mushroom shaped quarterfoil" with a large cytoplasmic region forming the cap and a smaller transmembrane region forming the stem (Figure 1.8A). The smaller transmembrane region forms a calcium conduction pore with a diameter of 2 to 3 nm to allow the movement of calcium out of the SR (Laporte *et al.,* 2004). As this pore region is critical for the functioning of the RyR channel, it is not surprising that this region is highly conserved between different RyR channel isoforms. That is not to say the cytoplasmic region is any less important. Consisting of 80% of the mass of RyR channel, the cytoplasmic region holds the binding sites for many modulators including "calcium, adenine nucleotides, calmodulin, FKBPs" (Wray and Burdyga, 2010; Figure 1.8B).

These binding sites are very important, especially for calcium, the primary trigger of RyR channel opening. During transient increases in cytoplasmic calcium (e.g. caused by opening of VOCCs), calcium can bind to RyR to stimulate opening of the channel and the release of calcium from the SR. When RyR is activated, considerable changes occur in the mushroom shaped structure with the four corners of the cytoplasmic region dipping down toward the SR while the central region lifts away (Samso *et al*., 2009). This calcium release event has been termed a "calcium spark". Calcium sparks generated just below the plasma membrane can activate BK_{Ca} channels and elicit spontaneous transient outward currents (STOCs; Jaggar *et al.* 1998, 2000). In intact arteries, STOCs can be electrically transformed through the passive cable properties of vascular smooth muscle cells into a sustained hyperpolarization that feeds back negatively upon constrictor responses (Jaggar *et al.* 1998; Knot *et al*. 1998).

In addition to activating BK_{Ca} channels, calcium sparks can also activate other neighbouring RyR channels leading to a cascade effect forming "calcium waves" to amplify the original calcium signal. Calcium waves are relative slow temporal events that generally propagate from one end of the cell to the other and are asynchronous among neighbouring smooth muscle cells (Boittin *et al.*, 1999; Jaggar & Nelson, 2000). In contrast to sparks, calcium waves have been proposed to facilitate arterial constriction through two possible mechanisms: 1. these events deliver a proportion of the calcium that controls the signalling

pathways associated with MLCK or MLCP (Kuo *et al.,* 2003; Lee *et al,.* 2005), 2. calcium waves activate an inward current to depolarize smooth muscle and subsequently elevate calcium influx through VOCCs (Gonzales-Cobos and Trebak*,* 2010). The association between contractile agonists and calcium waves is becoming accepted but it is less certain whether increases in intravascular pressure elicit a similar response. Indeed, published studies present conflicting findings ranging from no calcium waves to robust production (Miriel *et al.*, 1999; Jaggar, 2001; Zacharia *et al.*, 2007; Mufti *et al*., 2010).

1.12.5.2 RyR channel isoforms

RyR channels exist in several isoforms (RyR1, RyR2 and RyR3) with variable expression in different tissues. In smooth muscle cells, all three isoforms can be expressed and the relative proportion of expression of each is tissue- and species- dependent. For example, all three isoforms were detected in pulmonary arterial smooth muscle cells (Yang *et al*., 2007) but only RyR2 and RyR3 are found in mouse duodenal smooth muscles (Dabertrand *et al*., 2006). For cerebral arteries, RyR2 is 4 and 1.5 times more abundant than RyR1 and RyR3, respectively (Vaithianathan *et al*., 2010). Generally, RyR2 and RyR3 are the predominant isoforms in smooth muscle. Functionally, these isoforms are generally quite similar but a few functional differences may exist between them. For example, according to the [^{3}H]ryanodine binding studies reviewed by Meissner (1994) and Coronado *et al.* (1994), RyR1 and RyR2 are activated by

sub- to low μM levels of calcium and inactivated by μM to mM calcium. However, the RyR2 isoform is less sensitive to inactivation by calcium than RyR1. Comparing RyR3 to RyR1 and RyR2, RyR3 has the lowest calcium sensitivity of the three isoforms (Wray and Burdyga*,* 2010). Considering that calcium is the main trigger for RyR channel activation, these sensitivity differences can be very important functionally and may explain the variation in expression of these isoforms.

1.12.5.3 IP_3R structure

In addition to RyR channels, IP_3R is the alternate receptor channel responsible for the release of calcium from the SR. Structurally similar to the RyR, IP_3R channels are also tetramers but with much lower molecular mass of about 1.2 MDa (Foskett *et al*., 2007). IP_3Rs can be assembled either from identical subunits or from mixtures of the three isomers: IP_3R1, IP_3R2 and IP_3R3. These isomers share 60-80% overall amino acid similarity (Taylor *et al.,* 1999).

Structurally, the N-terminal portion of each subunit is a bulbous ligand-binding domain for IP_3 which makes up 24% of the receptor molecule (Laporte *et al.,* 2004). Connected to it is the largest component of the domain (60%) which also houses the binding sites for IP_3 and regulatory and modulator molecules such as calcium and ATP. Finally, the C-terminal is the portion that makes up the pore for calcium conduction (Figure 1.9). Four of these subunits come together to form a structure described as a four-fold symmetrical pinwheel of radial arms

projecting from a central square mass (Foskett *et al.,* 2007). This rather non-specific description is due to a lack of consensus among the three dimensional structures proposed by different groups based on electron microscopic single-particle analyses (Da Fonseca *et al*., 2003; Sato *et al.,* 2004; Serysheva *et al.,* 2003; Jiang *et al.,* 2002; Hamada *et al.,* 2003; Figure 1.10A & 1.10B).

1.12.5.4 Calcium release via IP_3R

Being a calcium release channel on the SR membrane, IP_3R are activated by both IP_3 and calcium. During agonist induced contraction, agonists binding to G-protein-coupled receptors will activate PLC at the plasma membrane which initiates hydrolysis of the membrane lipid phosphatidylinositol 4,5-bisphosphate to yield IP_3 and diacyglycerol (Berridge *et al.,* 1984). The water-soluble second messenger, IP_3, then freely diffuses in the cytoplasm and binds to IP_3Rs. IP_3R subunits each have a single high-affinity binding site and the IP_3R1 isoform is the most sensitive (Wokcikiewicz and Luo, 1998). IP_3 sensitivity can be modulated by the secondary activator calcium (Jean and Klee, 1986) which is both a modulator and an activator of IP_3R.

The effect of cytoplasmic calcium on release of calcium by IP_3R is biphasic: at low concentrations (sub-micromolar) it increases the release of calcium whereas at higher concentrations (micromolar or more) it significantly reduces the efflux of calcium (Lino, 1987, 1990; Marshall and Taylor, 1993). This interesting effect might serve as a feedback system to limit the release of

calcium when cytoplasmic concentrations are high. It is also of note to mention that the cross sensitivity of calcium for both RyR and IP_3R serves a very unique interaction to allow the coordinated release of calcium from SR. For instance, IP_3R can become activated by G-protein cascade and a "puff" of calcium from SR is released though the IP_3R channel. As RyR are sensitive to calcium, this puff can subsequently activate RyR channels to induce CICR which greatly increases cytoplasmic calcium and allows the activation of contractile machinery.

1.13 The SR and myogenic tone

As described above, there is wealth of knowledge on the structure and properties of the SR but the contribution of intracellular calcium stores in the myogenic response has remained poorly defined. This is partly due to the fact that active tone has an absolute requirement for extracellular calcium for activation of contractile proteins and maintenance of calcium stores. Arteries rapidly dilate toward their passive maximal diameter when perfused with solutions lacking calcium thus hampering the study of the functional role of the SR.

The first functional evidence for a role of the SR in myogenic tone came from Watanabe *et al.,* (1993) who showed that depletion of the SR slowed the rate of myogenic response in isolated skeletal arterioles and that ryanodine-sensitive SR function is involved in the myogenic response in the same vessels (Watanabe *et al.,* 1994). Around the same time, Osol *et al.,* (1993) demonstrated

that inhibition of PLC caused vasodilation of cannulated cerebral vessels. Moreover, Inscho *et al.* (1998) found that PLC inhibition attenuated pressure-induced contraction of renal afferent arterioles. More recently, Kotecha and Hill (2005) examined relationships between intraluminal pressure, membrane potential and myogenic tone in rat skeletal muscle arterioles. They showed that while depolarization mediated calcium entry makes a significant contribution to myogenic tone, an interaction between calcium entry and SR calcium release is necessary for maintenance of myogenic constriction.

In contrast, Knot and Nelson, (1998) suggested that rather than the SR contributing to myogenic constriction, localized, sub-membrane release of calcium from the SR via RyR regulates myogenic tone solely through activation of BK_{Ca} channels. As mentioned earlier (section 1.8.3) they proposed a model in which opening of these channels regulates membrane potential through tonic hyperpolarization, thus limiting calcium entry through L-type VOCCs. However, in the study described above by Kotecha and Hill (2005), inhibition of BK_{Ca} channels by ibertiotoxin had only a very modest effect on myogenic tone. Miriel *et al.,* (1999) showed that in isolated mesenteric arteries, calcium entry through VOCCs is obligatory for the myogenic response but suggested that intracellular calcium release events do not make a substantial contribution.

Thus, at the start of my project, it was clear that although calcium influx across the plasma membrane is essential for the myogenic response, there are

disparate reports regarding whether VOCC-mediated calcium entry is the prime determinant of myogenic tone. Data supporting the involvement of intracellular stores was provided, as was limited evidence for the involvement of mechanisms utilizing signaling pathways involving protein kinase C and Rho kinase (Lagaud *et al*., 2002). However, the existence of heterogeneity in the mechanisms underlying myogenic reactivity between tissues was becoming apparent and the contribution of non-voltage-dependent mechanisms to myogenic contraction was uncertain.

So, with this in mind, and given the importance of the myogenic response to autoregulation within the cerebral circulation, my first aim was to investigate the role of intracellular calcium stores in the myogenic response in isolated rat middle cerebral arteries. This vessel is one of the three major arteries that supplies blood to the cerebral cortex and is the most common of the three to be compromised during a cerebrovascular incident, thus, understanding the regulation of these arteries may provide information of future clinical importance (Porter, 2011).

1.14 Intracellular calcium stores in the vascular endothelium

It is of note that endoplasmic reticulum is also present within the vascular endothelium. It is less specialized than the smooth muscle cell SR and its main function is protein synthesis. However, they also can serve as an intracellular calcium store, one which plays a key role in the modulation of arterial diameter.

1.14.1 Endothelium-dependent modulation of vascular tone

The vascular endothelium regulates the contractile state of the underlying smooth muscle cells via the release of diffusible factors such as nitric oxide (NO) and via direct electrical coupling via gap junctions (endothelium-dependent hyperpolarization; EDH; reviewed by Feletou *et al.,* 2009). The generation of NO and the activation of EDH are both dependent on an increase in intracellular calcium levels within the endothelium. Activation of endothelial GPCRs such as the muscarinic acetylcholine receptor leads to generation of IP_3 which stimulates the release of calcium from the endoplasmic reticulum. The subsequent increase in intracellular calcium stimulates the production of NO by NO synthase (NOS) and activates intermediate (IK_{Ca}) and small (SK_{Ca}) - conductance calcium activated potassium channels to elicit hyperpolarization of the endothelial cell membrane potential. The hyperpolarization can spread to the smooth muscle cells through gap junctions to reduce the open probability of VOCCs and so reduce calcium influx and cause relaxation (Figure 1.11). Other putative pathways by which the endothelium can influence smooth muscle cell membrane potential involve the release of factors such as C-type natriuretic peptide and H_2O_2 which directly activate potassium channels on smooth muscle cells (Edwards *et al*., 2010; Campbell and Fleming, 2010).

Arachidonic acid metabolites such as prostacyclin from the cyclooxygenase (COX) pathway and products of cytochrome P450

monooxygenase epoxyeicosatrienoic acids (EETs) may also play a role in the endothelium dependent regulation of the contractile state of smooth muscle cells. However, inhibition of COX has been shown not to have significant effect on vascular tone in cerebral arteries (Thorin-Trescases and Bevan, 1998; Smeda *et al.,* 2010). EETs have been hypothesized to play a significant role in modulating tone in renal and coronary arteries through the activation BK_{Ca} (Félétou and Vanhoutte, 2006). Nonetheless, neither of these pathways involves activation of endothelial K_{Ca} channels nor are they closely associated with the endothelial endoplasmic reticulum and so will not be further discussed here. They have been extensively reviewed by Edwards *et al*. (2010) and Campbell and Fleming (2010).

Under normal conditions, the endothelial endoplasmic reticulum plays a key role in regulating intracellular calcium levels. Regulated release of calcium from this storage site in response to activation of GPCRs or mechanical forces such as changes in shear stress and flow leads to activation of vasodilator pathways which influence the contractile state of the underlying smooth muscle cells. However, loss of endothelial function including disruption of endoplasmic reticulum function, contributes to the etiology of cardiovascular disease.

Although the majority of studies of endothelium-dependent modulation of arterial diameter have focused on agents which directly activate the endothelium such as acetylcholine, it has long been known that arterial

vasoconstriction in response to agonists such as noradrenaline released from perivascular nerves is limited by the endothelium via a process termed myoendothelial feedback. Exactly how contraction of smooth muscle cells leads to activation of the endothelium is unclear but recent evidence from our lab and others supports the notion that myoendothelial feedback is due to flux of IP_3 through gap junctions leading to localized, IP_3-mediated release of calcium within spatially-restricted myoendothelial projections and subsequent activation of the NO and EDH pathways (Sandow *et al.,* 2009).

1.14.2 Endothelium-dependent modulation of the myogenic response

A large body of evidence supports the role of myoendothelial feedback in limiting agonist-evoked vasoconstriction but the role of the endothelium in modulating the myogenic response has been long debated. In isolated rat posterior cerebral, mesenteric and skeletal muscle arteries and rabbit ear arteries, myogenic tone was unaltered by removal of the endothelium (McCarron *et al.,* 1989; MacPherson *et al.,* 1991; Falcone *et al.,* 1991; Sun *et al.,* 1992). In contrast, in mouse mesenteric, guinea-pig coronary and cat skeletal muscle arteries, myogenic tone was enhanced by NOS inhibitors indicating that myoendothelial feedback may play a role modulating myogenic reactivity in these vessels (Ueeda *et al.,* 1992; Veerareddy *et al.,* 1994). However, in these latter studies, myogenic responses were assessed *in situ* and the possibility that

changes in flow over the surface of the endothelium was the stimulus for the release of NO rather than myogenic tone *per se* cannot be ruled out.

Thus, although it is clear that the endothelium is not required for the development of the myogenic response, it role in the modulation of myogenic tone is unclear. My second aim will be to investigate the role of the endothelium in regulating the myogenic response of middle cerebral arteries.

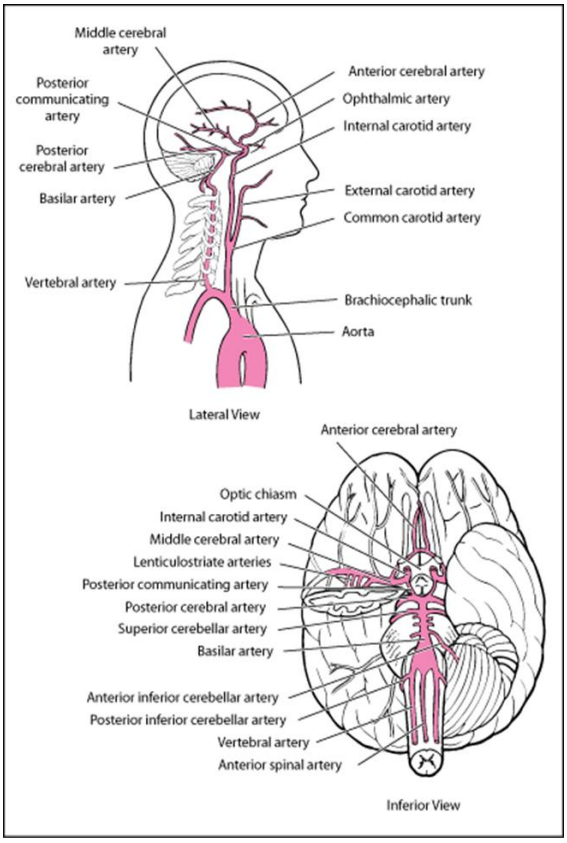
1.15 Hypothesis and aims

The overarching aim of my research is to investigate the role of intracellular calcium stores in the myogenic response of isolated middle cerebral arteries. To address this aim I have tested two hypotheses in middle cerebral arteries:

1. that the smooth muscle SR provides an internal source of calcium for the development of myogenic tone.

2. that the endothelium can modulate myogenic tone and that disruption of the endothelial endoplasmic reticulum will disturb myogenic reactivity.

A) B)



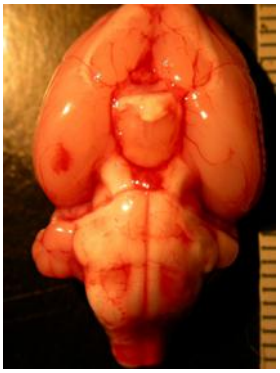


Figure 1.1 A: Diagram of the major arteries in the cerebral circulation network of the human brain. The principal arterial inflow for the cerebral circulation is through the two internal carotid and two vertebral arteries. These arteries carry blood to the basilar artery which then provides for the cerebral arteries on the two sides of the brain. The three pairs of cerebral arteries can then supply the left and right hemispheres of the cerebral cortex. From: Porter R. (2011) The Merck manual of diagnosis and therapy. Merck Research Laboratories.

Figure 1.1B: Bottom view of a rat brain with the anterior end toward the top and the posterior end toward the bottom of the page. Upon removal from the animal, the brain is placed in ice-cold, oxygenated physiological Krebs solution and experiments are carried out on the day the brain was dissected.

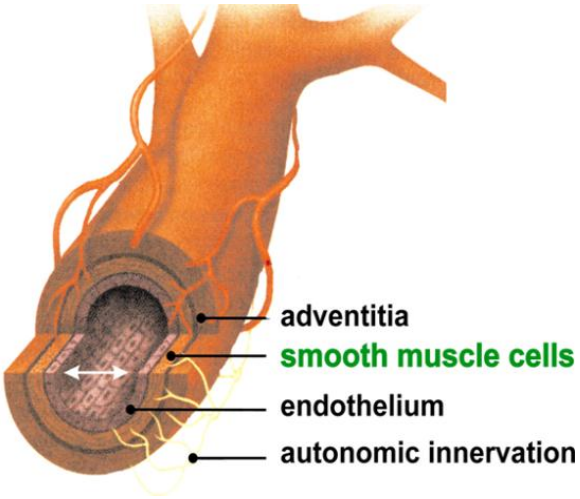


Figure 1.2: Structure of a resistance artery. These arteries are small arteries and arterioles with diameter less than 500 μm and are major contributors to regulation of peripheral vascular resistance. Smooth muscle cells make up the outer wall of the vessel and a single layer of endothelial cells lines the lumen.

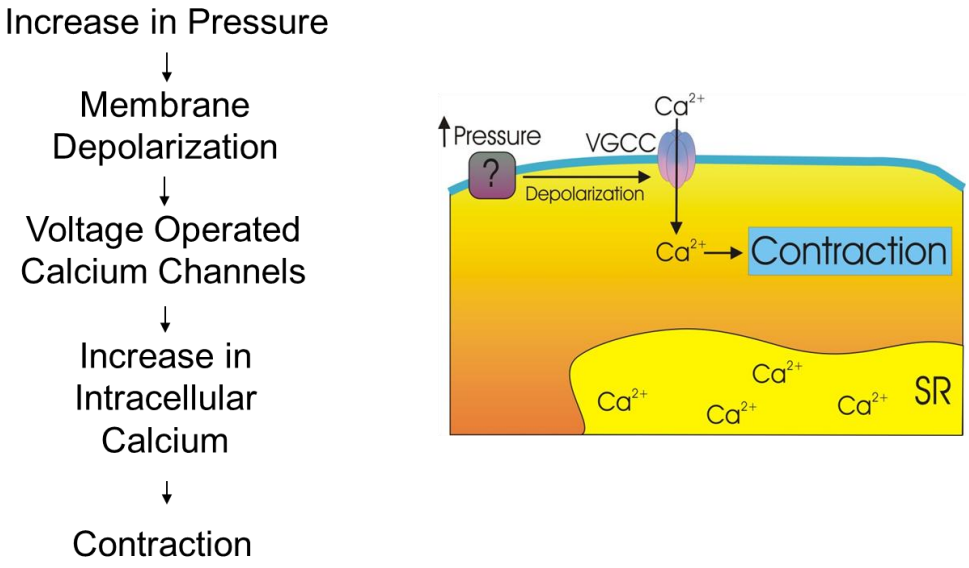


Figure 1.3: The currently accepted mechanism underlying the myogenic response. During increases in intraluminal pressure, in the smooth muscle cell, cell membrane becomes progressively depolarized. This depolarization leads to the opening of voltage-operated calcium channels (VOCC). The influx of extracellular calcium through the VOCCs interacts with the contractile machinery in the smooth muscle cells to elicit contraction. The role of the intracellular calcium stores in myogenic response has yet to be defined.

A) B)

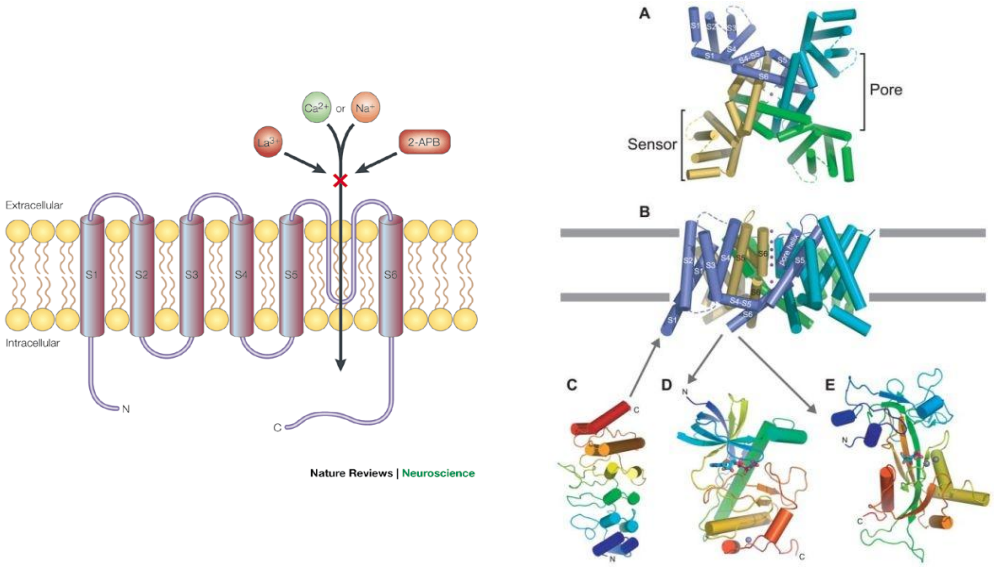


Figure 1.4 A: TRP channels. TRP channel subunits comprise six membrane-spanning (S1-S6) domains with a pore domain between the S5 and S6, and intracellular amino and carboxyl termini. TRP channels are non-selective cation channels. These channels respond to a number of stimuli including temperature, light, pressure and chemical. From: David EC, Loren WR, Carsten S. (2001) The trp ion channel family. *Nature Reviews*. 2: 387-396.

Figure 1.4 B: Each functional TRP channel consists of four subunits forming homomeric or heteromeric structures. In A, the channel is viewed from the intracellular side of the membrane, and in B, from its side. From: Liedtke WB, Heller S. (2007) Structural Insights into the Function of TRP Channels. Florida: CRC Press.

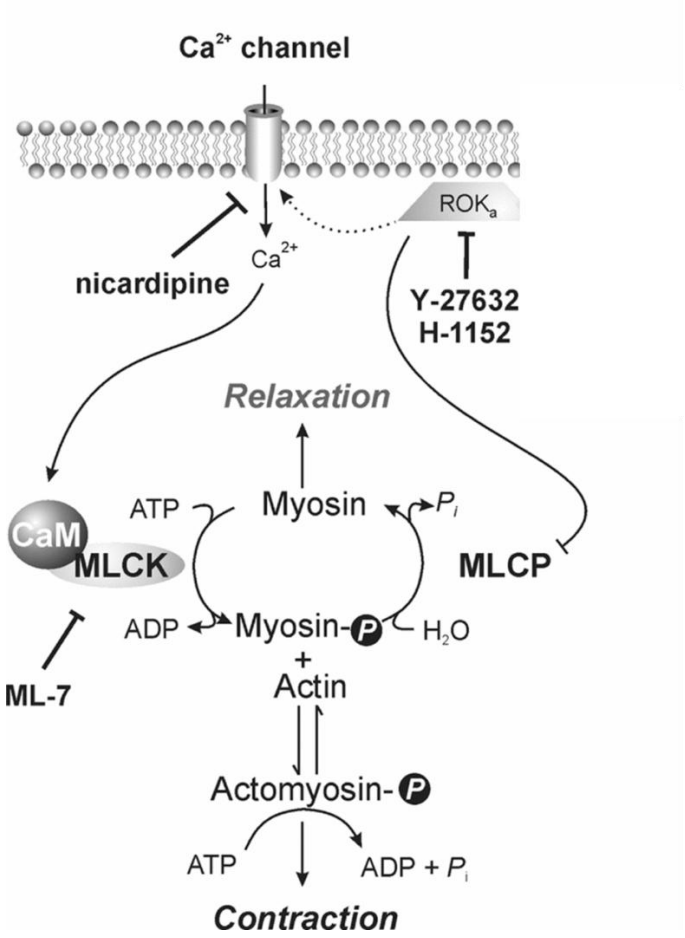


Figure 1.5: Schematic of pathways underlying smooth muscle contraction and relaxation. Cytoplasmic calcium binds to calmodulin and the resulting complex activates the calmodulin-dependent myosin light chain kinase (MLCK). MLCK then phosphorylates the myosin light chain (MLC20) on serine at position 19 which enables the molecular interaction of myosin with actin leading to contraction. Relaxation occurs when myosin light chain phosphatase (MLCP) removes high-energy phosphate from the MLC20 to reduce interaction with actin. Activation of MLCK and MLCP has opposing effects. The contractile state of smooth muscle can be modulated by Rho-Kinase (ROK) activity as indicated above. From: Wilson DP, Susnjar M, Kiss E, Sutherland C, Walsh MP. (2005) Thromboxane A2-induced contraction of rat caudal arterial smooth muscle involves activation of Ca^{2+} entry and Ca^{2+} sensitization: Rho-associated kinase-mediated phosphorylation of MYPT1 at Thr-855, but not Thr-697. *Biochem J.* 389: 763–774.

A) B)

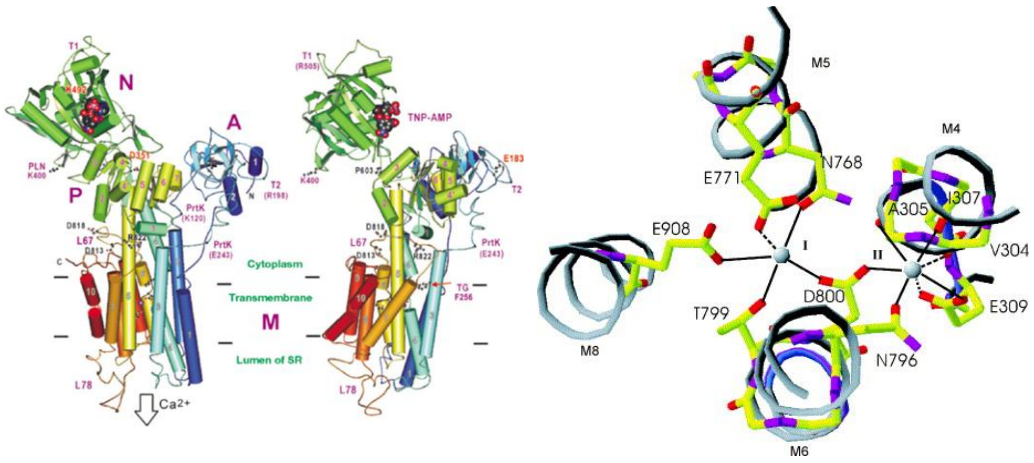


Figure 1.6A: SERCA is a 110-kDa protein that consists of a single polypeptide formed into four major domains: transmembrane (M) domain, actuator (A) domain, phosphorylation (P) domain and the nucleotide binding (N) domain. When SERCA is activated, two calcium ions move into the two binding sites in the M domain and is followed by the binding of ATP at the N domain. Autophosphorylation occurs at site SER 351 in the P domain leading to a large conformational change in the structure to facilitate the transport of calcium into the SR. The large movement which occurs during the catalytic cycle is believed to involve domain A. From: Toyoshima C, Nakasako M, Nomura H, Ogawa H. (2000) Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 A resolution. *Nature*. 405(6787): 647-55.

Figure 1.6B: Structure of SERCA viewing from the luminal side of the SR. In the center are the two binding sites of calcium being surrounded by the 4 α-helixes M4, M5, M6 and M8. From: Wuytack F, Raeymaekers L, Missiaen L. (2002) Molecular physiology of the SERCA and SPCA pumps. *Cell Calcium*. 32(5-6): 279-305.

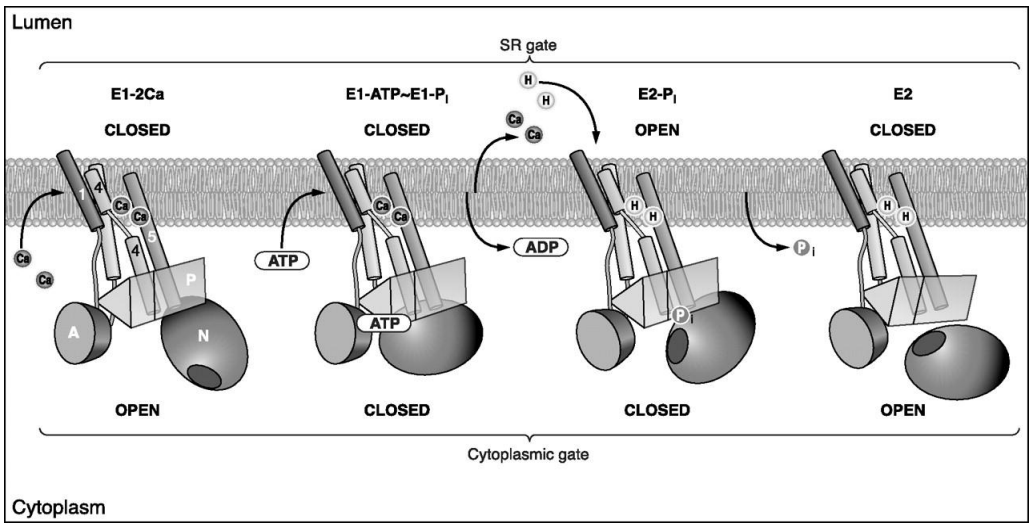


Figure 1.7: Schematic representation of the structural changes in SERCA and the accompanying catalytic cycle. In its low energy state, E1, the calcium binding site is only accessible from the cytoplasm side as indicated in the first configuration. Upon binding of calcium and ATP, autophosphorylation occurs and is accompanied by large changes in the SERCA structure resulting in the generation of an ADP molecule, the transport of calcium into the SR and the formation of an intermediate E2-P. This is shown in configuration two and three. In configuration four, protons are counter-transported as the enzyme reverts back to the E1 condition and the process may start again. From: Wray S, Burdyga T. (2010) Sarcoplasmic reticulum function in smooth muscle. *Physiol Rev*. 90(1): 113-78.

A) B)

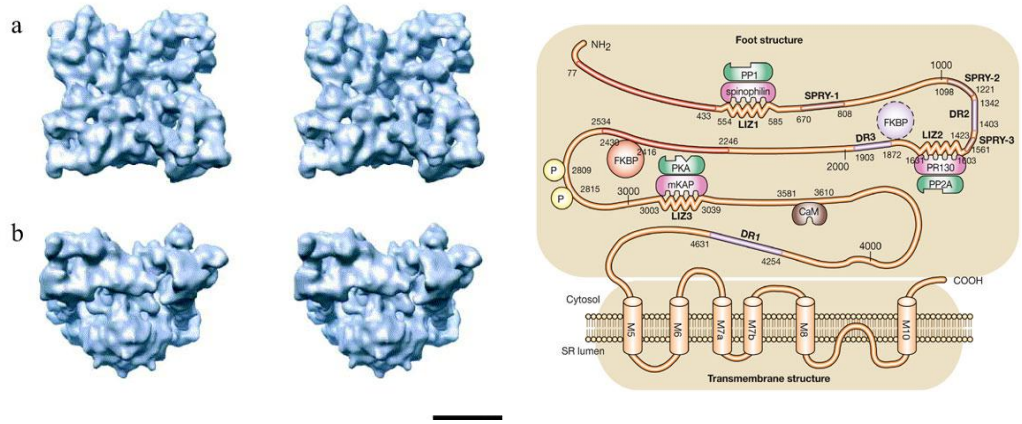


Figure 1.8A: The structure of RyR1 at 14 Å resolution. The structure is viewed from the cytoplasm (a) and from the side (b) with the cytoplasmic side facing upward. It forms a "mushroom shaped quarterfoil" with a large cytoplasmic region forming the cap and a smaller transmembrane region forming the stem. From: Seryshevaa I, Hamilton S, Chiua W, Ludtkea S. (2005) Structure of Ca^{2+} Release Channel at 14 Å Resolution. J Mol Biol. 345(3): 427-431.

Figure 1.8B: Consisting of 80% of the mass of RyR channel, the cytoplasmic region contains the binding sites for many modulators including calcium, adenine nucleotides, calmodulin and FKBPs. The smaller transmembrane region forms a calcium conducting pore to allow the movement of calcium out of the SR. From: Yano M, Yamamoto T, Ikeda Y, Matsuzaki M. (2006) Mechanisms of Disease: ryanodine receptor defects in heart failure and fatal arrhythmia. *Nat Clin Pract Cardiovasc Med*. 3(1): 43-52.

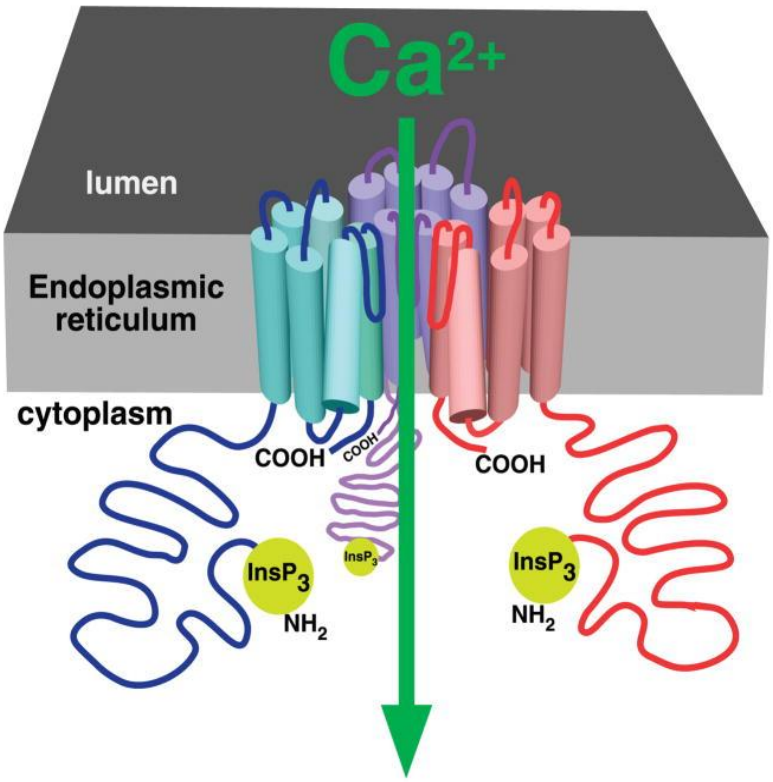


Figure 1.9: Schematic depiction of three of four IP_3R subunits of an IP_3R channel. Each subunit contains six transmembrane domains and part of the luminal loop connecting S5 and S6 of each monomer dips into the core to create a permeation pathway for calcium efflux during release. The bulky cytoplasmic N-terminal houses the binding domain of IP_3 and other regulator and modulatory molecules such as calcium and ATP. From: Foskett JK, White C, Cheung KH, Mak DO. (2007) Inositol trisphosphate receptor Ca^{2+} release channels. *Physiol Rev*. 87(2): 593-658.

A) B)

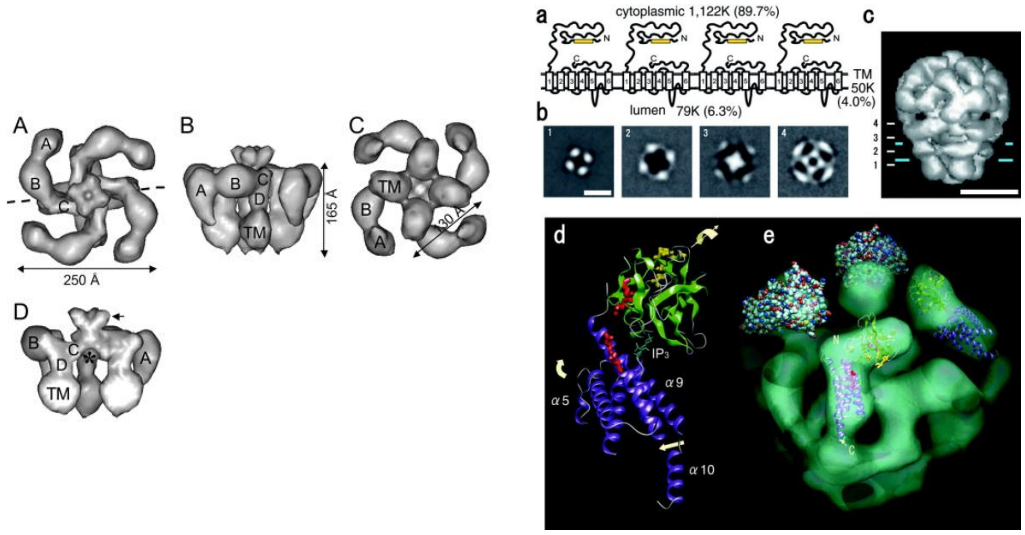


Figure 1.10: Structure of the IP_3R channel. Due to a lack of consensus among the three dimensional structures of the channel, several structural forms of it exist in the literature. In A) it is displayed as a four-fold symmetrical pinwheel of radial arms projecting from a central square mass, but in B) it is shown as a much denser unit with less arm extension from its core. From: Serysheva II, Bare DJ, Ludtke SJ, Kettlun CS, Chiu W, Mignery GA. (2003) Structure of the type 1 inositol 1,4,5-trisphosphate receptor revealed by electron cryomicroscopy. *J Biol Chem*. 278: 21319–21322. And Sato C, Hamada K, Ogura T, Miyazawa A, Iwasaki K, Hiroaki Y, Tani K, Terauchi A, Fujiyoshi Y, Mikoshiba K. (2004) Inositol 1,4,5-trisphosphate receptor contains multiple cavities and L-shaped ligand-binding domains. *J Mol Biol*. 336: 155–164.

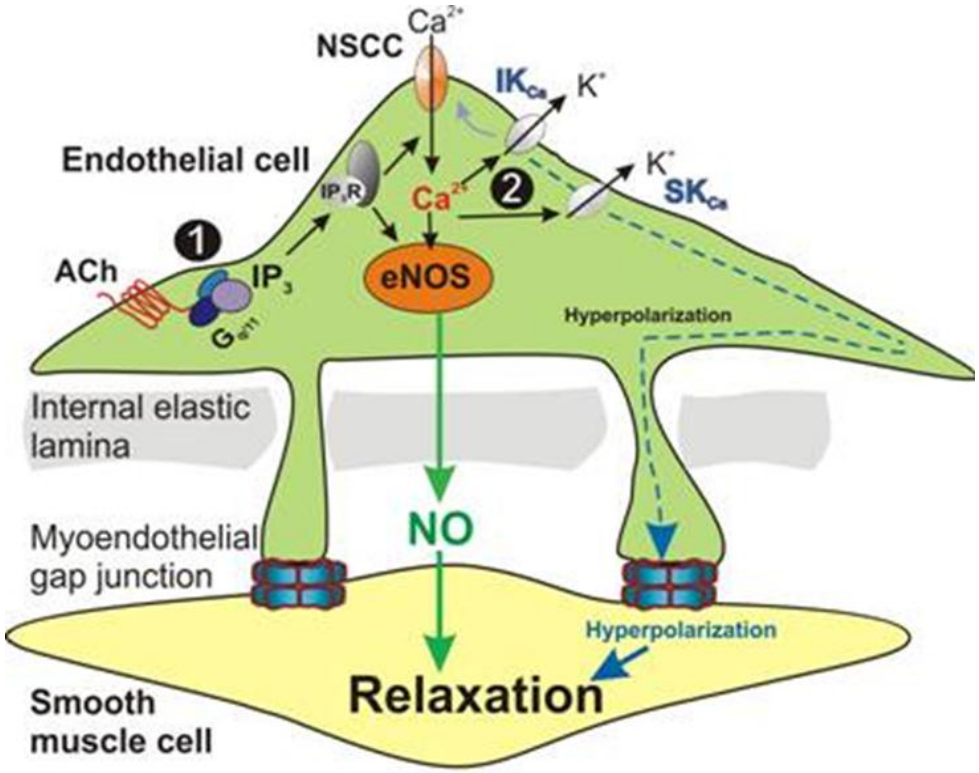


Figure 1.11: Schematic of pathways of endothelium-dependent vasorelaxation. Activation of endothelial GPCRs such as the muscarinic acetylcholine receptor leads to generation of IP_3 which stimulates the release of calcium from the endoplasmic reticulum. The subsequent increase in intracellular calcium stimulates the production of NO by NO synthase (NOS) and activates intermediate (IK_{Ca}) and small (SK_{Ca}) -conductance calcium activated potassium channels to elicit hyperpolarization of the endothelial cell membrane potential. The hyperpolarization can spread to the smooth muscle cells through gap junctions to reduce the open probability of VOCCs and so reduce calcium influx and cause relaxation.

Chapter 2: Methods

2.1 Animal care

Male Sprague Dawley rats (300-350g), fed standard chow and water *ad libitum*, were used for all experiments. Rats were housed in a facility accredited by the Canadian Council on Animal Care and the protocol for euthanasia was approved by the Animal Ethics Committee of the University of Alberta.

2.2 Pressure myography

Animals were euthanized via anaesthesia with isofluorane and followed by decapitation. The brain was carefully removed and placed in ice-cold, oxygenated physiological Kreb's solution containing (in mM) 118 NaCl, 25 $NaHCO_3$, 3.6 KCl, 1.2 $MgSO_4.7H_2O$, 1.2 KH_2PO_4, 11 glucose, 2.5 $CaCl_2$ and 0.02 EDTA. For calcium free solution, the recipe for Kreb's solution is followed with the omission of 2.5 $CaCl_2$ and added in its place 2 EGTA.

2-3 mm leak-free section of the middle cerebral artery distal to the Circle of Willis was isolated by clearing of surrounding tissue. The vessel was then mounted between two glass micropipettes in a 3 ml arteriograph chamber filled with Krebs's solution and secured with thin monofilament sutures (Figure 2.1A). The glass cannulae (borosilicate glass with OD of 1.2 mm and ID of 0.69 mm) were pulled using a Model P87 Flaming/Brown micropipette puller (Sutter Instruments, Novato, USA). The tips of the cannulae were polished using a sharpening stone.

After the vessels were secured, the arteriograph was placed on the stage of an inverted microscope (Nikon Eclipse, TE300). The vessels were connected to a peristaltic pump regulated by a pressure servo controller (Living Systems Instrumentation, St. Albans, Vermont) to allow manipulation of intraluminal pressure. Throughout the experiment, the vessel bath was oxygenated with 95% O_2/ 5% CO_2. Images of the vessel were captured using a Sony XC-73CE monochrome camera module and measurement of arterial diameter was achieved using an automated video dimension analyzer (Living Systems Instrumentation, St. Alban, Vermont). Diameter measurements were digitalized via a Powerlab data four channel acquisition system (AD Instruments, Colorado, USA) and all diameter and pressure measurements were recorded using the data acquisition software, LabChart 5, from AD Instruments (USA).

Initially the artery was pressurized to 80 mmHg in 20 mmHg steps; each pressure step was maintained for four minutes. After setting intraluminal pressure to 80 mmHg, the tissue was heated to 37 °C for one hour to allow for myogenic tone development (Figure 2.2A). This pressure was used because it approximates the mean arterial blood pressure of a rat *in vivo* (Faraci *et al.,* 1990). After the equilibration period, myogenic tone was assessed and vessels showing pronounced myogenic tone, as indicated by a diameter reduction of at least 100 μm at 80 mmHg, were used for experiments (Figure 2.1B).

Following the equilibration period, intravascular pressure was reduced to 20 mmHg for 15 minutes. A control pressure ramp was then conducted by increasing the pressure to 120 mmHg in increments of 20 mmHg. Each pressure step was held for 2-3 minutes or until the diameter response reached a plateau. The effect of pharmacological agents on myogenic reactivity was investigated by subjecting the arteries to the same pressure ramp in the presence and absence of the drugs. Drugs were either infused intraluminally (via the microcannulae) or extraluminally (by adding directly to the bath). All drug treatments were given for 15 minutes prior to the beginning of the pressure ramp except for N^G-Nitro-L-arginine methyl ester (L-NAME) which was given for 30 minutes. For a schematic representation of the pressure myography setup, see Figure 2.3.

2.3 Removal of the endothelium

In order to evaluate the role of the endothelium in modulating myogenic tone, some experiments were conducted using arteries in which the endothelial layer was removed. Once the vessel was mounted in the arteriograph and pressurized to 80 mmHg, a stream of air bubbles (0.35 ml) was passed through the lumen of the artery. The bath was then heated to physiological temperature of 35 °C and an hour allowed for equilibration and development of myogenic tone. A single dose of bradykinin (1 μM) was then used to assess the integrity of the endothelium. Successful removal of the endothelium was indicated by the lack of endothelium-dependent relaxation to bradykinin (Figure 2.2B).

2.4 Drug preparation

All chemicals and drugs were obtained from Sigma-Aldrich (Oakville, Canada) unless otherwise stated. All drugs were dissolved in water unless otherwise stated. Cyclopiazonic acid (CPA; Tocris, Ellisville, USA) was dissolved in DMSO (10 mM) and stored as a stock solution at -20 °C. Ryanodine (Tocris) was made weekly as a stock solution in DMSO (10 mM) and stored at room temperature. Xestospongin (Tocris; 10 mM) stock solution were made biweekly, dissolved in DMSO and stored in -20 °C. TRAM-34 (Tocris; 1 mM) were dissolved in DMSO and made bi-weekly and stored in room temperature. Apamin was dissolved in water as a stock solution (100 nM) and stored at -20 °C. Nifedipine (Sigma) was prepared daily as stock solution (1 mM) in DMSO. Bradykinin (Tocris) was prepared weekly as stock solution (10 mM) in water.

2.5 Data analysis and statistics

All data are presented as mean ± SEM. One-way analysis of variance (ANOVA) with Tukey's post-hoc test was used to determine differences in vessel diameter for multiple comparisons between experimental treatments. A paired Student's t-test was used to determine differences after drug treatments, where appropriate. Statistical calculations were performed using Prism 5 (Graphpad Software, La Jolla, USA). A level of $P<0.05$ is considered to be statistically significant (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). Figures of diameter response

traces were composed with Adobe Illustrator CS5 (Adobe Systems, San Jose, USA).

A) B)

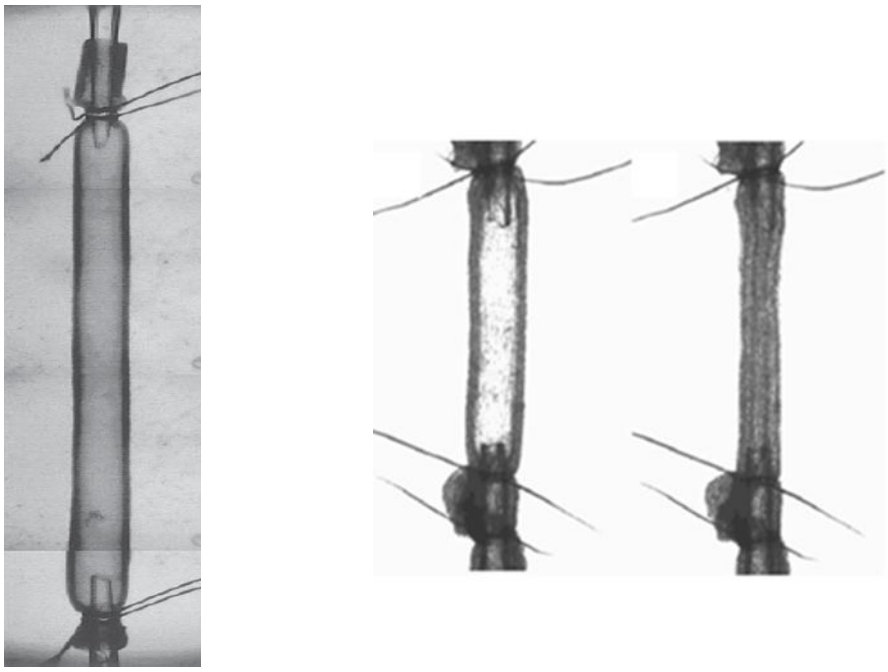
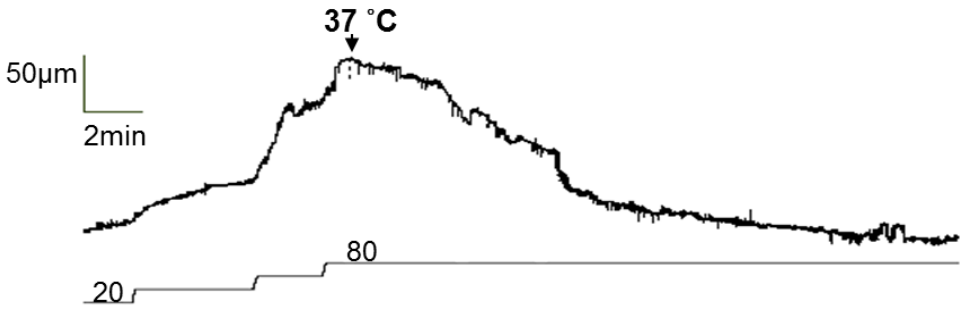


Figure 2.1A: Photograph of an artery mounted between two glass micropipettes in a 3 ml arteriograph chamber. The chamber was filled with Krebs's solution and the vessel was secured with thin monofilament sutures.

Figure 2.1B: An example demonstrating the diameter difference between a vessel before and after the development of myogenic tone. In both cases, the vessels were set at an intraluminal pressure of 80 mmHg. Only vessels with pronounced myogenic tone were used for experiments.

A)



B)

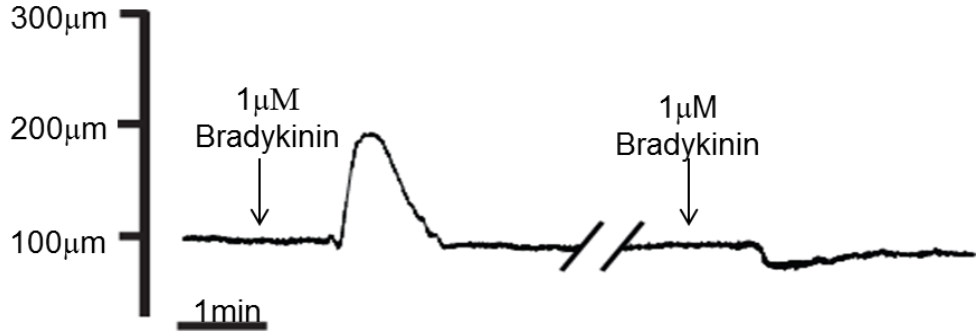


Figure 2.2A: Representative trace of pressure-diameter relationships for isolated segments of a rat middle cerebral artery during the development of myogenic tone. Intraluminal pressure was increased to 80 mmHg and the tissue was heated to 35 °C for one hour to allow for myogenic tone development. A spontaneous but gradual decrease in diameter was observed.

Figure 2.2B: Representative trace of the diameter responses to bradykinin (1 µM) for isolated segments of a rat middle cerebral artery. In endothelium intact arteries (on left), a transient dilation was observed following addition of bradykinin. In endothelium denuded arteries (on right), bradykinin did not elicit vasodilation but instead lead to a transient constriction.

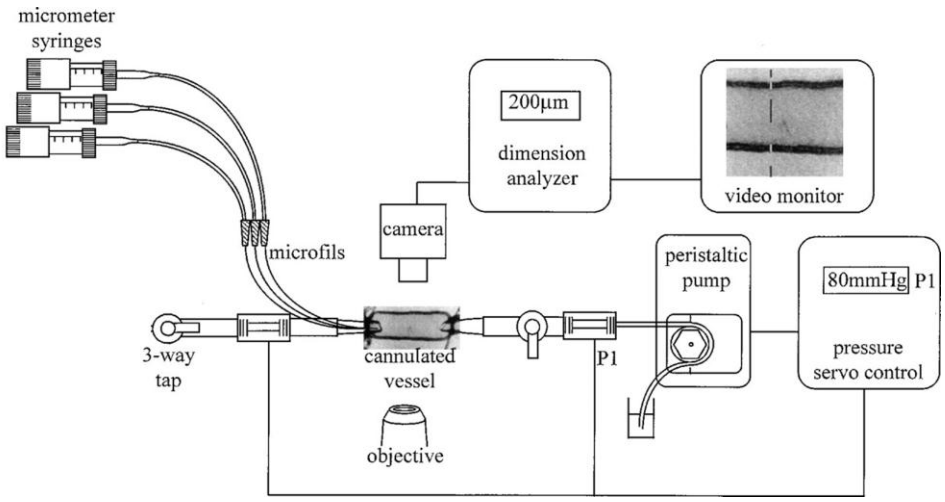


Figure 2.3: Schematic representation of pressure myography setup. Throughout the experiment, the diameter response of the vessel was monitored using the dimension analyzer and recorded in Chart. Drugs were either infused intraluminally (via the microcannulae) or extraluminally (by adding directly to the bath). The peristaltic pump regulated by a pressure servo controller allows the manipulation of intraluminal pressure. Adapted from: Doughty JM, Plane F, Langton PD. (1999) Charybdotoxin and apamin block EDHF in rat mesenteric artery if selectively applied to the endothelium. *Am J Physiol*. 276: H1107-12.

Chapter 3: Role of smooth muscle intracellular calcium stores in the myogenic response of isolated cerebral arteries

3.1 Introduction

As described in Chapter 1, although vascular smooth muscle cells contain well-defined calcium stores within the SR, the role of these stores in the myogenic response is ill-defined. While it is apparent that influx of extracellular calcium though VOCCs is essential for constriction in response to increases in intravascular pressure, limited functional evidence indicates that SR stores may also play an essential role.

Thus, the experiments described in this chapter were carried out to test the hypothesis that *the smooth muscle SR provides an internal source of calcium for the development of myogenic tone in middle cerebral arteries*.

3.2 Methods

All methods are described in Chapter 2. My approach in these experiments was to use endothelium-denuded arteries to investigate the role of SR function in the myogenic response in isolation from the influence of the endothelium. The role of SR in myogenic tone was investigated by applying pharmacological tools to actively interrupt SR function and observe the subsequent effect on pressure-induced changes in vessel diameter.

Four approaches were taken to disrupt SR function:

1) Inhibition of calcium release from SR using ryanodine (RyR channel inhibitor; 10 μM and 50 μM).
2) Direct depletion of SR stores using caffeine (RyR agonist; 1 mM and 10 mM).
3) Inhibition of calcium release from SR by xestospongin (IP_3R channel inhibitor; 10 μM).
4) Indirect depletion of the SR through the inhibition of calcium re-uptake using thapsigargin (500 nM) and CPA (10 μM), both inhibitors of SERCA.

3.3 Results

It is well established that extracellular calcium and VOCCs play an essential role in the myogenic response; application of an extracellular solution lacking calcium or inhibition of VOCCs abolishes myogenic tone. In such conditions, arteries dilate passively to increases in intravascular pressure (Davis and Hill, 1999). Therefore, initial experiments were carried out using the VOCC inhibitor nifedipine to demonstrate that, in my hands, cerebral arteries with myogenic tone could be obtained.

3.3.1 Effect of inhibition of VOCCs on myogenic tone

Under control conditions, when the intraluminal pressure was increased from 20 mmHg to 120 mmHg in 20 mmHg increments, only very small changes in

the diameter of middle cerebral arteries were observed; at an intravascular pressure of 20 mmHg, vessel diameter was 81.9±1.2 μm and at 120 mmHg it was 99.1±6.7 μm (n=6; Figure 3.1). Addition of nifedipine (1 μM) to vessels held at 20 mmHg resulted in an immediate dilation. Following a 15 minute equilibration period, application of the pressure ramp protocol resulted in passive dilation of the artery; at 20 mmHg the diameter was 129.2±10.6 μm (n=6; $p < 0.01$) and at 120 mmHg, the diameter was 236.1±7.7 μm (n=6; $p < 0.001$), more than twice the diameter observed at this pressure in the absence of nifedipine. The diameter measurements were statistically significant at all points between 20 to 120 mmHg (Figure 3.1).

Similarly, when the bathing solution was exchanged for Kreb's solution without calcium, passive dilation in response to increases in intravascular pressure were also observed; at 120 mmHg the diameter was 261±5.2 μm (n=6; $p < 0.001$; Figure 3.1).

The differences in vessel diameter between control and nifedipine or zero calcium conditions demonstrate that the vessels were able to generate an active myogenic response. Thus, in all subsequent studies, zero calcium solution was applied at the end of every experiment to reveal the maximum passive diameter of the vessels and thus confirm the presence of active myogenic tone.

3.3.2 Vehicle controls

As a number of the pharmacological agents to be used in my experiments (ryanodine, xestospongin, TRAM-34, CPA and thapsigargin) were to be dissolved in DMSO, prior to utilizing these drugs, I carried out experiments to ensure that this vehicle had no effect on myogenic response. In these experiments, bolus volumes of DMSO equivalent to those to be used in future experiments were applied to the tissues.

In these experiments, under control conditions, application of a pressure ramp from 20 to 120 mmHg resulted in no change in the diameter of the vessel as the pressure increased; at 20 mmHg the diameter was 71.9±9.3 μm (n=3) and at 120 mmHg the diameter was 68.3±4.5 μm (n=3; Figure 3.2). Application of 30 μl of DMSO to the tissue bath (this volume was chosen because this is the highest volume of DMSO that I may use at one time) for 15 minutes did not significantly affect the response of the arteries to increases in intravascular pressure: at 20 mmHg the diameter was 59.2±8.3 μm and at 120 mmHg it was 62.0±1.7 μm (n=3; $p > 0.05$; Figure 3.2). Following the addition of another 30 μl of DMSO, to give a total volume of 60 μl, the diameter at 20 mmHg was 51.3±2.6 μm and at 120 mmHg it was 62.2±2.8 μm (n=3; $p > 0.05$).

These results indicate that in middle cerebral arteries, myogenic response is not significantly affected by exposure to DMSO vehicle up to a volume of 60 μl (Figure 3.2).

3.3.3 Role of calcium regulation from SR in development of myogenic tone

3.3.3.1 Inhibition of SR calcium release via RyR channels

To investigate the role of SR in the myogenic response, ryanodine (10 μM) was used to close the RyR channel. Following construction of a control pressure-diameter ramp, tissues were pre-incubated with ryanodine (10 μM) for 15 minutes before the ramp was repeated. In the presence of ryanodine, at lower pressures, 20-60 mmHg, myogenic tone was maintained but rapid oscillatory events were observed. At 40 mmHg, the diameter was 75.5±4.8 μm and 72.4±5.0 μm in the absence and presence of ryanodine, respectively (n=6; $p > 0.05$). In contrast, at higher pressures of 80-120 mmHg, significant loss of myogenic tone was recorded. Under control conditions, the diameter at 100 mmHg was 77.4±5.49 μm (n=6) whereas in the presence of ryanodine, the diameter was 135.0±11.1 μm (n=6; $p < 0.01$). Due to the transient nature of the diameter changes observed in the presence of ryanodine, the measurements were taken as either an average of the peak and trough or the plateau response when the diameter response stabilized. A representative trace of the response of a vessel to increases in pressure in the absence and presence of ryanodine is shown in Figure 3.3.

At low micromolar and sub-micromolar concentrations, ryanodine locks the ryanodine channel into a sub-conductance state whereas at high micromolar concentrations, it completely closes the channel. In the literature, there are

variations in the reported concentration at which the two effects are observed. According to Herrmann-Frank *et al*. (1991), low micromolar concentration of ryanodine did not induce a sub-conducting state in RyR channels from canine and porcine aortic microsomal protein fractions. However, in bovine coronary arteries, 0.1-10 μM ryanodine was reported to induce a sub-conductance state in RyR channels and 20 & 50 μM closed it (Li *et al*., 2001). In cerebral arteries, 10 μM ryanodine was sufficient to block the release of calcium from RyR channels (Jaggar *et al.*, 1998; Wellman *et al.*, 2001). Due to these discrepancies, I also conducted another group of experiments utilizing a higher concentration of ryanodine to ensure the effect we saw at 10 μM was due to the closing of the RyR channels. In arteries treated with 50 μM ryanodine, a very similar effect to that observed with 10 μM ryanodine was seen. Large oscillations in diameter were detected following the addition of ryanodine and these oscillations persisted throughout the duration of the pressure ramp. At pressure ranges between 20-60 mmHg, no significant differences were detected between control conditions and ryanodine treatment. The diameter of the vessel at 60 mmHg was 70.4±5.7 μm and 98.2±3.5 μm in the absence and presence of ryanodine ($n=3$; $p > 0.05$). Above 80 mmHg, significant differences in arterial diameter were observed in the absence and presence of ryanodine; at 80 mmHg, control diameter was 69.9±4.6 μm ($n=3$) and in the presence of ryanodine it was 128.6±5.9 μm ($n=3$; $p < 0.05$). Figure 3.4b is a representative trace showing the

complete loss of myogenic tone as the diameter fluctuated wildly during pressure increases.

3.3.3.2 Depletion of calcium stores by caffeine

To further investigate the role of SR in the development of myogenic response, caffeine was utilized to actively deplete the SR stores. Caffeine is an alkaloid from plants which can increase the RyR calcium sensitivity; hence, basal cytoplasmic calcium level is sufficient to stimulate channel opening. Following construction of a control pressure-diameter ramp, tissues were pre-incubated with caffeine (1 mM) for 15 minutes before the ramp was repeated. Immediately upon the addition of caffeine to arteries held at 20 mmHg, a transient contraction was observed which was quickly followed by a rapid dilation. The average diameter at 20 mmHg increased from 84.2±8.7 μm to 115.6±10.6 μm (n=5; $p < 0.01$). This dilation however was not sustained in four out of five experiments and in those vessels, by the end of the 15 minute equilibration period, the average diameter had returned to 90.4±11.5 μm (n=4; $p > 0.05$). Construction of a pressure ramp showed no significant differences in the diameter response between control and caffeine-treated vessels. At 80 mmHg, the mean control diameter was 95.8±6.7 μm (n=5) and in the presence of caffeine it was 104.3±12.9 μm (n=5; $p > 0.05$; Figure 3.5).

Upon seeing the strong but transient effect of caffeine on diameter when first added, I reviewed the literature and repeated the experiments in the

presence of a higher concentration, 10 mM caffeine. In the presence of this higher concentration, a more sustained dilation was observed following the addition of caffeine; at the end of the 15 minute equilibration period, the mean diameter response at 20 mmHg was a sustained increase to 96.3±9.6 μm from 80.8±11.6 μm (n=5). However, the change was not significantly different ($p > 0.05$). During subsequent application of the pressure ramp, very rapid passive dilations were observed at each pressure step; at 80 mmHg, under control conditions the diameter was 95.8±6.7 μm whereas in the presence of caffeine it was 182.6±12.1 μm (n=5; $P < 0.001$; Figure 3.6).

3.3.3.3 Effect of inhibition of BK_{Ca} channels on caffeine induced dilation

The previous findings of Nelson *et al.,* (1997) led to the suggestion that release of sub-plasmalemmal calcium sparks from RyR-sensitive stores modulates the myogenic response by activation of BK_{Ca} channels to elicit smooth muscle hyperpolarization and so limit calcium entry through VOCCs. Thus, the effect of the BK_{Ca} channel inhibitor IbTX on the pressure-diameter relationship of middle cerebral arteries and on the effect of caffeine was investigated.

Following construction of a control pressure-diameter ramp, tissues were pre-incubated with IbTX (100 nM) for 15 minutes before the ramp was repeated. The presence of IbTX did not significantly alter the myogenic response to pressure steps between 20 and 120 mmHg. At 80 mmHg, vessel diameter was 77.5±3.9 μm under control conditions and 80.4±1.3 μm (n=3; $P > 0.05$) in the

presence of IbTX. Representative traces and mean data showing the pressure-diameter relationship for middle cerebral arteries in the absence and presence of IbTX are shown in Figure 3.7.

The effect of IbTX on the actions of caffeine was also investigated to determine if the loss of myogenic tone could be attributed to activation of BK_{Ca} channels. Following construction of a control pressure-diameter ramp, tissues were pre-incubated with IbTX (100 nM) for 15 minutes and then caffeine (10 mM) was added for a further 15 minutes before the ramp was repeated. As in the absence of IbTX, addition of caffeine caused an immediate dilation of the blood vessel. During the subsequent pressure ramp, very rapid passive dilations were observed at each pressure increment; at 80 mmHg, under control conditions the diameter was 77.5±3.9 μm and in the presence of caffeine and IbTX it was 187.0±16.4 μm (n=3; $P < 0.01$). Representative traces and mean data showing the pressure-diameter relationship for middle cerebral arteries in the absence and presence of caffeine and IbTX are shown in Figure 3.7.

3.3.3.4 Inhibition of IP_3-mediated calcium release

Previous studies have suggested that IP_3-mediated calcium release may contribute to the myogenic response (Potocnik and Hill, 2001) and so the effect of the IP_3R inhibitor, xestospongin (10 μM) on the pressure-diameter response of isolated middle cerebral arteries was investigated. Following construction of a control pressure-diameter ramp, cerebral arteries were pre-incubated with

xestospongin for 15 minutes before the ramp was repeated. Upon addition of xestospongin to arteries held at 20 mmHg, no changes in the diameter were detected. Construction of a pressure ramp in the presence of xestospongin revealed that the pressure-diameter relationship was not significantly different and myogenic response was maintained; at 80 mmHg the vessel diameter was 92.6±5.5 μm and 100.3±14.4 μm (n=3; P >0.05) in the absence and presence of xestospongin, respectively (Figure 3.8).

3.3.3.5 Disruption of calcium reuptake through SERCA

SERCA is the main calcium reuptake mechanism for refilling the intracellular SR calcium stores in smooth muscle cells. In order to further investigate the role of the smooth muscle SR in the myogenic response, functioning of SR was disrupted by blocking SERCA using CPA or thapsigargin. This approach should lead to eventual depletion of the SR calcium stores.

Following construction of a control pressure-diameter ramp, cerebral arteries were pre-incubated with CPA (10 μM) for 15 minutes before the ramp was repeated. CPA is a mycotoxin produced by some strains of mold which is thought to reduce SERCA activity by reversibly reducing its affinity (K_D) for ATP. Upon the addition of CPA to arteries held at 20 mmHg, a small transient constriction was observed in one out of four of our experiments. No other changes in vessel diameter were observed during the 15 minutes equilibration period. Construction of a pressure ramp in the presence of CPA revealed that

the pressure-diameter relationship was not significantly different and myogenic tone was maintained; at 80 mmHg the vessel diameter was 73.5±8.3 μm and 79.3±10.6 μm (n=4; p >0.05) in the absence and presence of CPA, respectively. Representative traces and mean data showing the pressure-diameter relationship for middle cerebral arteries in the absence and presence of CPA are shown in Figure 3.9.

To confirm the findings with CPA, experiments were also carried out with thapsigargin (500 nM), another inhibitor of SERCA. Unlike CPA, thapsigargin is a plant-derived sesquiterpene lactone which irreversibly locks SERCA in a "dead-end complex" in the catalytic cycle to inhibit SERCA activity. Following construction of a control pressure-diameter ramp, cerebral arteries were pre-incubated with thapsigargin (500 nM) for 15 minutes before the ramp was repeated. Upon the addition of thapsigargin to arteries held at 20 mmHg, no immediate change in vessel diameter was observed. In the presence of thapsigargin, the pressure-diameter relationship was not significantly different from control (p >0.05). Under control conditions, the vessel diameter at 80 mmHg was 77.3±5.1 μm (n=6) and in the presence of thapsigargin, the diameter was 68.6±4.5 μm (n=6; p >0.05). Representative traces and mean data showing the pressure-diameter relationship for middle cerebral arteries in the absence and presence of thapsigargin are shown in Figure 3.10.

3.4 Discussion:

These data indicate that the release of calcium from the SR through RyR channels contributes to the myogenic response in rat isolated middle cerebral arteries. However, inhibition of SERCA within the smooth muscle cells did not alter myogenic reactivity, suggesting that this pump does not contribute to regulation of myogenic reactivity. Also, the lack of effect of IbTX, a selective inhibitor of BK_{Ca} channels, on the myogenic response indicates that the postulated negative feedback mechanism by which activation of BK_{Ca} channels limits myogenic reactivity does not play a major role in these arteries.

The smooth muscle cell SR is a major source of calcium for agonist-induced constriction of blood vessels. It is well established that endogenous agonists, such as norepinephrine and angiotensin, induce contraction though the GPCR cascade involving PLC–mediated cleavage of phosphatidylinositol 4,5-bisphosphate and subsequent formation of IP_3 and DAG. IP_3 then stimulates release of calcium from the SR with the resultant increase in cytoplasmic calcium which activates the contractile machinery (reviewed by Webb, 2003).

The evidence on the role of SR calcium stores in myogenic vasoconstriction is more disparate. Hence, my hypothesis in undertaking this study was that *the smooth muscle SR provides an internal source of calcium for the development of myogenic tone in middle cerebral arteries.* In some myogenically active vessels such as rabbit cerebral arteries (Knot and Nelson,

1998) and rat skeletal muscle arteries (Kotecha and Hill, 2005), the pressure-diameter relationship is biphasic with passive dilation at lower pressure (up to 40-50 mmHg) followed by active constriction at higher pressures (>60 mmHg), such that vessel diameter at 120 mmHg is comparable to that seen at 20 mmHg. As shown in Figure 3.1, rat middle cerebral arteries do not show passive dilation and vessel diameter is maintained across the entire pressure range (20-120 mmHg). The fact that the observed effect is active myogenic tone and not just vessel stiffness is demonstrated when on removal of extracellular calcium or addition of the VOCC blocker nifedipine, under which conditions passive dilation is seen across the entire pressure range. Within the literature, there is significant variation in the reported level of myogenic response seen in rat cerebral arteries and also in how it is described (e.g. % active tone, % passive dilation). This makes inter-study comparisons difficult. Nonetheless, a recent study by Mufti *et al*., (2010) showed a similar pattern of maintenance of the arterial diameter across the pressure range of 20-100 mmHg in rat isolated posterior cerebral arteries as those found in my study. Also, comparison of the active and passive pressure-diameter relationship curves obtained during my experiments show that, for example, at 80 mmHg, the vessel diameter is 38.5% of the passive diameter. This indicates a strong myogenic response, stronger than in many published studies and indicative of healthy tissues.

In order to investigate the role of RyR channels in the myogenic response, experiments were carried out using ryanodine. Ryanodine, a plant-derived alkaloid from members of the genus *Ryania,* has complex pharmacology. Its effect on the conductance of RyR channels is highly concentration-dependent. At low, sub-micromolar concentrations, it locks the channel into a sub-conductance state; at high micromolar concentrations, it completely closes the channel (Rousseau *et al.,* 1987; Meissner *et al.,* 1994). This concentration-dependence may be due to the presence of two binding sites on RyR channels; a high affinity site (K_D ~ 1-10 nM) binding to which results in channel activation or sub-conductivity, and a low affinity site (K_D ~ 1-10 μM) binding to which leads to channel inhibition (Sutko *et al.,* 1994). Commonly, ryanodine is used to deplete SR calcium stores by causing the RyR channels to remain in a semi-conducting state. However, in the present study, I am interested in finding out whether the SR is involved in the myogenic response through the release of calcium through the RyR channels. Thus, concentrations in the mid-micromolar range were used to inhibit the release of calcium from the SR by closing the channel.

Application of ryanodine (10 and 50 μM) had a biphasic effect on the myogenic response; at lower pressures rapid oscillatory events were observed but vessel diameter was maintained. At higher pressures, rapid oscillatory events persisted and a significant loss of myogenic tone was observed (Figure 3.3 & 3.4). There may be two possible explanations for such an effect. Firstly, at the

lower pressure range, the degree of active tone (as determined by the difference between pressure-diameter relationships in the presence and absence of zero extracellular calcium) is less and so a smaller amount of calcium is required for maintaining a constant diameter. Hence, the vessel can still maintain its diameter even if calcium release via the RyR channels is reduced. Secondly, an alternate explanation might be due to the use dependence property of ryanodine's actions (Laporte *et al.,* 2004). High affinity binding occurs when the RyR channels are in the open state. At lower pressure range, RyR channels are less active. Thus, high affinity binding does not occur and myogenic tone can be maintained. As the pressure increases, more RyR channels become activated and subsequently blocked as ryanodine begins binding to them with high affinity, resulting in the loss of myogenic tone in the upper part of the pressure range.

Thus, these findings indicate that blocking of RyR channels compromises the myogenic response and so calcium release via these channels may be important for myogenic constriction. Very few studies have examined the effects of ryanodine in myogenically active arteries, especially in cerebrals. Watanabe *et al.,* (1993) found that ryanodine (10 μM) caused constriction of rat skeletal muscle arterioles held at a constant intravascular pressure of 40 mmHg. The diameter gradually decreased over 15-20 minutes to give a new steady state diameter which was smaller than control. In the same study, ryanodine did not significantly alter the diameter of myogenically active mesenteric arterioles. In

hamster cremaster arterioles (Westcott and Jackson, 2011), rat urethral arterioles (Borisova *et al.,* 2009), human mesenteric arteries (Coats *et al.,* 2001) and rat posterior cerebral arteries (McCarron *et al.,* 1997), myogenic response was not found to be affected by ryanodine. In contrast, in a more recent study of posterior rat cerebral arteries pre-treated with ryanodine (50 μM), passive dilation was observed to pressure steps up to 60 mmHg, indicating a loss of myogenic response (Mufti *et al.,* 2010). Different from my data, at higher pressures no further dilation was observed in their study and vessel diameter was maintained. The authors' speculated that at higher intravascular pressures the contractile apparatus is more sensitized to calcium and so a reduction in RyR channel-mediated calcium release has less effect. Lastly, Löhn *et al.,* (2001) claimed that myogenic reactivity was reduced in isolated cerebral arteries from mice lacking the RyR3 channel sub-type. However, examination of their data shows that the cerebral vessels from their wild-type mice lacked myogenic tone thus making interpretation of their data rather difficult.

The contention that RyR channel-mediated calcium release may be important for myogenic constriction is in contrast to the findings of Nelson *et al.* (1997). They suggested that in rabbit cerebral arteries, rather than contributing to constriction, release of sub-plasmalemmal calcium sparks through RyR channels plays a modulatory role in the myogenic response by activating BK_{Ca} channels to limit calcium entry through VOCCs (see section 1.8.3). In my

experiments, IbTX, the selective inhibitor of BK_{Ca} channels did not significantly alter the pressure-diameter relationship in rat isolated middle cerebral arteries indicating that calcium spark-activated BK_{Ca} channel does not play a role in regulating the myogenic response in rat middle cerebral arteries (Figure 3.7). Addition of IbTX from the same aliquots enhanced spontaneous contractile activity in rat isolated colon, demonstrating that they are pharmacologically active (personal communication Dr. Paul Kerr).

Although widely quoted as an important mechanism in limiting myogenic constriction, most studies on RyR-mediated calcium sparks have actually been carried out using isolated vascular smooth muscle cells (Bonev *et al.,* 1997; Jaggar *et al.,* 2002) or in unpressurized vessels (Curtis *et al.,* 2004; Gollasch *et al.,* 1998; Jaggar and Nelson*,* 2000) and so most of the work done were not in myogenically active tissues. Furthermore, recent functional studies have also shown limited effects of BK_{Ca} channel blockade on myogenic tone and have suggested that the "fine-tuning" role of calcium spark-activated BK_{Ca} channel activity may contribute to differing degrees between vascular beds (Chlopicki *et al.,* 2001; Kotecha and Hill, 2005). Ahmed *et al.,* (2004) showed that activation of BK_{Ca} channels does not contribute to regulation of myogenic tone in porcine cerebral arteries. Westcott and Jackson (2011) showed that ryanodine-sensitive calcium sparks were present in myogenically active hamster cremaster muscle arteries but not in downstream arterioles from the same preparation (Westcott

and Jackson, 2011). Furthermore, ryanodine-sensitive sparks were not found in myogenic urethral arterioles (Borisova *et al.,* 2009), again supporting the proposal that the contribution of this mechanism to modulation of the myogenic response is not uniform across the vasculature.

Ryanodine has several perceived advantages over other agents used to deplete the SR calcium store; ryanodine retains a high target selectivity and its mechanism of action is well documented in the cardiovascular literature (Dibb *et al.* 2007; Gyorke and Terentyev, 2008). However, in the present study, further support for the proposal that RyR channel-mediated calcium release contributes to myogenic vasoconstriction was obtained by depleting these stores using caffeine, a plant-derived alkaloid which acts on RyR channels. At a concentration of 1 mM, caffeine caused a transient dilation when applied to the arteries but did not alter the myogenic response. In contrast, at a higher concentration (10 mM), caffeine greatly reduced myogenic tone and the arteries dilated passively at each pressure step (Figure 3.5 & 3.6). Pre-incubation of arteries with the BK_{Ca} channel inhibitor IbTX did not alter the effect of caffeine and so it is unlikely to involve activation of these channels (Figure 3.7).

As with ryanodine, few studies have actually examined the effects of caffeine on myogenically active arteries even though a large number of studies have examined its effects in isolated smooth muscle cells and extrapolated to make conclusions regarding the physiological role of calcium stores (e.g. Gollasch

et al., 1998). In line with my observation, caffeine (20 mM) caused a large dilation of myogenically active rat cremaster arterioles held at 70 mmHg (Potocnik and Hill, 2001) and a lower concentration (1 μM) caused dilation of pressurized porcine newborn cerebral arteries (Ahmed *et al.,* 2004). However, interpretation of the effects of caffeine is difficult as there are reports of multiple targets for its actions including inhibition of VOCCs and inhibition of phosphodiesterase enzymes (Laporte *et al.,* 2004).

It is well established that following activation of GPCRs, IP_3-mediated calcium release is a major contributor to vasoconstriction. In contrast, data describing the importance of IP_3 in the myogenic response is limited. Narayanan *et al.* (1994) demonstrated in renal arcuate arteries that both IP_3 and DAG increase as intraluminal pressure is raised and the putative PLC inhibitor, U-73122, has been reported to inhibit myogenic responsiveness in rat cerebral (Osol *et al.,* 1993) and renal (Inscho *et al.,* 1998) arterioles, and in human sub-cutaneous resistance arteries (Coats *et al.,* 2001). However, U-73122 has recently been shown to deplete smooth muscle SR calcium stores via inhibition of SERCA (Macmillan and McCarron, 2010).

In my experiments, xestospongin, an inhibitor IP_3 receptors, did not alter the pressure-diameter relationship in cerebral arteries (Figure 3.8). As a control, the same batch of xestospongin caused inhibition of myoendothelial feedback in phenylephrine-induced vasoconstriction of mesenteric arteries (Kerr *et al*., 2011;

Figure 3.11). Kotecha and Hill (2005) also found that in rat skeletal muscle arterioles blockade of IP_3 receptors with 2-aminoethoxydiphenyl borate (2-APB) was without effect on myogenic reactivity. In contrast, in rat cremaster muscle feed arteries and arterioles, xestospongin, 2-APB, and the inhibition of PLC caused vasodilation (Westcott and Jackson, 2010). In the same vessels, Potocnik and Hill (2001) found that 2-APB totally inhibited the mechanical response to an acute intraluminal pressure step from 50 to 120 mmHg (loss of myogenic reactivity) but only exerted a modest dilator effect on the same arterioles maintained at 70 mmHg conditions (maintenance of myogenic tone). The authors suggested that the IP_3 receptors, or related mechanism, are involved in the initiation of the myogenic response; once steady-state contraction is achieved, the requirement for IP_3 receptor-mediated mechanisms is reduced. These findings may highlight the contrasting roles played by RyRs and IP_3Rs in myogenic tone in different vessels.

As SERCA is the main calcium reuptake mechanism for refilling the intracellular SR calcium stores in smooth muscle cells, the effect of blocking SERCA on myogenic responses was investigated. As shown in Figures 3.9 and 3.10, inhibition of SERCA by addition of either CPA or thapsigargin did not significantly affect the pressure-diameter relationship, i.e. the myogenic response was not changed. One reason for this could have been that only a small amount of SR calcium release was required and that inhibiting SERCA for

the duration of my experiments did not sufficiently deplete the stores. However, extending the pre-incubation time with CPA to 60 minutes still did not cause any alteration in the myogenic response. It was reported that in cultured smooth muscle cells the basal leak from SR is approximately 22% per minute and so the SR in my vessels should be depleted within a few minutes (Missiaen *et al.,* 1996). Nonetheless, similar findings were observed by Knot *et al.,* (1995) in rat posterior cerebral arteries and Takenaka *et al*., (1998) in rat interlobular arteries. However, more recently, Mufti *et al*., (2010) showed that in the rat posterior cerebral artery, application of thapsigargin, in the presence of the VOCC channel blocker diltiazem, caused further loss of myogenic tone. Unfortunately, the effects of thapsigargin alone were not shown. In rat cremaster arteries, addition of thapsigargin to vessels held at 70 mmHg caused a biphasic change in diameter with an initial phase of dilation followed by constriction to 60% of basal diameter over a period of about 5 minutes (Potocnik and Hill, 2001).

In conclusion, my data indicate that intracellular SR calcium stores in smooth muscle cells are important in myogenic constriction in rat middle cerebral arteries. I have shown that RyR channels are required for generation of myogenic response such that inhibition of these channels will lead to a dysregulation of diameter control. In contrast, I found no evidence for a role of IP_3 receptors, SERCA or BK_{Ca} channels.

A)

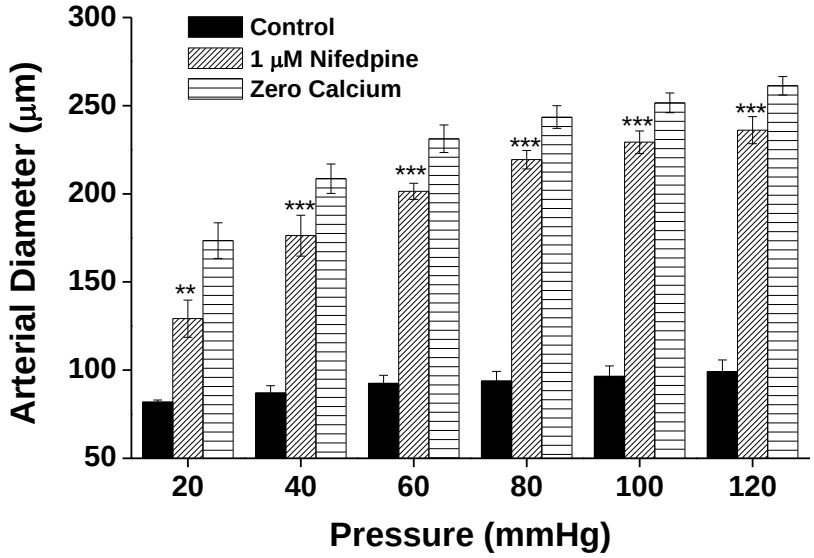
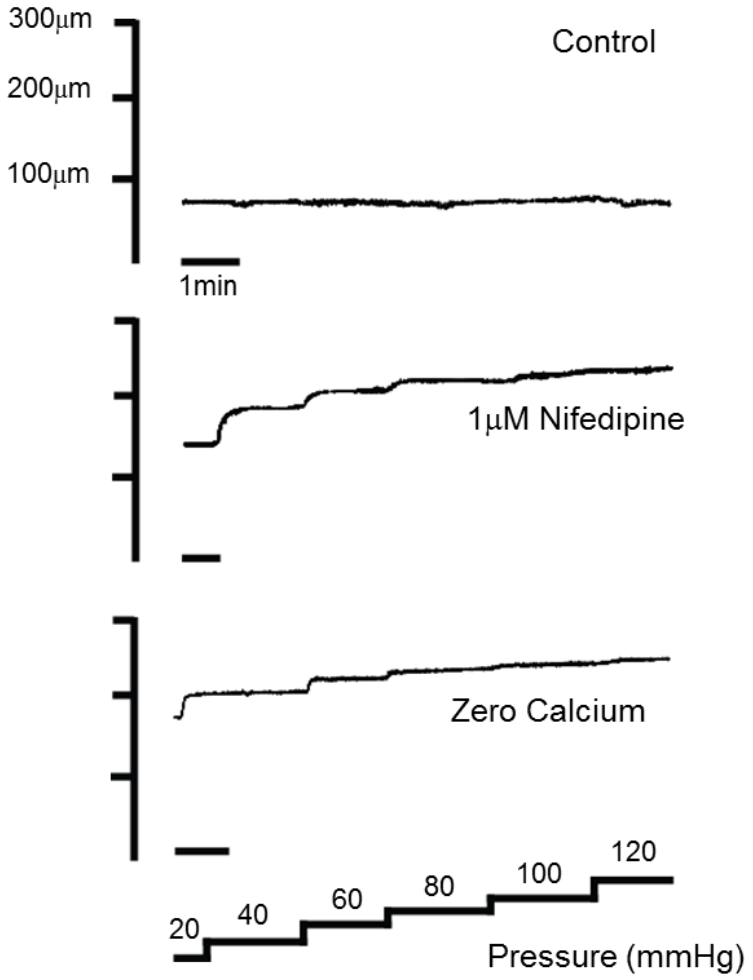


Figure 3.1: Myogenic reactivity of rat isolated middle cerebral arteries.

A) Mean pressure-diameter relationships for isolated segments of rat middle cerebral artery mounted in a pressure myograph and subjected to increases in pressure from 20-120 mmHg in 20 mmHg increments. Mean responses are shown in the presence and absence of nifedipine (1 μM) or zero extracellular calcium (passive response). Values are presented as mean ± SEM (n=6). When compared to control, a level of P <0.05 is considered to be statistically significant (* p <0.05, **p <0.01, ***p <0.001).

B)



B) Representative traces of pressure-diameter relationships for isolated segments of a rat middle cerebral artery in the presence and absence of nifedipine and zero extracellular calcium.

A)

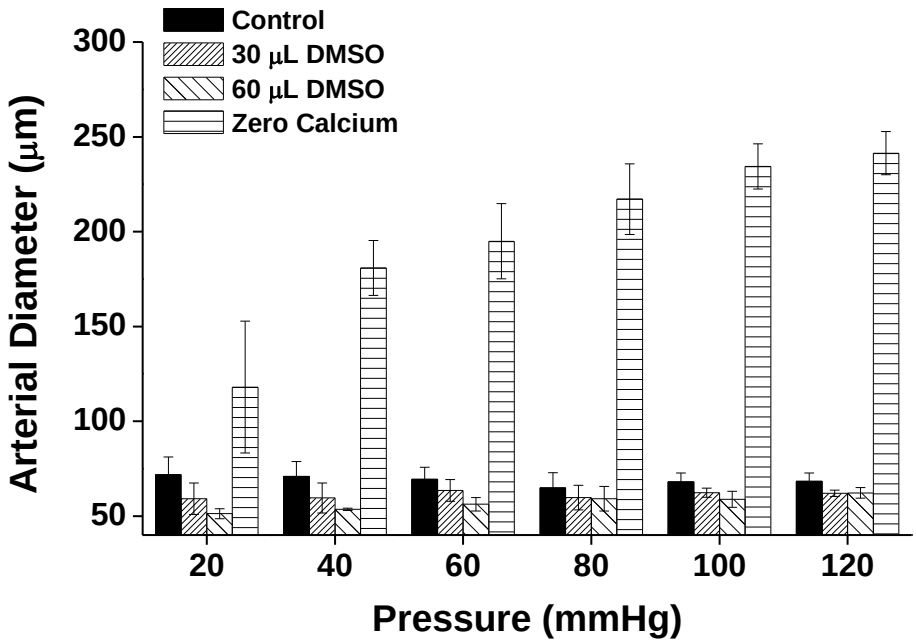
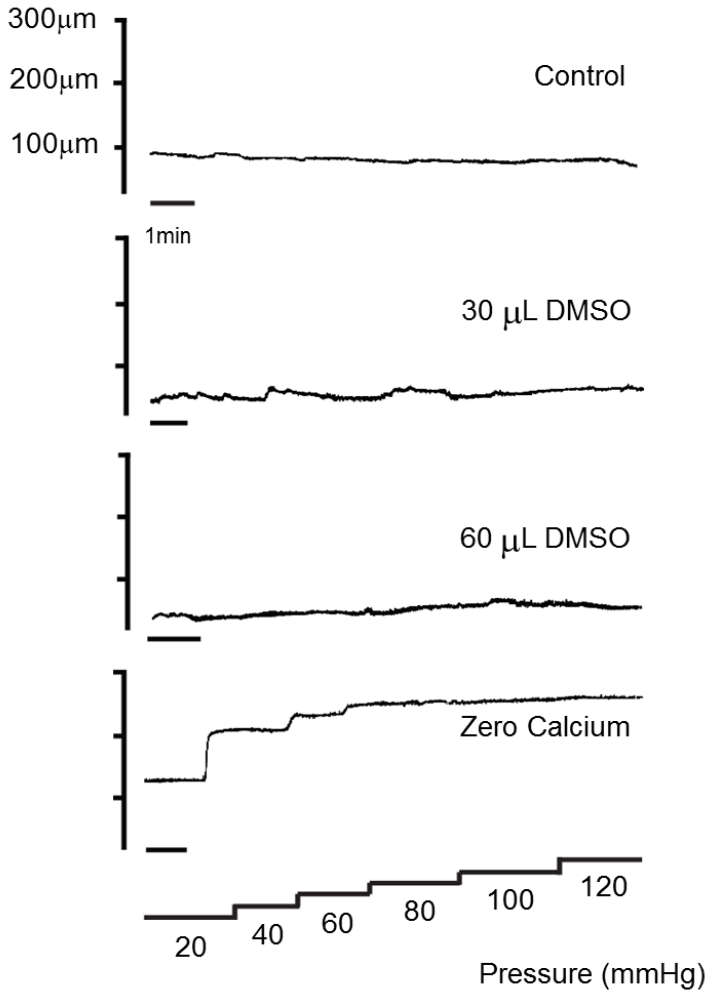


Figure 3.2: DMSO had no effect on the myogenic response of rat isolated middle cerebral arteries.

A) Mean pressure-diameter relationships for isolated segments of rat middle cerebral artery mounted in a pressure myograph and subjected to increases in pressure from 20-120 mmHg in 20 mmHg increments. Mean responses are shown in the presence and absence of DMSO (30 μl and 60μl) or zero extracellular calcium (passive response). Values are presented as mean ± SEM (n=3).

B)



B) Representative traces of pressure-diameter relationships for isolated segments of a rat middle cerebral artery in the presence and absence of DMSO and zero extracellular calcium.

A)

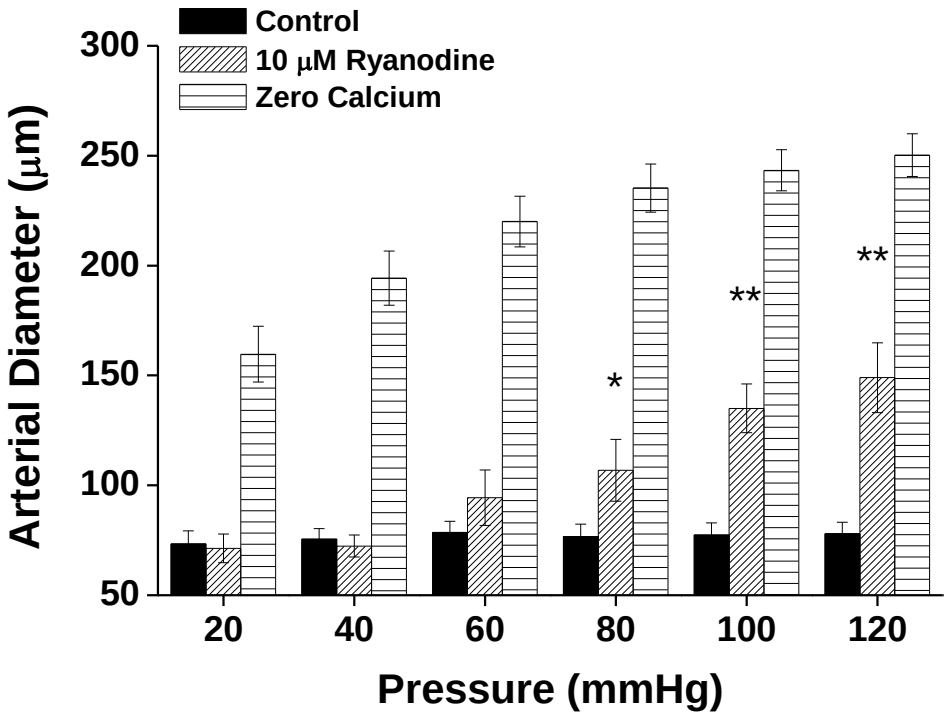
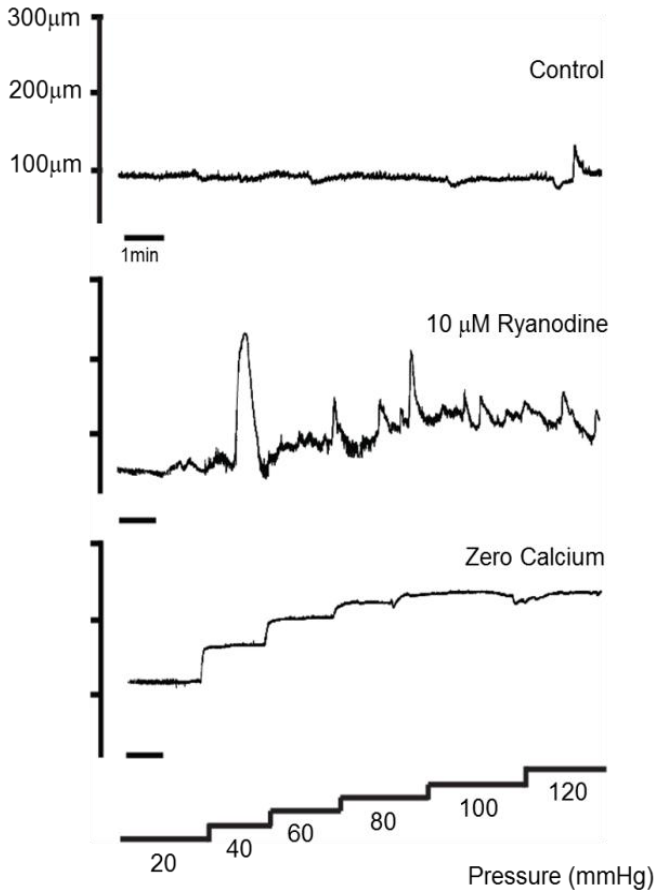


Figure 3.3: Calcium release from the SR through RyR channel is required for the myogenic response of rat isolated middle cerebral arteries.

A) Mean pressure-diameter relationships for isolated segments of rat middle cerebral artery mounted in a pressure myograph and subjected to increases in pressure from 20-120 mmHg in 20 mmHg increments. Mean responses are shown in the presence and absence of ryanodine (an inhibitor of RyR; 10 μM) or zero extracellular calcium (passive response). Values are presented as mean ± SEM (n=6). When compared to control, a level of P <0.05 is considered to be statistically significant (*p <0.05, **p <0.01, ***p <0.001).

B)



B) Representative traces of pressure-diameter relationships for isolated segments of a rat middle cerebral artery in the presence and absence of ryanodine and zero extracellular calcium.

A)

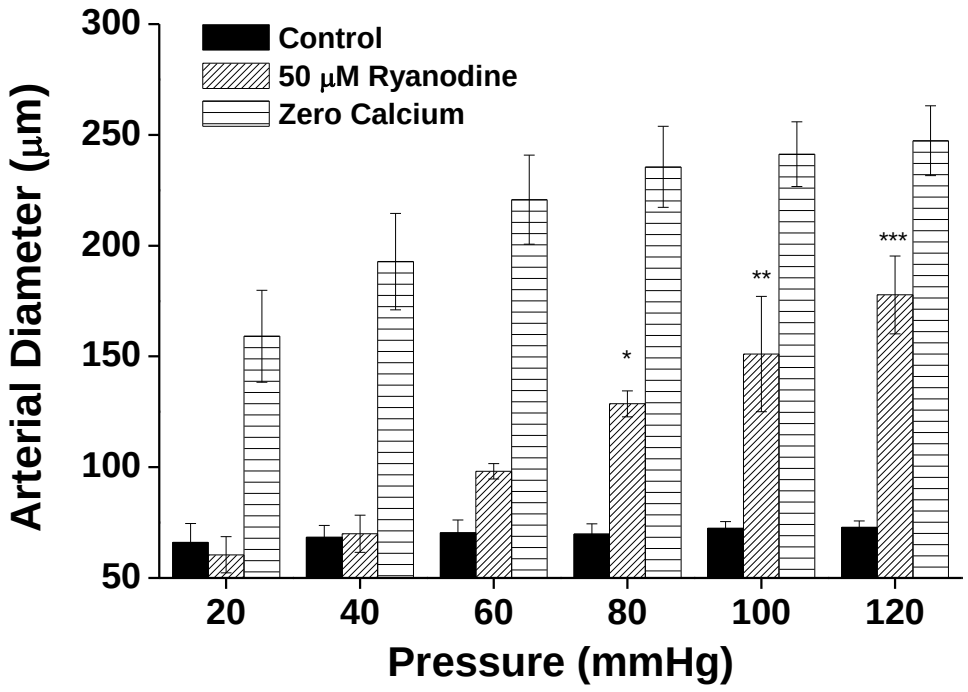
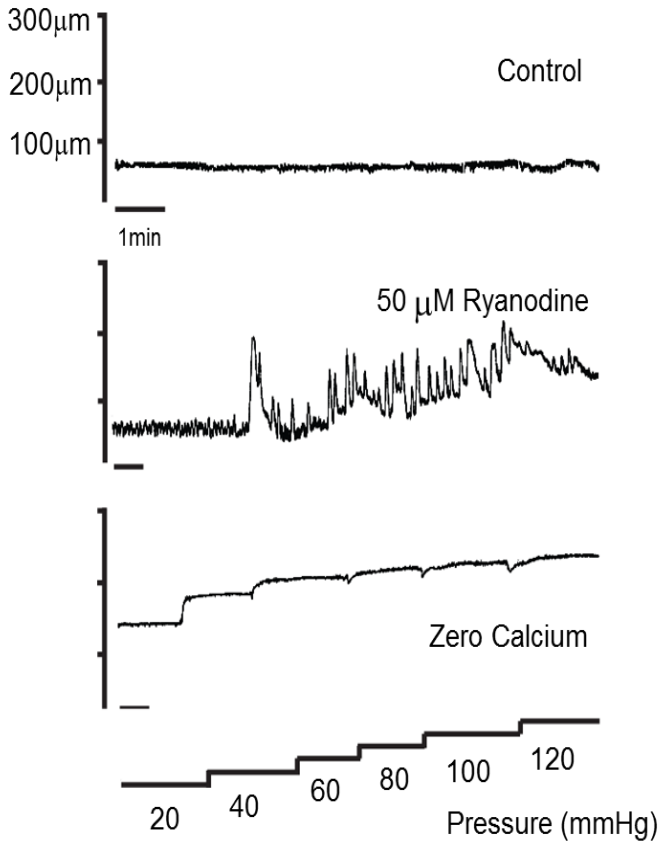


Figure 3.4: Calcium release from SR through RyR channel is required for the myogenic response of rat isolated middle cerebral arteries.

A) Mean pressure-diameter relationships for isolated segments of rat middle cerebral artery mounted in a pressure myograph and subjected to increases in pressure from 20-120 mmHg in 20 mmHg increments. Mean responses are shown in the presence and absence of ryanodine (an inhibitor of RyR; 50 μM) or zero extracellular calcium (passive response). Values are presented as mean ± SEM (n=3). When compared to control, a level of P <0.05 is considered to be statistically significant (*p <0.05, **p <0.01, ***p <0.001).

B)



B) Representative traces of pressure-diameter relationships for isolated segments of a rat middle cerebral artery in the presence and absence of ryanodine and zero extracellular calcium.

A)

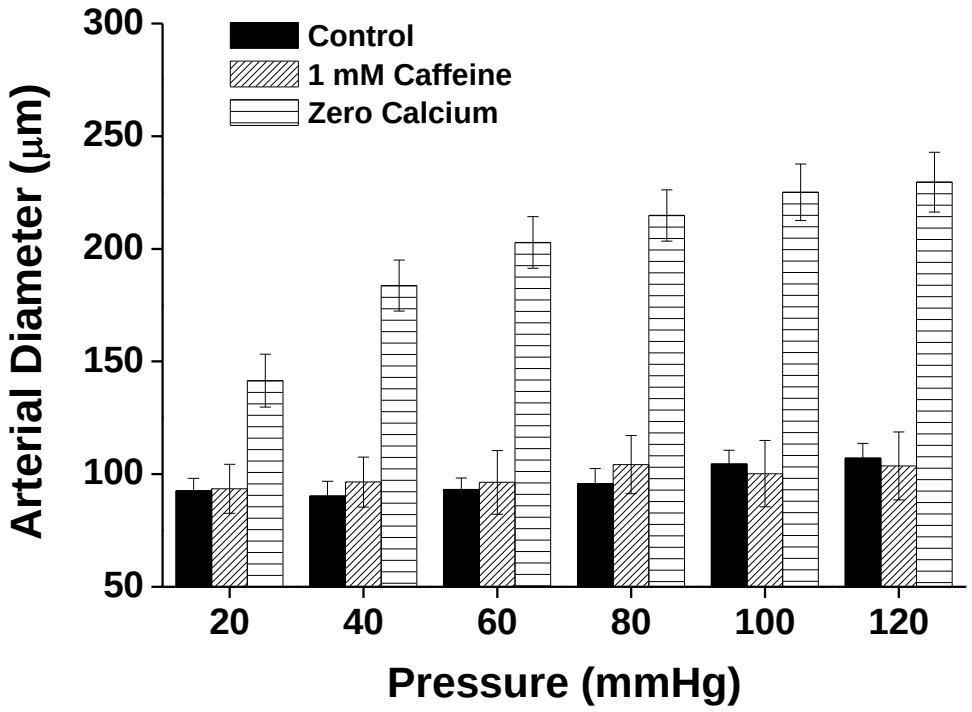
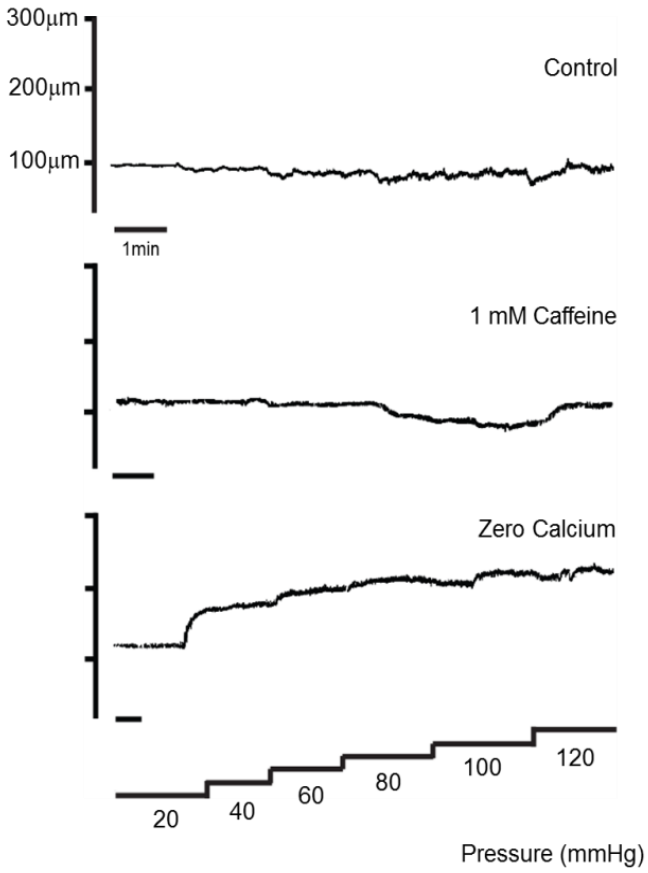


Figure 3.5: Release/depletion of calcium from SR (1 mM caffeine) had no effect on the myogenic response of rat isolated middle cerebral arteries.

A) Mean pressure-diameter relationships for isolated segments of rat middle cerebral artery mounted in a pressure myograph and subjected to increases in pressure from 20-120 mmHg in 20 mmHg increments. Mean responses are shown in the presence and absence of caffeine (an agonist of RyR; 1 mM) or zero extracellular calcium (passive response). Values are presented as mean ± SEM (n=5).

B)



B) Representative traces of pressure-diameter relationships for isolated segments of a rat middle cerebral artery in the presence and absence of caffeine and zero extracellular calcium.

A)

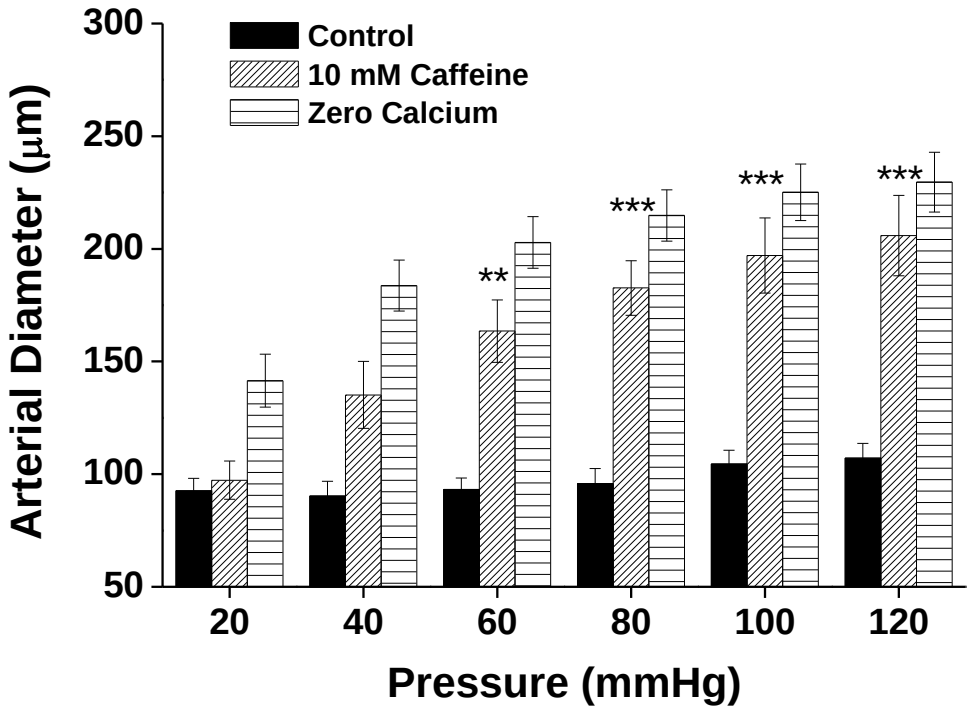
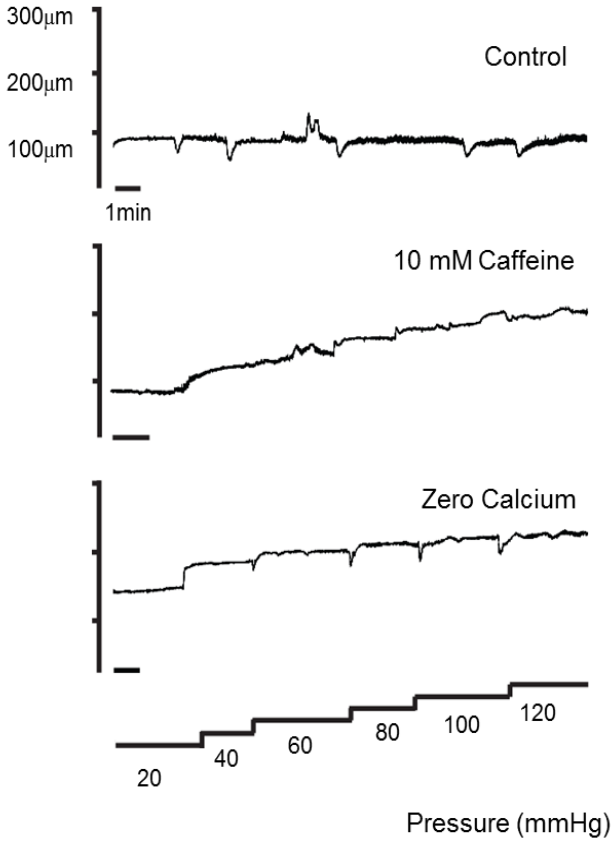


Figure 3.6: Release/depletion of calcium from the SR (10 mM caffeine) led to a loss in myogenic response of rat isolated middle cerebral arteries.

A) Mean pressure-diameter relationships for isolated segments of rat middle cerebral artery mounted in a pressure myograph and subjected to increases in pressure from 20-120 mmHg in 20 mmHg increments. Mean responses are shown in the presence and absence of caffeine (an agonist of RyR; 10 mM) or zero extracellular calcium (passive response). Values are presented as mean ± SEM (n=5). A level of $P < 0.05$ is considered to be statistically significant when compared to control (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

B)



B) Representative traces of pressure-diameter relationships for isolated segments of a rat middle cerebral artery in the presence and absence of caffeine and zero extracellular calcium.

A)

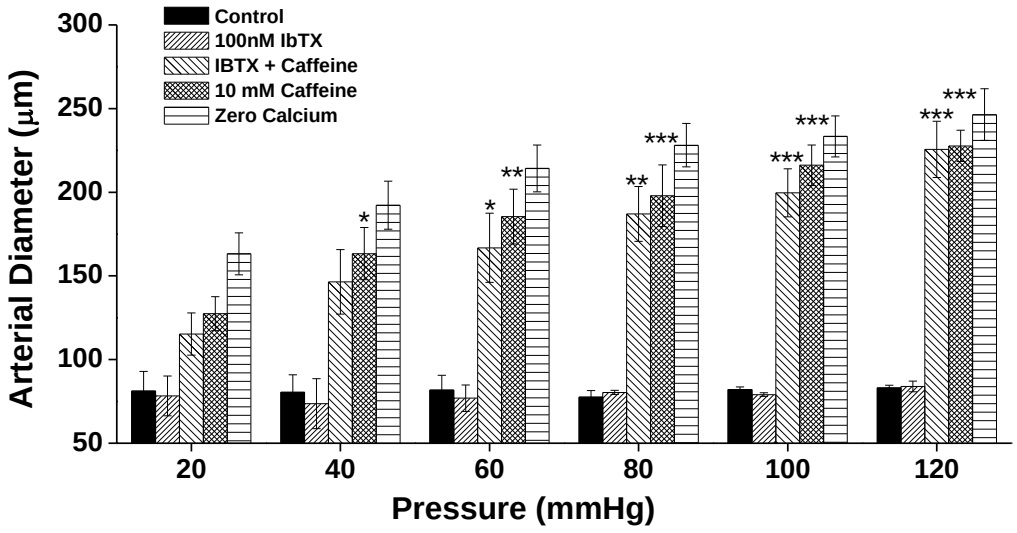
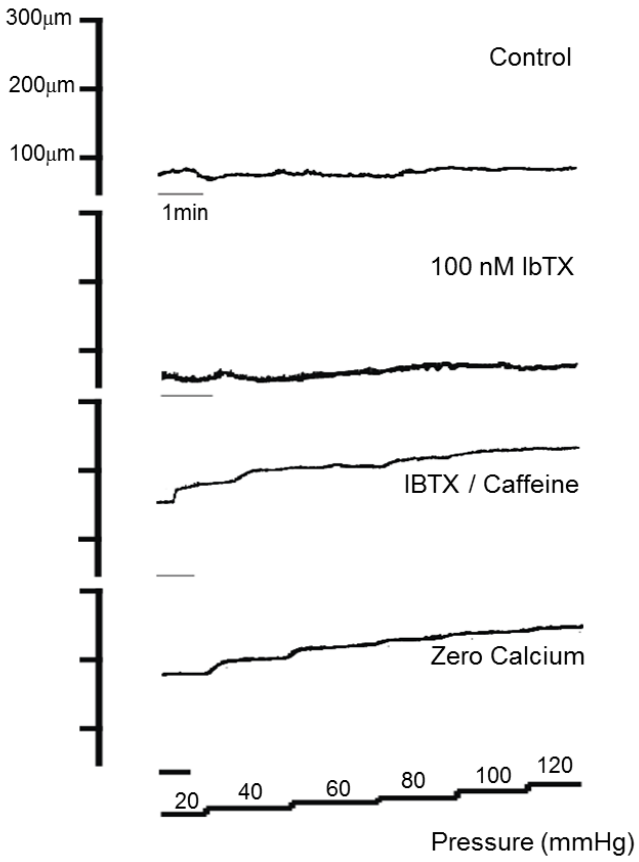


Figure 3.7: Caffeine-induced loss of the myogenic response was not due to the activation of BK_{Ca} channels.

A) Mean pressure-diameter relationships for isolated segments of rat middle cerebral artery mounted in a pressure myograph and subjected to increases in pressure from 20-120 mmHg in 20 mmHg increments. Mean responses are shown in the presence and absence of caffeine (10 mM), with (n=3) or without IbTX (an inhibitor of BK_{Ca} channels; 100 nM; n=6). Mean response of IbTX (100 nM; n=3) alone and zero extracellular calcium (passive response) are also included. Values are presented as mean ± SEM. A level of $P < 0.05$ is considered to be statistically significant when compared to control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

B)



B) Representative traces of pressure-diameter relationships for isolated segments of a rat middle cerebral artery in the presence and absence of IbTX, caffeine with IbTX, and zero extracellular calcium.

A)

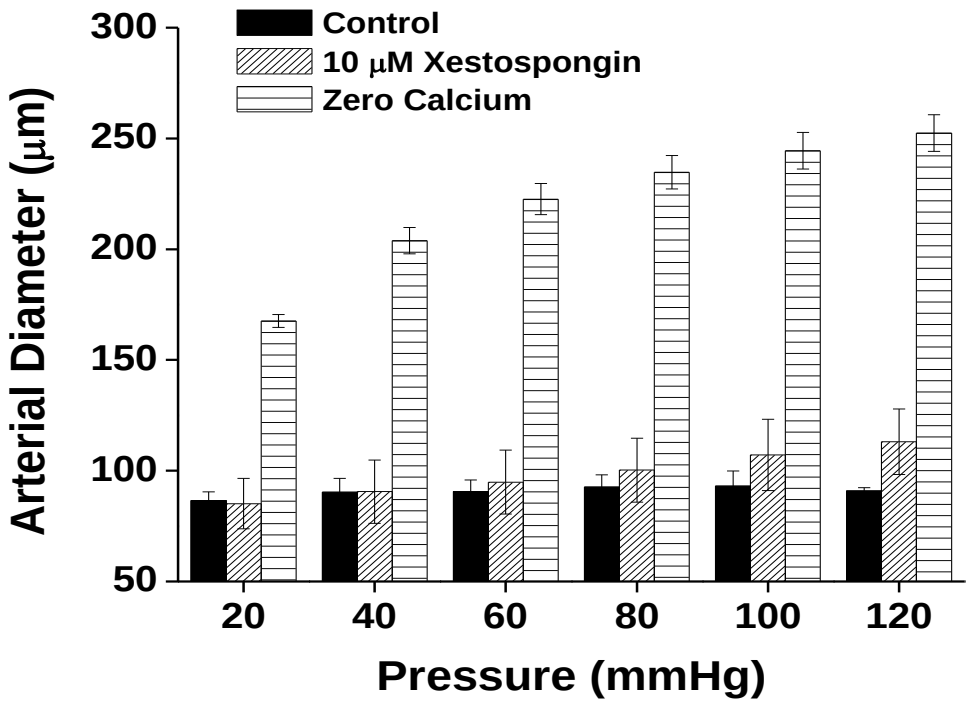
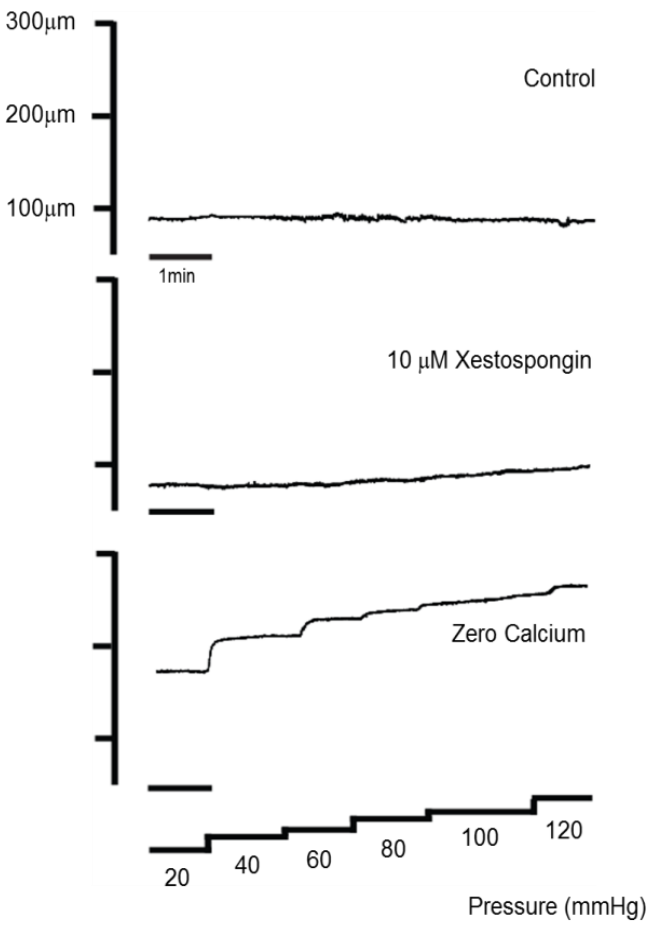


Figure 3.8: Calcium release from SR through IP_3R is not required in the myogenic response of rat isolated middle cerebral arteries.

A) Mean pressure-diameter relationships for isolated segments of rat middle cerebral artery mounted in a pressure myograph and subjected to increases in pressure from 20-120 mmHg in 20 mmHg increments. Mean responses are shown in the presence and absence of xestospongin (an inhibitor of IP_3R; 10 μM) or zero extracellular calcium (passive response). Values are presented as mean ± SEM (n=3).

B)



B) Representative traces of pressure-diameter relationships for isolated segments of a rat middle cerebral artery in the presence and absence of xestospongin and zero extracellular calcium.

A)

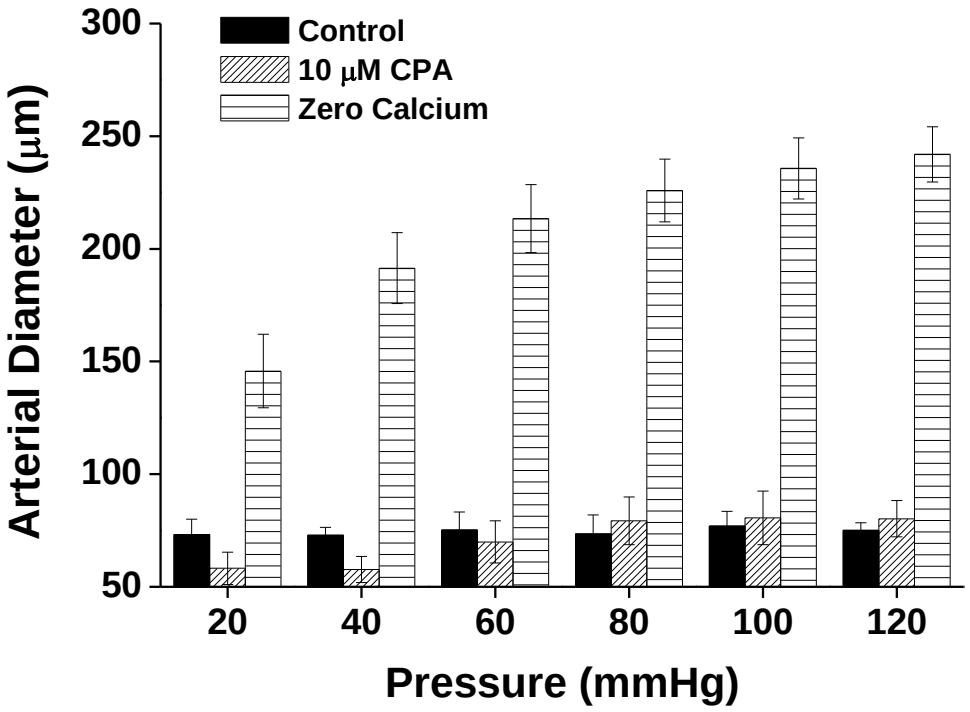
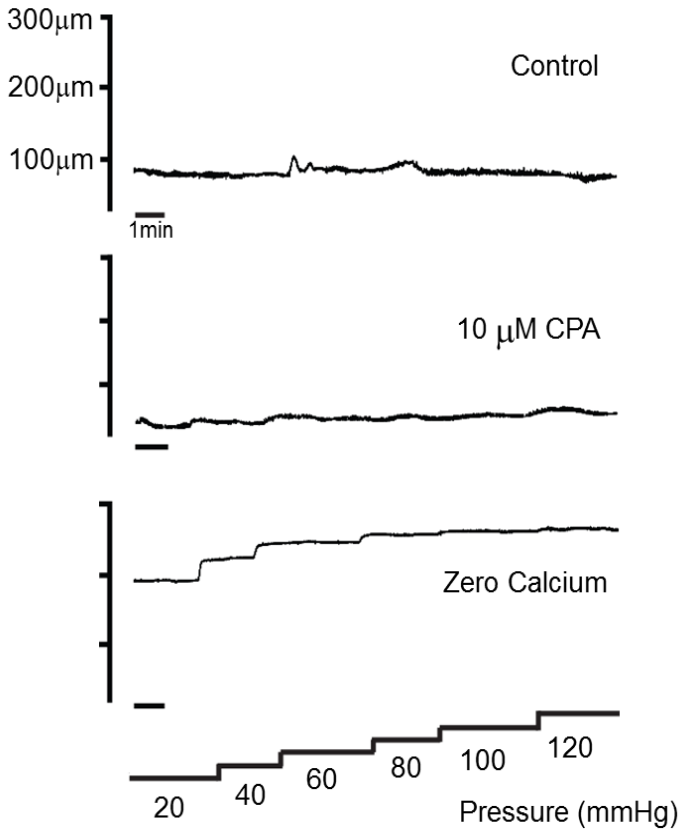


Figure 3.9: Inhibition of SERCA had no effect on the myogenic response of rat isolated middle cerebral arteries.

A) Mean pressure-diameter relationships for isolated segments of rat middle cerebral artery mounted in a pressure myograph and subjected to increases in pressure from 20-120 mmHg in 20 mmHg increments. Mean responses are shown in the presence and absence of CPA (an inhibitor of SERCA; 10 μM) or zero extracellular calcium (passive response). Values are presented as mean ± SEM (n=4).

B)



B) Representative traces of pressure-diameter relationships for isolated segments of a rat middle cerebral artery in the presence and absence of CPA and zero extracellular calcium.

A)

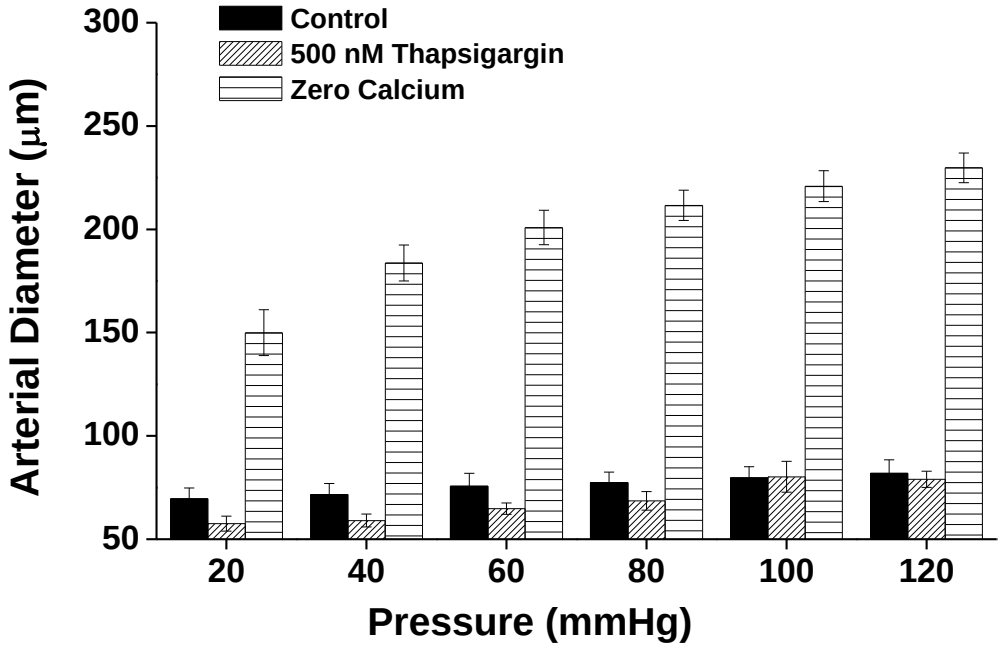
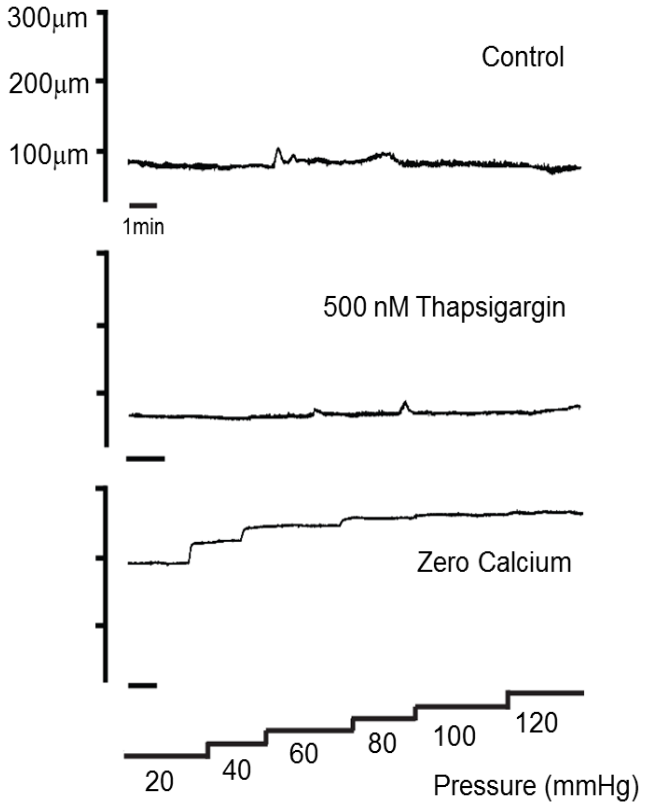


Figure 3.10: Inhibition of SERCA had no effect on the myogenic response of rat isolated middle cerebral arteries.

A) Pressure-diameter relationships for isolated segments of rat middle cerebral artery mounted in a pressure myograph and subjected to increases in pressure from 20-120 mmHg in 20 mmHg increments. Mean responses are shown in the presence and absence of thapsigargin (an inhibitor of SERCA; 500 nM) or zero extracellular calcium (passive response). Values are presented as mean ± SEM (n=6).

B)



B) Representative traces of pressure-diameter relationships for isolated segments of a rat middle cerebral artery in the presence and absence of thapsigargin and zero extracellular calcium.

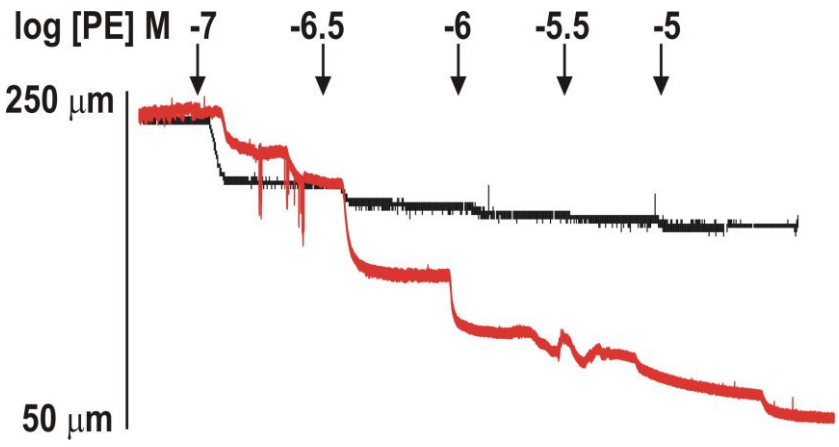


Figure 3.11: Positive control demonstrating xestospongin used was pharmacologically active.

Representative traces of pressure-diameter relationships for isolated segments of a rat mesenteric artery. Phenylephrine (PE) evoked vasoconstriction was enhanced due to inhibition of myoendothelial feedback by intraluminal administration of xestospongin (red). Response in the absence of xestospongin is also displayed in black. From previous work done by Dr. Plane.

Chapter 4: Modulation of cerebral artery myogenic response by the endothelium

4.1 Introduction:

As shown in the previous chapter, pharmacological disruption of SR stores in vascular smooth muscle inhibits the myogenic response of rat isolated middle cerebral arteries in the absence of endothelium. However, physiologically, all arteries in the body have an intact endothelial cell layer which modulates the contractile state of the underlying smooth muscle cells via release of NO and activation of EDH. As described in Chapter 1, agonist-evoked vasoconstriction is limited by the endothelium via a process termed myoendothelial feedback. Nonetheless, the role of the endothelium in modulating the myogenic response has been debated. Activation of both NO and EDH-mediated pathways is dependent on increases in calcium within endothelial cells. As in smooth muscle cells, endothelial cell calcium levels are tightly regulated; calcium is stored within endoplasmic reticulum stores and disruption of these stores can significantly alter endothelial function.

Thus, the experiments described in this chapter were carried out to test the hypothesis that *the endothelium can modulate myogenic tone and that disruption of the endothelial endoplasmic reticulum will disturb myogenic reactivity in these vessels.*

4.2 Methods

All methods are as described in Chapter 2. My approach in these experiments was to use endothelium-intact arteries to investigate the role of the endothelium in modulating the myogenic response of isolated middle cerebral arteries. Comparison of the effect of pharmacological agents which disrupt calcium stores in intact and denuded arteries can be used to determine the role of endothelial versus smooth muscle intracellular calcium stores.

4.3 Results

4.3.1 Endothelium-dependent modulation of myogenic tone

There were no significant differences in the myogenic response of endothelium intact and endothelium denuded arteries. In endothelium intact arteries, at 20 mmHg the diameter was at 62.9±3.2 μm (n=4) and at 120 mmHg it was 79.9±8.0 μm (n=4). After the endothelium was removed, the diameter of the same vessels at 20 mmHg was 66.3±6.0 (n=4; $p > 0.05$) and at 120 mmHg it was 75.5±3.4 (n=4; $p > 0.05$; Figure 4.1). The presence of the endothelium was confirmed by the presence of dilation to addition of bradykinin (1 μM; Figure 2.2B) and endothelium removal was confirmed by the absence of such dilation.

The lack of modulation of myogenic tone by the endothelium in middle cerebral arteries was further confirmed by examining the effects of NOS and EDH inhibitors on the myogenic response. Following construction of a control

pressure-diameter ramp, tissues were pre-incubated with L-NAME (100 μM; NOS inhibitor; 30 minutes), apamin (50 nM; SK_{Ca} channel inhibitor; 15 minutes) and TRAM-34 (IK_{Ca} channel inhibitor; 15 minutes), either alone, or in combination before the ramp was repeated. In the presence of the inhibitors alone, or together, the pressure-diameter relationships was unaltered; at 80 mmHg, the control diameter was 81.1±7.0 μm and in the presence of all three inhibitors it was 81.4±7.7 μm (n=7; p >0.05). Representative traces and mean data showing the pressure-diameter relationship for endothelium-intact middle cerebral arteries in the absence and presence of L-NAME, apamin and TRAM-34 are shown in Figure 4.2.

4.3.2 Inhibition of SR calcium release via RyR channels

To determine whether the endothelial endoplasmic reticulum could modulate the effects of RyR channel activation on myogenic response, intact arteries were exposed to ryanodine. Following construction of a control pressure-diameter ramp, tissues were pre-incubated with ryanodine (10 or 50 μM) for 15 minutes before the ramp was repeated.

Under control conditions, the vessels used in these experiments maintained a constant diameter in response to increasing pressure steps (20-120 mmHg, 20 mmHg increments); at 20 mmHg, the diameter was at 74.2±5.0 μm and 91.0±8.1 μm at 120 mmHg (n=6). In the presence of ryanodine 10 μM, the diameter response was not significantly different from control between 20-80

mmHg but strong transient spikes were detected in the diameter response. At 60 mmHg, the diameter was 82.1±6.8 μm and 91.4±12.5 μm (n=6; p >0.05) in the absence and presence of ryanodine, respectively. Between 100-120 mmHg, myogenic tone was reduced and the diameter fluctuated as pressure increased. Under control conditions, at 100 mmHg the vessel diameter was 88.1±8.4 μm and in the presence of ryanodine it was 131.4±15.1 μm (n=6; p <0.01). Representative traces and mean data showing the pressure-diameter relationship for endothelium-intact middle cerebral arteries in the absence and presence of 10 μM ryanodine are shown in Figure 4.3.

In the presence of 50 μM ryanodine, the vessel diameter was unstable as pressure increased and there were large cyclical fluctuations in the diameter. Between 20 to 60 mmHg, the average diameter was not significantly different from control but there were many spikes in the diameter response. Under control conditions, the diameter at 60 mmHg was 82.1±6.8 μm and in the presence of 50 μM ryanodine, it was 91.3±7.1 μm (n=6; p >0.05). For all pressures from 80 to 120 mmHg, the differences in diameter between control and ryanodine-treatment were statistically significant (80 mmHg: p <0.05, 100 mmHg: p <0.01, 120 mmHg: p <0.001). At 100 mmHg, the diameter was increased from 88.1±8.4 μm under control conditions to 142.0±15.9 μm in the presence of ryanodine (n=6; p <0.01). Representative traces and mean data showing the pressure-diameter relationship for endothelium-intact middle

cerebral arteries in the absence and presence of 50 μM ryanodine are shown in Figure 4.3. The effects of ryanodine (10 μM and 50 μM) in these endothelium-intact arteries were not significantly different to those observed in endothelium-denuded arteries (Figure 3.3 & 3.4).

4.3.3 Depletion of endoplasmic reticulum calcium stores by caffeine

As in denuded vessels, the second method used to disrupt the function of SR stores was to actively deplete them by activating the RyR channel. Following construction of a control pressure-diameter ramp, tissues were pre-incubated with caffeine (10 mM) for 15 minutes before the ramp was repeated.

Under control conditions, the diameter of the endothelium-intact arteries was maintained as intravascular pressure was increased from 20 to 120 mmHg; at 20 mmHg, diameter was 71.8±3.0 μm, and at 120 mmHg diameter was 79.6±5.1 μm (n=6). Upon application of caffeine to arteries held at 20 mmHg, an immediate dilation was observed with the diameter increasing from 68.2±4.9 μm to 134.2±8.6 μm (n=6; $p < 0.001$). This dilation persisted and at the end of the 15 minute incubation period vessel diameter was 123.3±11.9 μm (n=6; $p < 0.01$). In the presence of caffeine the vessels dilated passively as pressure was increased from 20 to 120 mmHg. The resulting pressure-diameter relationship was significantly different from control values at all pressure steps ($p < 0.001$; Figure 4.4). At 80 mmHg, the control diameter was 74.8±4.8 μm and in the presence of

caffeine it was 197.9±18.4 μm (n=6; p <0.001). When compared to the effects of caffeine on the myogenic response of denuded arteries, the two were not significantly different (p >0.05; Figure 3.6 & 4.4). Representative traces and mean data showing the pressure-diameter relationship for endothelium-intact middle cerebral arteries in the absence and presence of caffeine are shown in Figure 4.4.

4.3.4 Disruption of calcium reuptake via SERCA

SERCA is the main calcium reuptake mechanism for refilling the intracellular calcium stores in both smooth muscle and endothelial cells. As described in Chapter 3, in endothelium-denuded arteries, block of SERCA by application of either CPA or thapsigargin did not affect the myogenic response in isolated middle cerebral arteries. As SERCA is also an important regulator of intracellular calcium levels in the endoplasmic reticulum of endothelial cells, the effect of blocking endothelial SERCA on the pressure-diameter response of endothelium-intact arteries was investigated.

Following construction of a control pressure-diameter ramp, cerebral arteries were pre-incubated with CPA (10 μM) for 15 minutes before the ramp was repeated. Under control conditions, the diameter of the endothelium-intact arteries was maintained as intravascular pressure was increased from 20 to 120 mmHg; at 20 mmHg vessel diameter was 90.4±5.0 μm, and at 120 mmHg diameter was 112.5±5.2 μm (n=6). Immediately upon the addition of CPA to vessels held at 20 mmHg, the arterial diameter began to oscillate and continued

to do so throughout the 15 minutes incubation period. Furthermore, in the presence of CPA, myogenic response was not maintained as intravascular pressure was increased from 20 to 120 mmHg. Dilation was observed at each pressure step and the pressure-diameter response was significantly different from control; at 80 mmHg, the diameter increased from 110.0±6.5 μm under control conditions to 195.5±9.4 μm (n=6; $p < 0.001$) in the presence of CPA. Representative traces and mean data showing the pressure-diameter relationship for endothelium-intact middle cerebral arteries in the absence and presence of CPA are shown in Figure 4.5.

To further support these findings, experiments were carried out using thapsigargin. Following construction of a control pressure-diameter ramp, endothelium-intact cerebral arteries were pre-incubated with thapsigargin (500 nM) for 15 minutes before the ramp was repeated. Under control conditions, the diameter of the endothelium-intact arteries was maintained as intravascular pressure was increased from 20 to 120 mmHg; at 20 mmHg, diameter was 75±4.4 μm, and at 120 mmHg diameter was 88.9±7.8 μm (n=6). Addition of thapsigargin to arteries held at 20 mmHg induced similar oscillations in diameter to those observed in the presence of CPA; there were strong contractions quickly followed by relaxations. This rhythmic activity persisted for the duration of the 15 minutes incubation period. On subsequent application of the pressure ramp, large fluctuations in the vessel diameter were observed in response to each

pressure step. At 80 mmHg, the vessel diameter under control conditions was 86.1±7.6 μm but in the presence of thapsigargin it was 210.6±15.0 μm (n=6; p <0.001). Representative traces and mean data showing the pressure-diameter relationship for endothelium-intact middle cerebral arteries in the absence and presence of thapsigargin are shown in Figure 4.6.

Thus, both CPA and thapsigargin caused endothelium-dependent inhibition of myogenic response. In endothelium-denuded arteries, block of SERCA had no effect on myogenic response. To further elucidate the underlying mechanisms of the effect, inhibitors of NOS and EDH on the actions of CPA in endothelium-intact arteries was investigated.

4.3.4.1 Role of NO in CPA-induced loss of myogenic tone

As described in section 4.3, inhibition of endothelium-derived NO did not significantly alter the myogenic response in isolated middle cerebral arteries. However, NOS is activated by increases in endothelial calcium. Hence, to determine whether production of NO could contribute to the endothelium-dependent loss of myogenic tone caused by CPA, the effect of CPA was examined in the presence of the NOS inhibitor, L-NAME. Following construction of a control pressure-diameter ramp, endothelium-intact cerebral arteries were pre-incubated with L-NAME (100 μM) for 30 minutes before the ramp was repeated. As described earlier, L-NAME had no significant effect on the pressure-diameter response of middle cerebral arteries; at 80 mmHg the vessel diameter was

94.9±6.4 μm and 85.0±6.4 μm (n=4; p >0.05) in the absence and presence of L-NAME respectively. In the continued presence of L-NAME, CPA (10 μM) induced a gradual dysregulation of myogenic tone in the form of either spikes or rhythmic oscillations in the vessel diameter. However, during the subsequent pressure ramp from 20 to 120 mmHg, vessel diameter was only statistically different from control at 120 mmHg (149.3±11.3 μm, p <0.05). Comparison of the effect of CPA alone with CPA and L-NAME together showed statistical difference between the two groups at all pressure steps; at 80 mmHg, diameter of arteries exposed to CPA alone was 195.5±9.4 μm (n=6) and this was reduced to 114.1±14.8 μm (n=6; p <0.001) by the presence of L-NAME. Representative traces and mean data showing the pressure-diameter relationship for endothelium-intact middle cerebral arteries in the absence and presence of L-NAME and CPA are shown in Figure 4.7.

4.3.4.2 Role of EDH in CPA-induced loss of myogenic tone

As inhibition of NO production did not completely prevent the effect of CPA on the myogenic response, experiments were carried out to investigate whether the EDH pathway was involved. This pathway is dependent upon activation of endothelial SK_{Ca} and IK_{Ca} channels and so the effects of apamin and TRAM-34, selective inhibitors of these respective channels, on CPA-induced loss of myogenic tone was examined.

As described earlier, apamin and TRAM-34 had no significant effect on the pressure-diameter response of middle cerebral arteries; at 80 mmHg the vessel diameter was 80.3±8.4 μm and 85.8±11.5 μm (n=5; $p > 0.05$) in the absence and presence of apamin and TRAM-34, respectively. In the continued presence of apamin and TRAM-34, addition of CPA to endothelium-intact arteries maintained at 20 mmHg caused significant, transient vasoconstriction; before addition of CPA, the diameter was 73.8±9.2 μm and immediately after application of CPA the diameter was 35.8±6.9 μm (n=5; $p < 0.01$). However, by the end of the 15 minute incubation period, vessel diameter returned to the pre-CPA level. During subsequent application of a pressure ramp, myogenic tone was maintained and the pressure diameter relationship was not significantly different to that observed under control conditions; at 80 mmHg was 80.3±8.4 μm and 76.7±8.9 μm (n=5; $p > 0.05$) in absence and presence of CPA, apamin and TRAM-34, respectively. Representative traces and mean data showing the pressure-diameter relationship for endothelium-intact middle cerebral arteries in the absence and presence of apamin, TRAM-34 and CPA are shown in Figure 4.8. Furthermore, application of apamin and TRAM-34 during CPA-induced oscillation in arterial diameter inhibited the oscillations and restored normal diameter control. As shown in Figure 4.9, CPA-induced oscillations stopped quickly (<3 min) upon addition of both apamin and TRAM-34. However, addition of just one of the inhibitors did not alter the oscillations (Figure 4.9).

The NO and EDH pathways can act in a co-operative manner to regulate the contractile state of underlying smooth muscle cells and thus the effect of combined blockade of these pathways on CPA-evoked loss of myogenic reactivity was investigated. As described earlier, the combination of L-NAME, apamin and TRAM-34 had no significant effect on the pressure-diameter response of middle cerebral arteries; at 80 mmHg the vessel diameter was 81.1±7.0 μm and 81.4±7.7 μm (n=7; p >0.05) in the absence and presence of L-NAME, apamin and TRAM-34 respectively. In the continued presence of these inhibitors, addition of CPA to endothelium intact arteries held at 20 mmHg did not significantly alter the pressure-diameter response compared to control conditions; at 80 mmHg the vessel diameter was 81.1±7.0 μm and 71.5±4.5 μm (n=7, p >0.05), in the absence and presence of the drug treatments. Representative traces and mean data showing the pressure-diameter relationship for endothelium-intact middle cerebral arteries in the absence and presence of the combination of L-NAME, apamin, TRAM-34 and CPA are shown in Figure 4.10.

4.3.4.3 Role of gap junctions in CPA-induced loss of myogenic tone

EDH-dependent modulation of smooth muscle function is mediated through electrical coupling via gap junctions. Thus, the effect of inhibiting gap junction function with 18α-glycyrrhetinic acid on the endothelium-dependent actions of CPA was investigated. Following construction of a control pressure-diameter ramp, endothelium-intact cerebral arteries were pre-incubated with

18α-glycyrrhetinic acid (10 μM) for 15 minutes before the ramp was repeated. Application of 18α-glycyrrhetinic acid to arteries maintained at 20 mmHg had no effect on the pressure-diameter response of middle cerebral arteries; at 80 mmHg the diameter was 74.2±9.6 μm and 74.0±7.6 μm (n=4; p >0.05) in the absence and presence of 18α-glycyrrhetinic acid, respectively. In the continued presence of 18α-glycyrrhetinic acid, addition of CPA to arteries held at 20 mmHg induced large oscillations in the diameter similar to those seen in the presence of CPA alone. During the subsequent pressure ramp from 20 to 120 mmHg, the oscillations persisted but the vessel diameter was only statistically different from control at 80-120 mmHg (p <0.01, Figure 4.10A). Comparison of the effect of CPA alone with CPA and 18α-glycyrrhetinic together showed statistical difference between the two groups at all pressure steps except 120 mmHg. At 80 mmHg, diameter of arteries exposed to CPA alone was 195.5±9.4 μm (n=6) and this was reduced to 126.4±12.6 μm (n=4; p <0.001) by the presence of 18α-glycyrrhetinic. When L-NAME, TRAM-34 and apamin were added in addition to 18α-glycyrrhetinic acid, CPA did not induce changes in the pressure-diameter response. The diameter at 80 mmHg was 74.2±9.6 μm under control conditions and 77.9±1.9 μm (n=4; p >0.05) under drug treatment. Representative traces and mean data showing the pressure-diameter relationship for endothelium-intact middle cerebral arteries in the absence and presence of the combination

of L-NAME, apamin, TRAM-34, 18α-glycyrrhetinic acid and CPA are shown in Figure 4.11.

4.3.4.4 Source of calcium for CPA-induced disruption of myogenic response

Depletion of stores within cells stimulates calcium influx via capacitative (or store-operated) calcium entry. In order to determine whether calcium influx contributes to the actions of agents which deplete calcium stores, experiments may be carried out in preparations bathed in calcium-free solution. However, evaluating the functional role of such mechanisms in isolated arteries is complicated by that fact that removal of extracellular calcium causes loss of the myogenic response such that increases in intravascular pressure result in passive dilation of vessels (Figure 3.1). To circumvent this issue, experiments were carried out in which the solution within the lumen of the vessel was replaced with Kreb's buffer lacking calcium.

Isolated arteries were mounted in the pressure myograph as previously described (Chapter 2). Once myogenic tone had developed, 300 μl of zero calcium Kreb's buffer was perfused into the vessel lumen. Once the vessel diameter had restabilized, CPA (10 μM) was applied to the artery. Immediately, oscillations in tone were observed and subsequent application of a pressure ramp showed that the myogenic response was lost; at 80 mmHg the vessel diameter was increased from 81.2+5.8 um to 158.1+-17.5um (n=4; $p < 0.05$). This effect was not significantly different from that observed in tissues in which CPA

was applied to arteries in which normal (calcium-containing) Kreb's was present in the lumen (Figure 4.12). In addition, as a control (n=4), 300 μl of regular Kreb's solution was used to perfused into the vessel lumen in place of the 300 μl zero calcium Kreb's and the experiment repeated. This was to ensure flow does not contribute to the observed effect. There was no significant difference between this control and the treatment where zero calcium Kreb's was perfused into the lumen (Figure 4.13).

4.4 Discussion

These data indicate that under normal conditions, the endothelium does not play a key role in modulating the myogenic response of isolated middle cerebral arteries. However, inhibition of the endothelial SERCA pump causes loss of the myogenic response due to activation of the NO and EDH pathways.

In resistance arteries, the endothelium is a key regulator of vascular tone and hence blood flow. This regulation is primarily achieved via the release of NO and activation of the EDH pathway whereby K_{Ca} channel-mediated endothelial hyperpolarization is transferred to the underlying smooth muscle cells via gap junctions.

Arterial vasoconstriction in response to agonists is limited by the endothelium via a process termed myoendothelial feedback (Sandow *et al*., 2009). This can be demonstrated functionally as an increase in constriction

caused by inhibitors of the NO and EDH pathways or by endothelial removal. The role of myoendothelial feedback in regulating myogenic responses has been debated with significant variation shown between studies (McCarron *et al.,* 1989; MacPherson *et al.,* 1991; Ueeda *et al.,* 1992; Veerareddy *et al.,* 1994). My data show that removal of the endothelium or inhibition of the NO and/or EDH pathways did not significantly alter the pressure-diameter relationship of rat isolated middle cerebral arteries. Thus, it appears the myoendothelial feedback is not an important factor in regulating myogenic reactivity of these vessels. This finding is in line with published data which indicate that in isolated rat posterior cerebral, mesenteric and skeletal muscle arteries and rabbit ear arteries, myogenic tone was unaltered by removal of the endothelium (McCarron *et al.,* 1989; MacPherson *et al.,* 1991; Falcone *et al.,* 1991; Sun *et al.,* 1992; Lagaud *et al.,* 1999; Oyabe *et al.,* 2000). Marrelli (2002) and Cipolla *et al.,* (2009) reported that L-NAME enhanced constriction of rat middle cerebral arteries but the vessels used in those studies showed very little myogenic tone; for example in the report by Marelli, at 85 mmHg the vessel diameters were ~220 μm and the passive diameter at the same pressure was ~280 μm. McNeish *et al.* (2005) reported that in rat middle cerebral arteries inhibition of NOS and/or endothelial K_{Ca} channels had no effect on myogenic reactivity of vessels which showed a comparable degree of myogenic reactivity to that observed in my study.

Recent data indicate that myoendothelial feedback is mediated by flux of IP_3 from the smooth muscle to the endothelium (Sandow *et al.,* 2009). Thus, the lack of endothelial modulation of myogenic reactivity observed in this study may be explained by the absence of IP_3 generation during the myogenic response. As discussed in section 3.5, although it is well established that IP_3-mediated calcium release is a major contributor to vasoconstriction elicited by GPCR agonists, data supporting a role for generation of IP_3 within smooth muscle cells during the myogenic response is limited (Narayanan *et al.,* 1994; Osol *et al*., 1993; Inscho *et al*., 1998). In my experiments, xestospongin, an inhibitor IP_3 receptors, did not alter the pressure-diameter relationship in cerebral arteries suggesting that IP_3 is not a major factor in the myogenic response of these vessels. Thus, the lack of endothelial modulation of myogenic tone in middle cerebral arteries may support the contention that vasoconstrictor responses which do not involve IP_3 generation are not limited by myoendothelial feedback.

The endothelial endoplasmic reticulum plays a key role in regulating intracellular calcium levels. Regulated release of calcium from this storage site in response to activation of GPCRs or mechanical forces such as shear stress leads to activation of the NO and EDH pathways. The effect of ryanodine and caffeine on the pressure diameter relationship of middle cerebral arteries was the same in endothelium-intact as in denuded arteries. This may not be surprising given recent studies showing that ryanodine had no effect on endothelial calcium

levels in rat (Kansui *et al.,* 2008) and mouse mesenteric arteries (Ledoux *et al.,* 2008). In the latter study, no RyR isoforms could be detected in endothelial cells leading to the suggestion that they do not normally express RyR channels. However, the presence of RyRs has been shown in endothelial cells from rabbit aortic (Wang *et al.,* 1995; Rusko *et al.,* 1995; Liang *et al.,* 2004) and human mesenteric artery endothelial cells (Köhler *et al.,* 2001). In isolated bovine aortic endothelial cells, calcium release via RyR channels has been linked to NOS activation (Paltauf-Doburzynska *et al,.* 1998) but functional evidence supporting this has yet to be published.

SERCA plays an important role in regulating endothelial calcium levels. In my experiments, inhibition of SERCA by CPA and thapsigargin caused a loss of myogenic control in endothelium-intact vessels. This endothelium-dependent loss of myogenic tone caused by CPA was due to NO and EDH. A previous study showed that in rat mesenteric arteries, thapsigargin caused an endothelium-dependent inhibition of noradrenaline-evoked contraction (Nilsson *et al.,* 1998). Thapsigargin induced a large hyperpolarization of the smooth muscle of intact, but not denuded arteries, the time course of which correlated with the time course of inhibition of contraction to noradrenaline suggesting a role for EDH but this was not investigated further.

CPA has been shown to elicit endothelium-dependent relaxations of isolated blood vessels. In cultured endothelial cells, SERCA inhibition by both

thapsigargin and CPA caused a rise in intracellular calcium levels which remained elevated over basal values for several minutes and was substantially inhibited in the absence of extracellular calcium (Gericke *et al.,* 1993; Schilling *et al.,* 1992). It has been proposed that CPA and thapsigargin selectively activate endothelial K_{Ca} channels to elicit EDH-mediated vasorelaxation by promoting capacitative calcium entry *via* store-operated calcium channels (Fukao *et al.,* 1995). EDH-mediated relaxations to CPA have been reported in rat mesenteric (Fukao *et al.,* 1995), mouse mesenteric artery (Dora *et al.,* 2003) and human iliac artery (Chaytor *et al.,* 2005). However, in rabbit aorta (Matsuyama *et al.,* 1993) and rat superior mesenteric artery (Stankevicius *et al.,* 2006) CPA-evoked relaxations were due to NO, and in rabbit iliac artery, reductions in arterial tone elicited by CPA were due to both NO and EDH (Fernandez-Rodriguez *et al.,* 2009).

In my experiments, inhibition of NOS by L-NAME caused a significant reduction in the actions of CPA whereas inhibition of EDH by blockers of endothelial SK_{Ca} and IK_{Ca} channels, alone or with L-NAME, abolished it. Thus it appears that in rat middle cerebral arteries, CPA can modulate myogenic tone by activating both NO and EDH-mediated pathways through its action on SERCA. The majority of the published literature has focused on the essential role of endothelial SK_{Ca} and IK_{Ca} channels in EDH-mediated responses. Indeed, inhibition of a functional vascular response by the combination of apamin and TRAM-34 has been described as the “gold standard” for defining the contribution of EDH

(Busse *et al.,* 2002). However, evidence is accumulating that activation of these channels also contributes to production of NO. Acetylcholine-evoked relaxation of rat aorta (Qiu and Quilley, 2001) and basilar arteries (Plane and Cole, 2006), which can be fully accounted for by release of endothelium-derived NO, is inhibited by blockers of endothelial K_{Ca} channels. Also, pharmacological activation of these channels causes NO production in a wide range of vessels including porcine retinal arterioles (Dalsgaard *et al.,* 2010), rat superior mesenteric artery (Stankevicius *et al.,* 2011), dog coronary artery (Kurian *et al.*, 2011) and rat cremaster arterioles (Sheng *et al.,* 2009) as well as in cultured endothelial cells (Sheng and Braun, 2007). Thus, my observation that there is overlap between the actions of L-NAME and the potassium channel blockers apamin and TRAM-34 in preventing the loss of myogenic tone caused by CPA, may reflect a functional link between activation of SK_{Ca} and IK_{Ca} channels and NO in this vessel.

Transfer of membrane hyperpolarization from endothelial to smooth muscle cells via gap junctions is regarded as the primary mechanism to account for EDH-dependent modulation of vascular tone (Edwards *et al.,* 2010). Gap junction function is difficult to assess in intact arteries but 18α-glycyrrhetinic acid has been shown to selectively inhibit EDH-mediated responses in a large number of vessels (see for example, Griffiths *et al.,* 2004; Morton *et al.*, 2010). My data with 18α-glycyrrhetinic acid demonstrated a partial reduction of CPA-evoked

myogenic tone loss. This coincides with my finding that the NO pathway is only partially responsible for the CPA effect, and further support the notion that NO and EDH pathways act cooperatively in mediating the actions of CPA. Our lab has previously used 18α-glycyrrhetinic acid to block communication between endothelial and smooth muscle cells in the rat basilar artery (Allen *et al.*, 2002) but to date, the effects of this agents on EDH-mediated responses has not been reported in the rat middle cerebral artery.

In cultured endothelial cells, SERCA inhibition causes a rise in calcium levels which is dependent on calcium influx, presumably due to capacitative (store-operated) calcium entry (Gericke *et al.,* 1993; Schilling *et al.,* 1992). As exposing intact arteries to solutions lacking calcium inhibits all tone development, I endeavoured to examine the functional consequences of removing extracellular calcium on CPA-induced loss of myogenic tone by replacing the solution in the lumen of the arteries with one containing zero calcium. This approach had no effect on the actions of CPA possibly because although calcium was removed from the luminal surface of the endothelium, there was sufficient calcium at the abluminal surface within extracellular fluid to provide calcium influx.

In conclusion the data from these experiments show that although the endothelium does not modulate the myogenic response of rat isolated middle cerebral arteries under normal conditions, disruption of endothelial calcium

handling can lead to loss of myogenic reactivity through activation of the NO and EDH pathways. The myogenic response is a key auto-regulatory mechanism to maintain cerebral blood flow within a narrow range in the face of increases in blood pressure. Several pathological conditions have been associated with loss of myogenic tone. In endothelium-intact rat middle cerebral arteries, ischemia/reperfusion causes loss of myogenic tone and flaccid vascular paralysis that results in loss of autoregulation (Cipolla *et al.*, 1997; Palomares and Cipolla, 2011). The mechanism underlying this loss of myogenic reactivity is unclear but separate studies have shown that ischemia/reperfusion is associated with significant potentiation of EDH-dependent vasodilation of rat middle cerebral and dog coronary arteries (Marrelli *et al.,* 1999; Chan and Woodman, 1999). In middle cerebral arteries, the ischemia/reperfusion-induced enhancement of EDH-mediated dilation was associated with augmented endothelial calcium levels due to fundamental changes in calcium handling (Marrelli, 2002). The exact mechanisms were not investigated but the author suggested that there could be a greater release of calcium release from internal stores or enhanced calcium influx.

To date, the role of the endothelium in the loss of myogenic tone has yet to be investigated. However, taken together, my findings that disruption of endothelial calcium handling leads to loss of myogenic tone and published observations of enhanced EDH and calcium levels in ischemia/reperfusion in

cerebral arteries, may support the contention that disruption of endothelial calcium handling may contribute to the loss of myogenic reactivity in pathological states such as ischemia/reperfusion. In support of this, loss of autoregulation (myogenic tone) in a rat model of ischemic acute renal failure was attributed to increased production of NO and EDH (Guan *et al.*, 2006).

A)

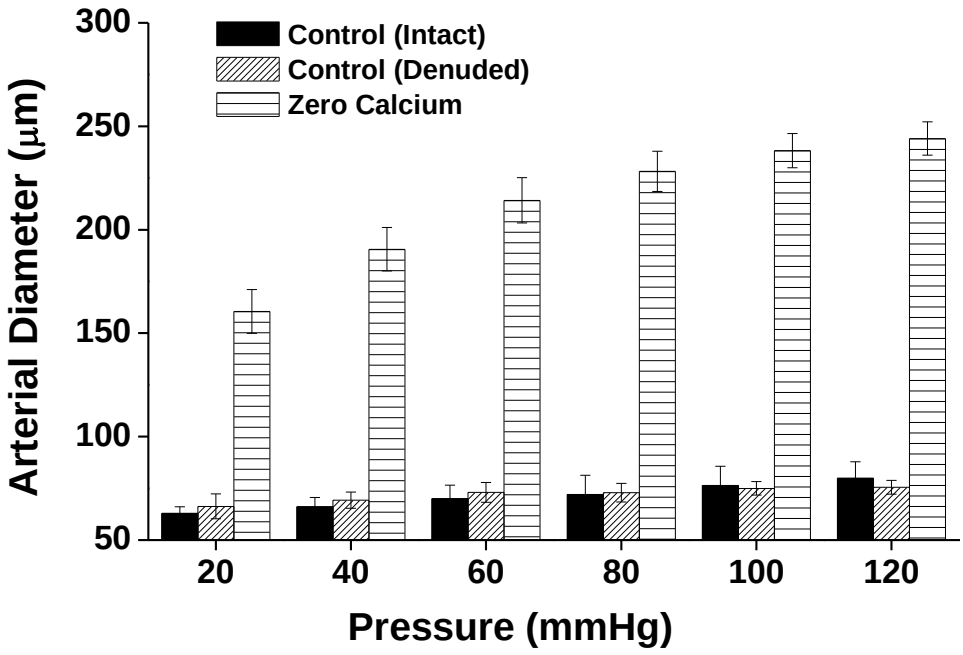
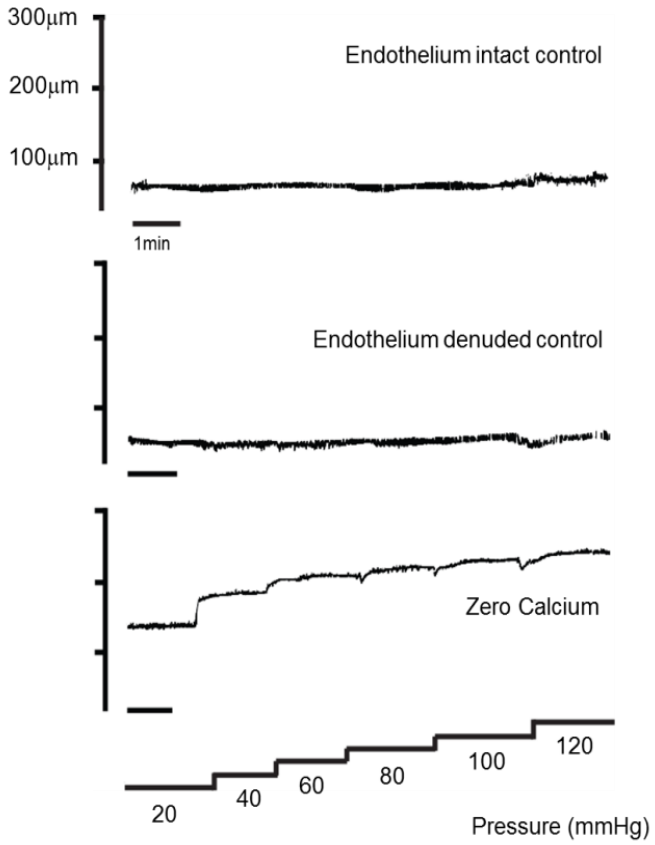


Figure 4.1: Myogenic response of rat isolated middle cerebral arteries was unaffected by endothelium removal.

A) Mean pressure-diameter relationships for isolated segments of rat middle cerebral artery mounted in a pressure myograph and subjected to increases in pressure from 20-120 mmHg in 20 mmHg increments. Mean diameter response of endothelium denuded arteries in zero extracellular calcium is also included (passive response). Values are presented as mean ± SEM (n=4).

B)



B) Representative traces of pressure-diameter relationships for isolated segments of a rat middle cerebral artery with endothelium intact, endothelium denuded and in zero extracellular calcium.

A)

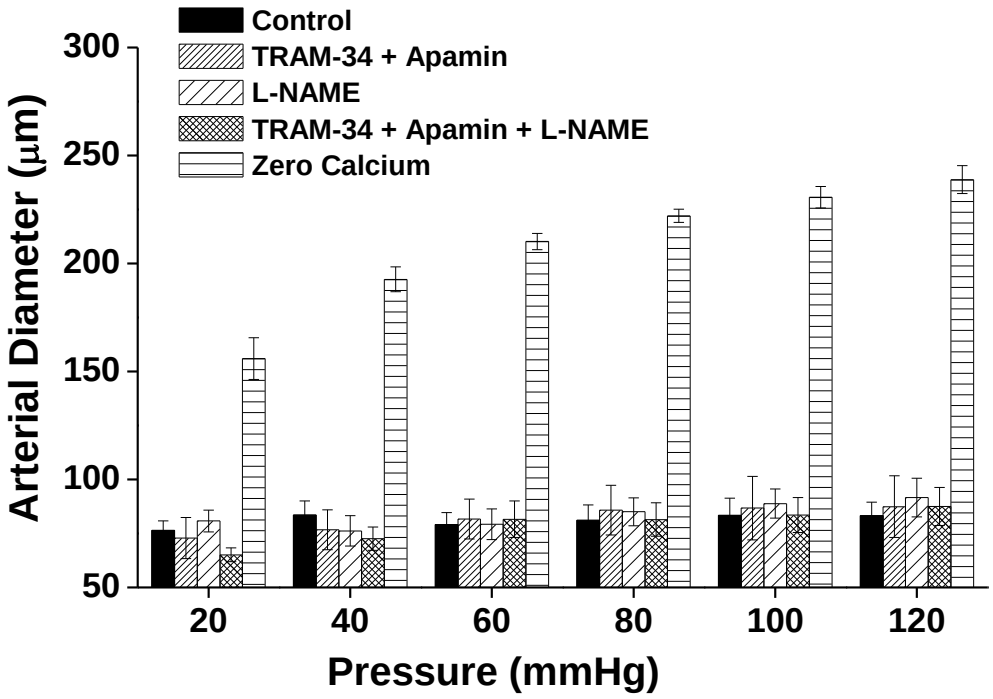
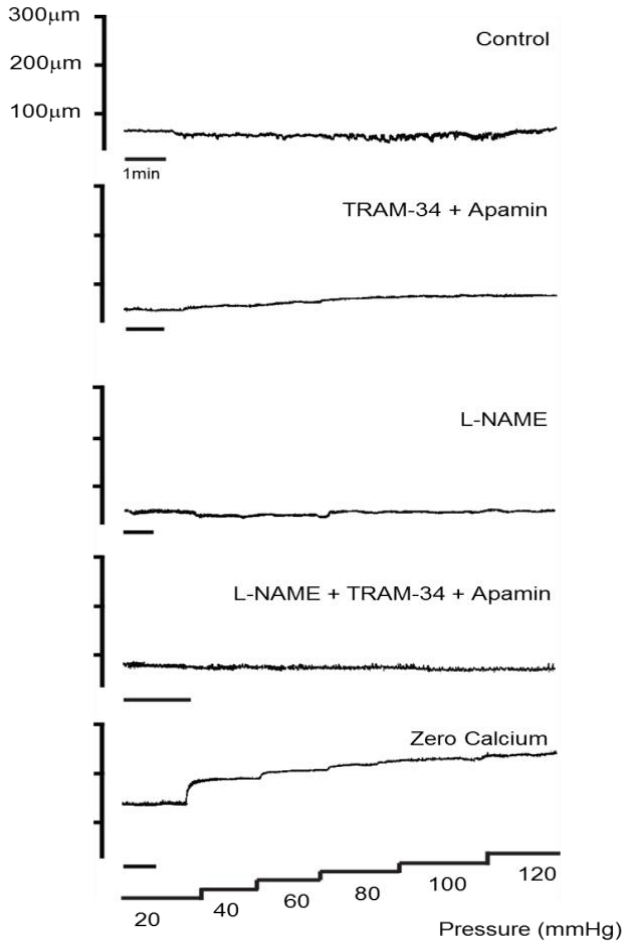


Figure 4.2: Myogenic response of rat isolated middle cerebral arteries was not modulated by NOS or EDH.

A) Mean pressure-diameter relationships for isolated segments of rat middle cerebral artery mounted in a pressure myograph and subjected to increases in pressure from 20-120 mmHg in 20 mmHg increments. Mean responses are shown in the presence and absence of L-NAME (100 μM), TRAM-34 (1 μM) and apamin (50 nM) alone or in combination. Mean response of zero extracellular calcium (passive response) is included. Values are presented as mean ± SEM (L-NAME n=4; TRAM-34 + apamin n=4; all three n =7).

B)



B) Representative traces of pressure-diameter relationships for isolated segments of a rat middle cerebral artery in presence and absence of inhibitors and in zero extracellular calcium.

A)

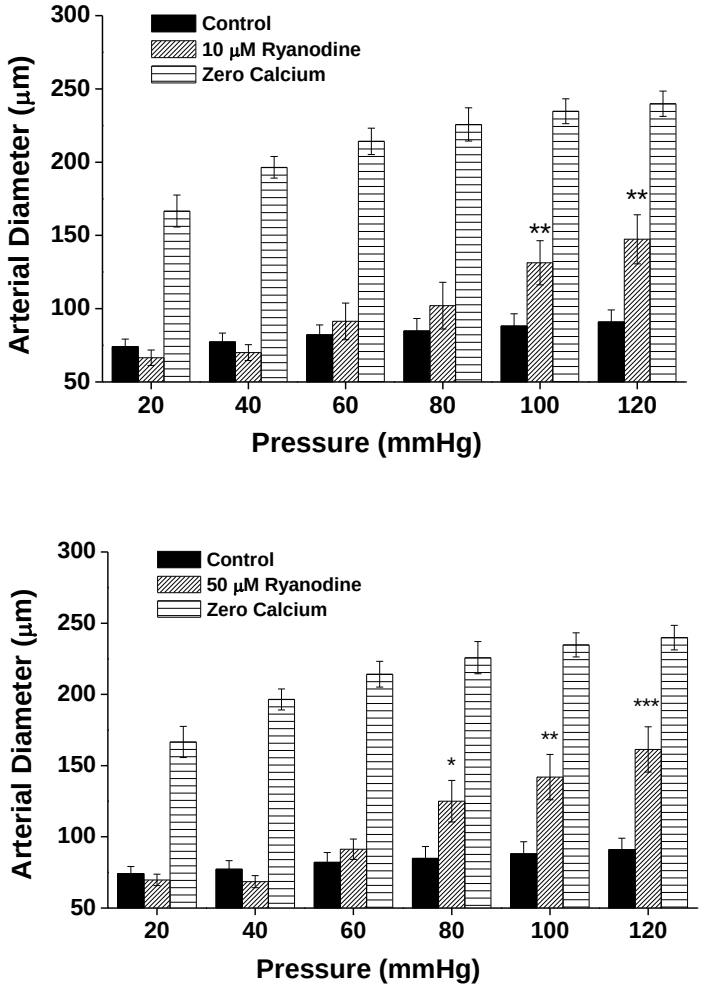
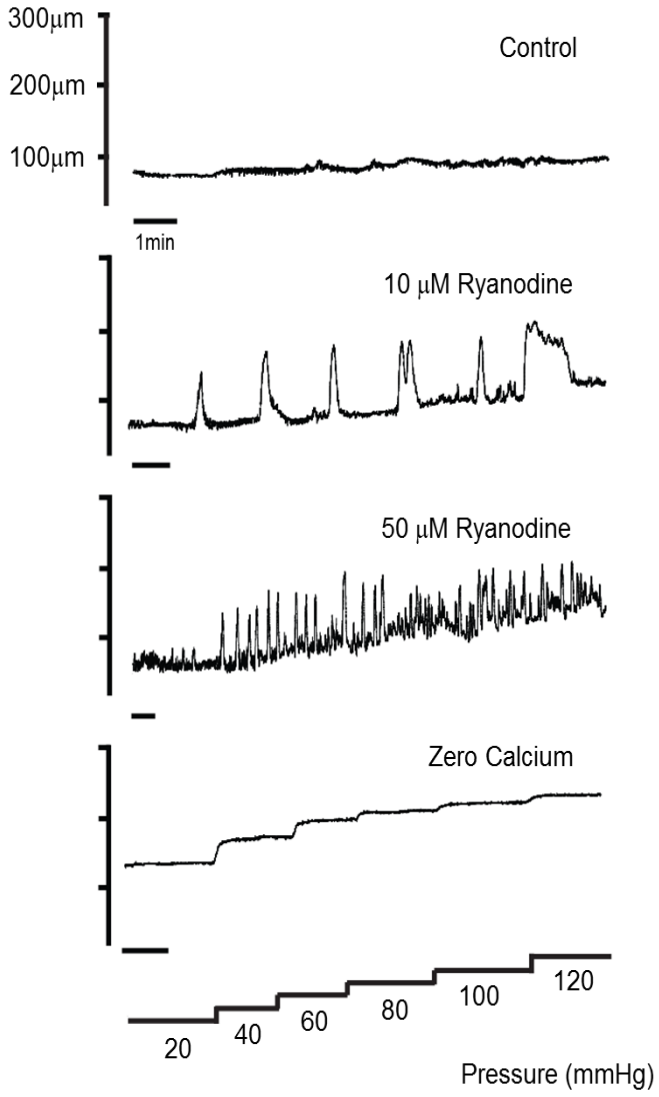


Figure 4.3: Calcium release from endoplasmic reticulum through RyR channel was required in the myogenic response of rat isolated middle cerebral arteries.

A) Mean pressure-diameter relationships for isolated segments of rat middle cerebral artery mounted in a pressure myograph and subjected to increases in pressure from 20-120 mmHg in 20 mmHg increments. Mean responses are shown in the presence and absence of ryanodine (an inhibitor of RyR; 10 μM; 50 μM) or zero extracellular calcium (passive response). Values are presented as mean ± SEM (n=6). When compared to control, a level of $P < 0.05$ is considered to be statistically significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

B)



B) Representative traces of pressure-diameter relationships for isolated segments of a rat middle cerebral artery in the presence and absence of ryanodine and zero extracellular calcium.

A)

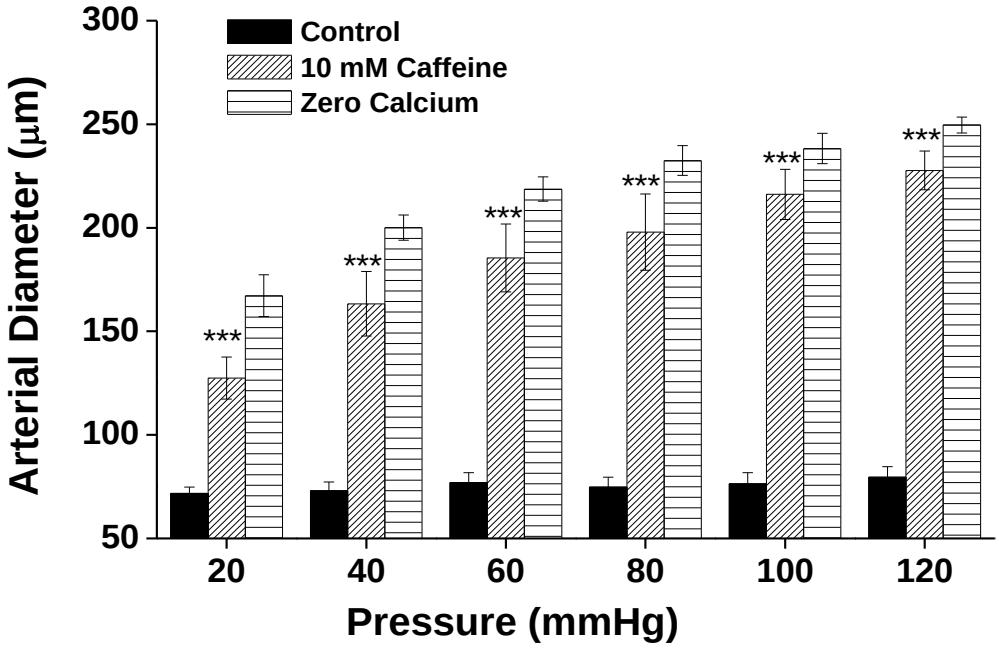
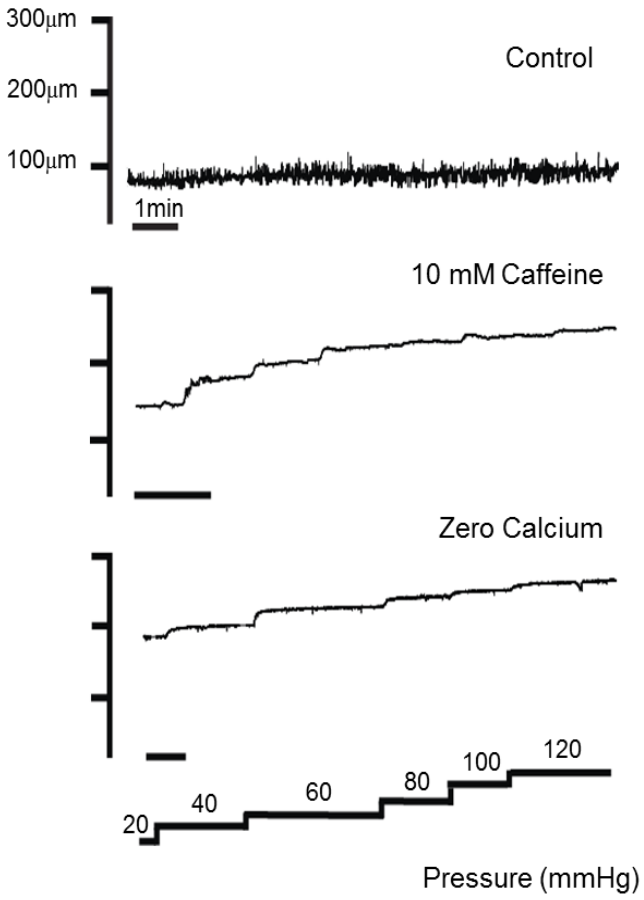


Figure 4.4: Release/depletion of calcium from endo/SR (10 mM caffeine) led to a loss in myogenic response of rat isolated middle cerebral arteries.

A) Mean pressure-diameter relationships for isolated segments of rat middle cerebral artery mounted in a pressure myograph and subjected to increases in pressure from 20-120 mmHg in 20 mmHg increments. Mean responses are shown in the presence and absence of caffeine (an agonist of RyR; 10 mM) or zero extracellular calcium (passive response). Values are presented as mean ± SEM (n=6). When compared to control, a level of P <0.05 is considered to be statistically significant (*p <0.05, **p <0.01, ***p <0.001).

B)



B) Representative traces of pressure-diameter relationships for isolated segments of a rat middle cerebral artery in the presence and absence of caffeine and zero extracellular calcium.

A)

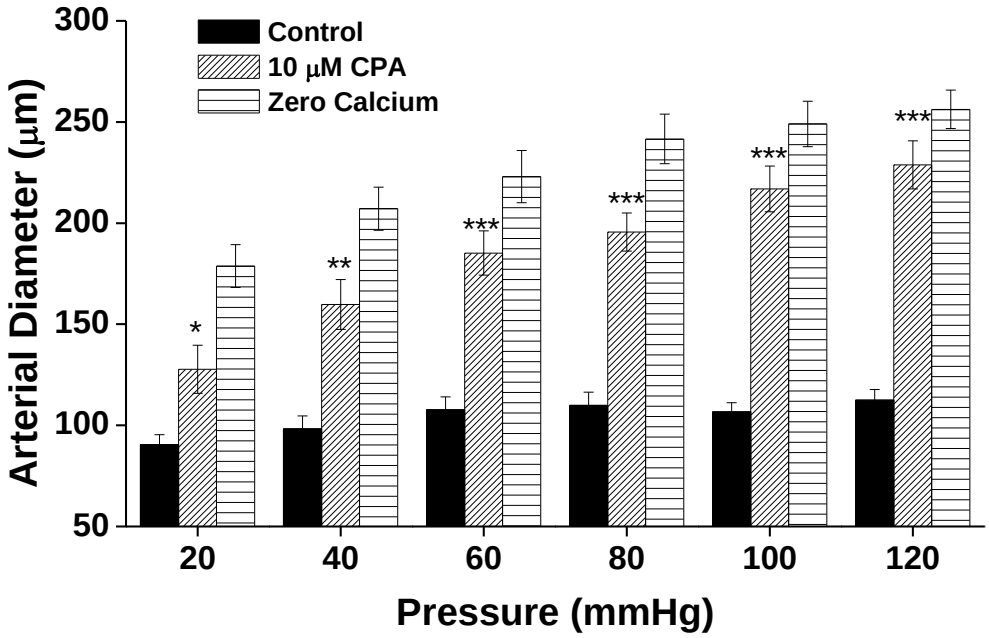
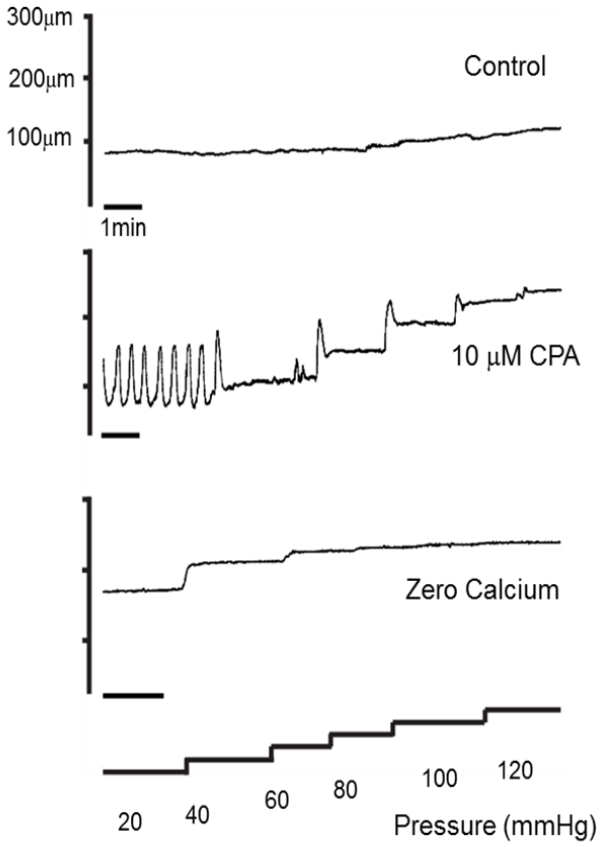


Figure 4.5: Inhibition of SERCA led to an endothelium-dependent loss of myogenic response.

A) Mean pressure-diameter relationships for isolated segments of rat middle cerebral artery mounted in a pressure myograph and subjected to increases in pressure from 20-120 mmHg in 20 mmHg increments. Mean responses are shown in the presence and absence of cyclopiazonic acid (CPA; an inhibitor of SERCA; 10 μM) or zero extracellular calcium (passive response). Values are presented as mean ± SEM (n=6). When compared to control, a level of P <0.05 is considered to be statistically significant (*p <0.05, **p <0.01, ***p <0.001).

B)



B) Representative traces of pressure-diameter relationships for isolated segments of a rat middle cerebral artery in the presence and absence of CPA and zero extracellular calcium.

A)

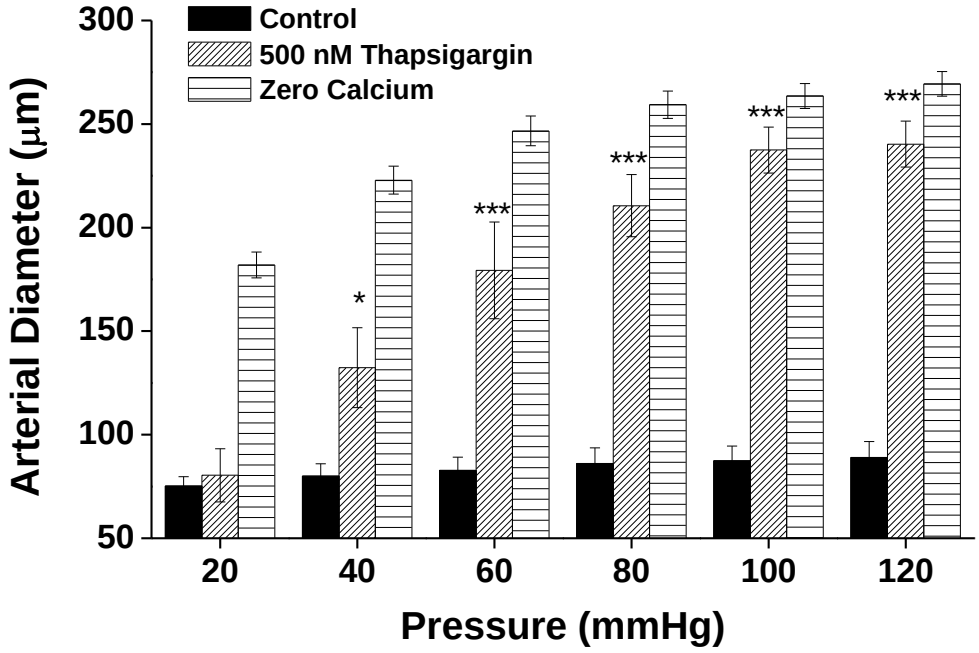
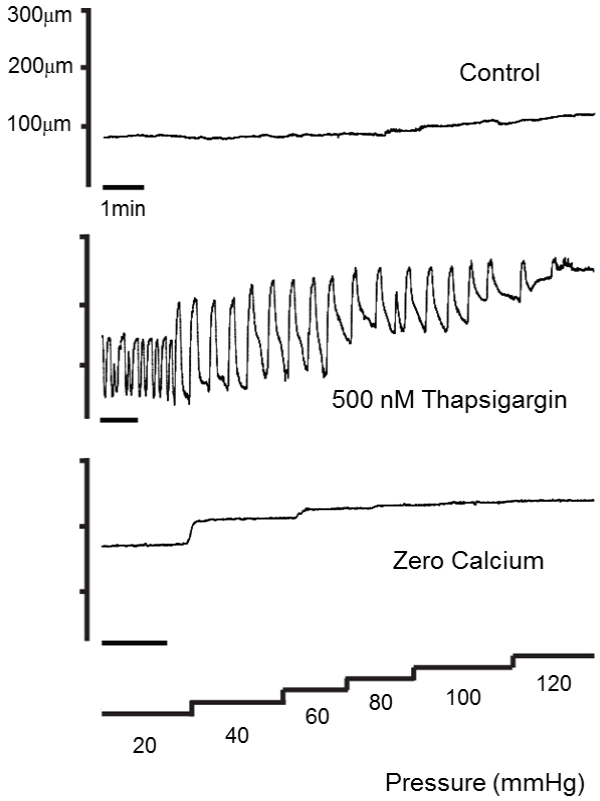


Figure 4.6: Inhibition of SERCA led to an endothelium-dependent loss of myogenic response.

A) Mean pressure-diameter relationships for isolated segments of rat middle cerebral artery mounted in a pressure myograph and subjected to increases in pressure from 20-120 mmHg in 20 mmHg increments. Mean responses are shown in the presence and absence of thapsigargin (an inhibitor of SERCA; 500 nM) or zero extracellular calcium (passive response). Values are presented as mean ± SEM (n=6). When compared to control, a level of P <0.05 is considered to be statistically significant (*p <0.05, **p <0.01, ***p <0.001).

B)



B) Representative traces of pressure-diameter relationships for isolated segments of a rat middle cerebral artery in the presence and absence of thapsigargin and zero extracellular calcium.

A)

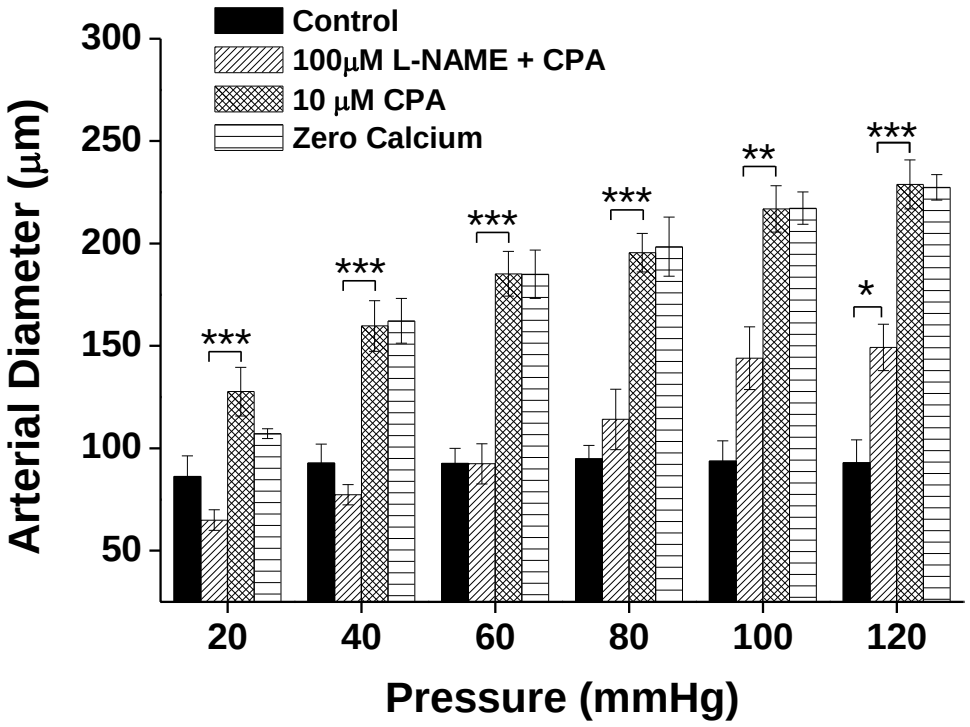
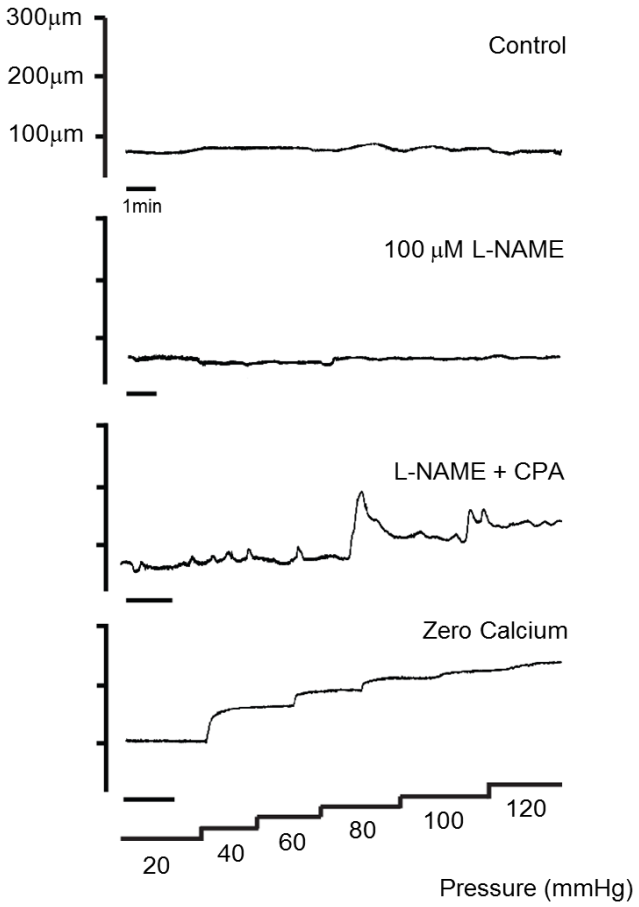


Figure 4.7: CPA-induced inhibition of myogenic response was mediated by activation of the NO pathway.

A) Mean pressure-diameter relationships for isolated segments of rat middle cerebral artery mounted in a pressure myograph and subjected to increases in pressure from 20-120 mmHg in 20 mmHg increments. Mean responses are shown in the presence and absence of CPA (10 μM), with (n=4) and without (n=6) L-NAME (an inhibitor of NOS; 100 μM). Response of zero extracellular calcium (passive response) is included. Values are presented as mean ± SEM. A level of $P < 0.05$ is considered to be statistically significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

B)



B) Representative traces of pressure-diameter relationships for isolated segments of a rat middle cerebral artery in the presence and absence of L-NAME, CPA with L-NAME, and zero extracellular calcium.

A)

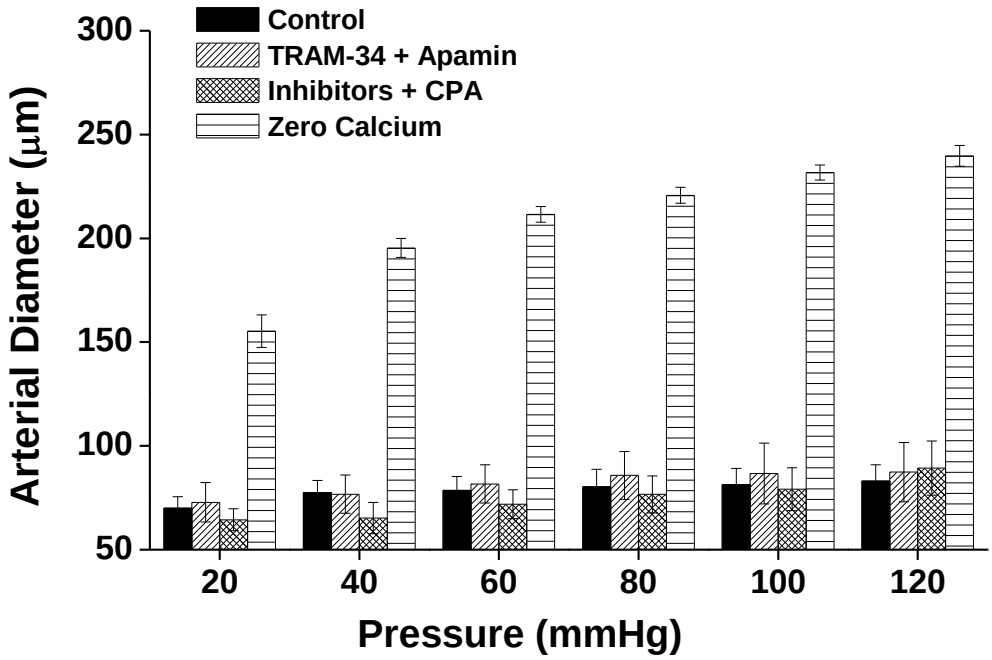
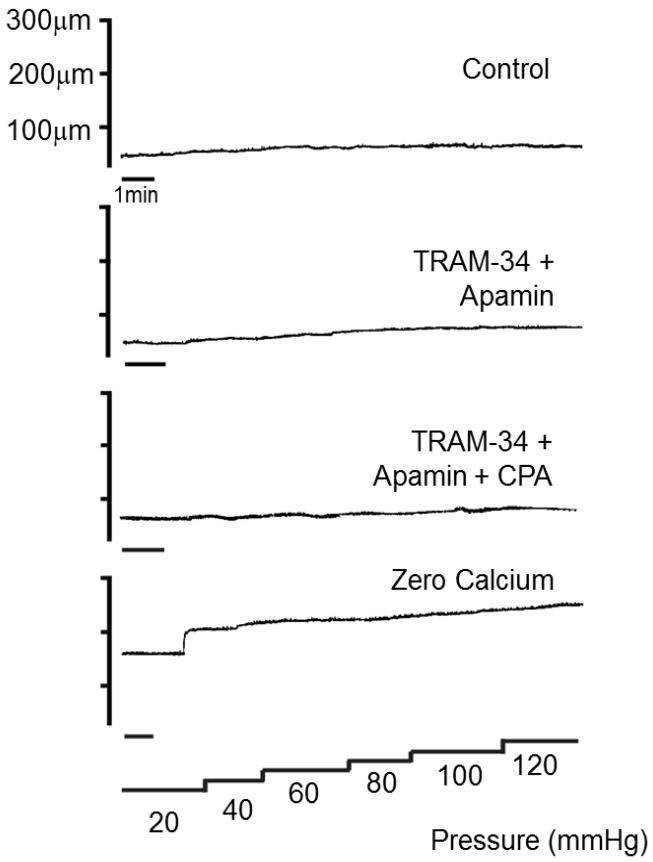


Figure 4.8: CPA-induced inhibition of myogenic response was mediated by activation of the EDH pathway.

A) Pressure-diameter relationships for isolated segments of rat middle cerebral artery mounted in a pressure myograph and subjected to increases in pressure from 20-120 mmHg in 20 mmHg increments. The endothelium is intact in these arteries. Mean responses are shown in the presence and absence of CPA (10 μM) with inhibitors TRAM-34 (1 μM) and apamin (50 nM). The response of TRAM-34 and apamin in combination is also included. Zero extracellular calcium shows passive response. Values are presented as mean ± SEM (n=4).

B)



B) Representative traces of pressure-diameter relationships for isolated segments of a rat middle cerebral artery in the presence and absence of TRAM-34 & apamin, CPA with TRAM-34 & apamin, and zero extracellular calcium.

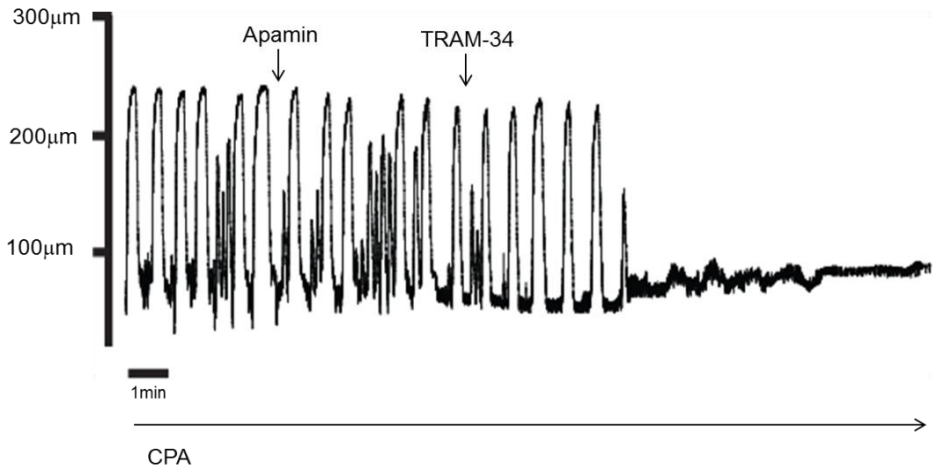


Figure 4.9: CPA-evoked inhibition of myogenic response was mediated by activation of the EDH pathway.

Representative trace of the diameter response to CPA (10 μM) with TRAM-34 (1 μM) and apamin (50 nM). A loss of myogenic tone was induced by CPA (10 μM) but the effect was reversed through the inhibition of SK_{Ca} and IK_{Ca} by apamin (50 nM) and TRAM-34 (1 μM). The intraluminal pressure was held at 80 mmHg during the experiments (n=4).

A)

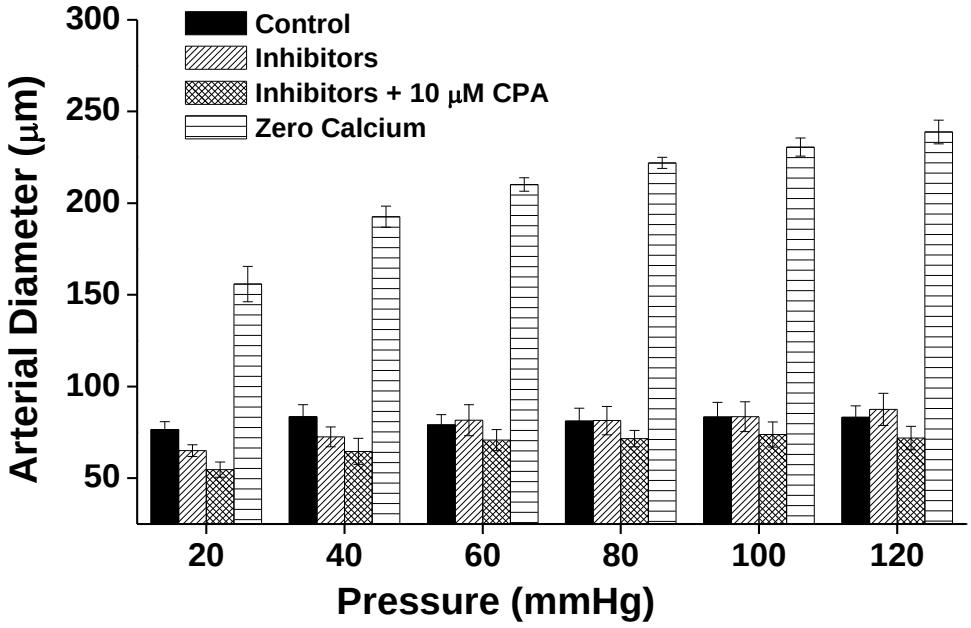
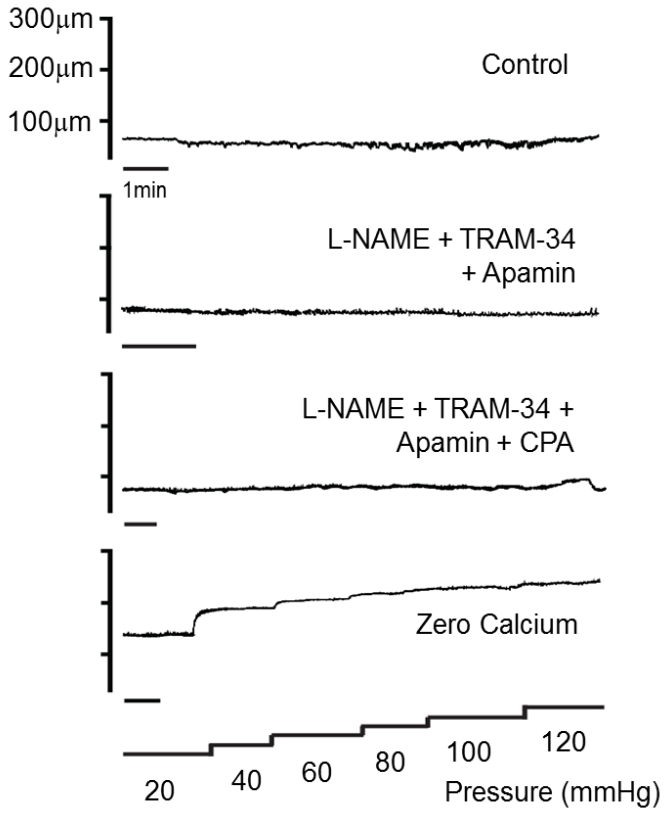


Figure 4.10: CPA-evoked inhibition of myogenic response was mediated by activation of the EDH and NO pathways.

A) Mean pressure-diameter relationships for isolated segments of rat middle cerebral artery mounted in a pressure myograph and subjected to increases in pressure from 20-120 mmHg in 20 mmHg increments. Mean responses are shown in the presence and absence of CPA (10 μM) with L-NAME (an inhibitor of nitric oxide synthase; 100 μM), TRAM-34 (an inhibitor of IK_{Ca}; 1 μM) and apamin (an inhibitor of SK_{Ca}; 50 nM). The response of L-NAME, TRAM-34 and apamin in combination is also included. Zero extracellular calcium shows passive response. Values are presented as mean ± SEM (n=5).

B)



B) Representative traces of pressure-diameter relationships for isolated segments of a rat middle cerebral artery in the presence and absence of inhibitors, CPA with inhibitors, and zero extracellular calcium.

A)

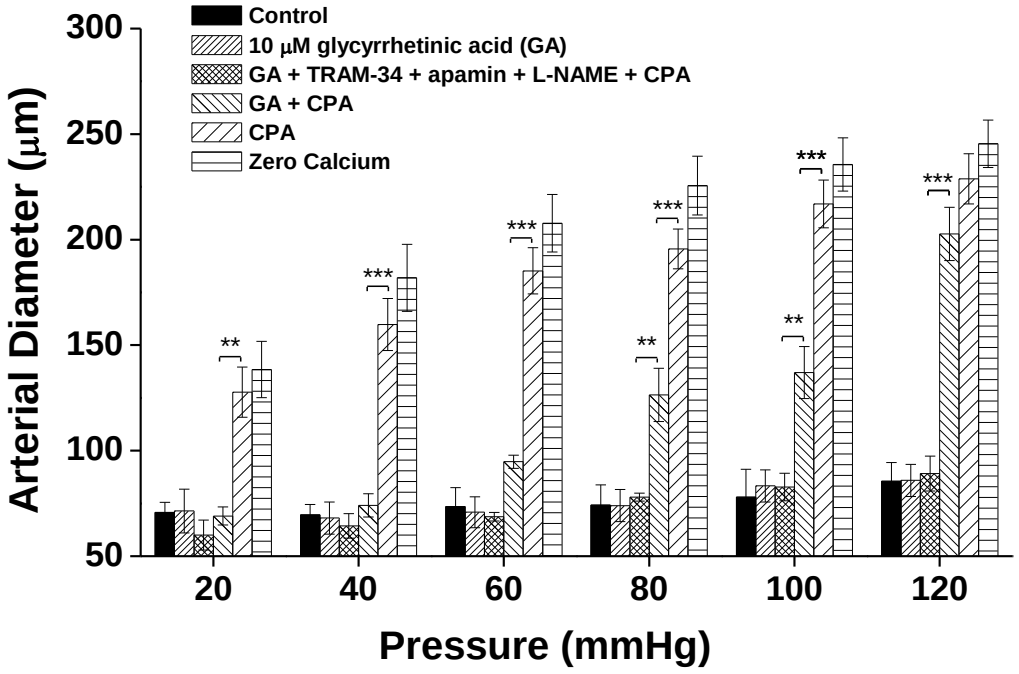
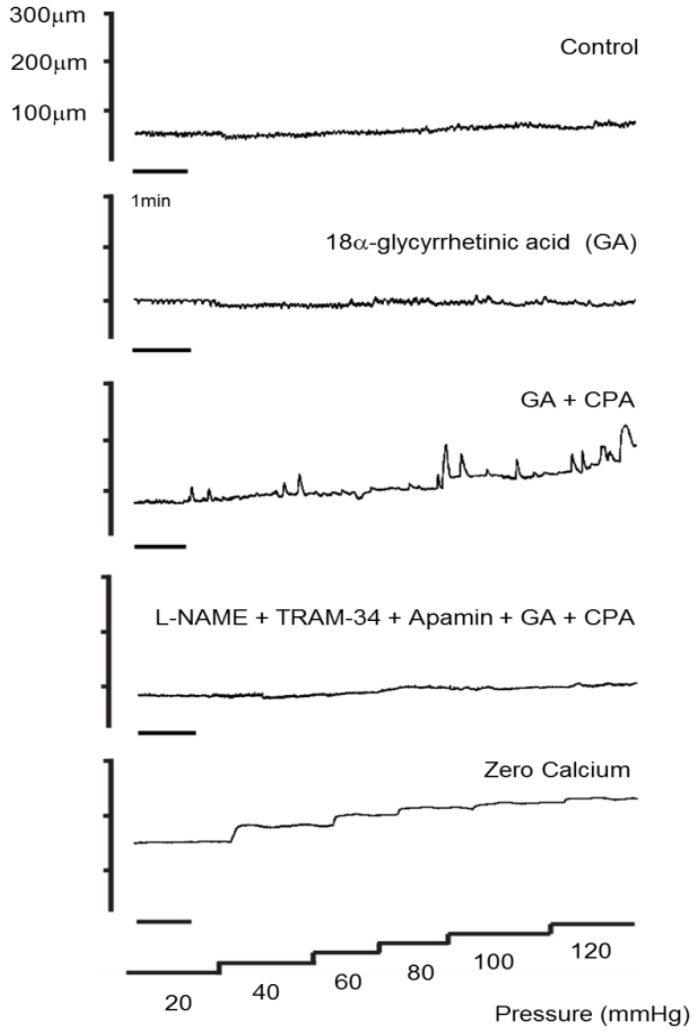


Figure 4.11: CPA-evoked inhibition of the myogenic response was partially reversed by inhibitor of endothelial- smooth muscle communication.

A) Mean pressure-diameter relationships for isolated segments of rat middle cerebral artery mounted in a pressure myograph and subjected to increases in pressure from 20-120 mmHg in 20 mmHg increments. Mean responses are shown in the presence and absence of 18α-glycyrrhetinic acid (10 μM), TRAM-34 (1 μM), apamin (50 nM), L-NAME (100 μM) and CPA (10 μM) alone or in combination. Mean response of zero extracellular calcium (passive response) is included. Values are presented as mean ± SEM (GA n=4; inhibitors+CPA n=4, GA+CPA n=4, CPA n=6). A level of $P < 0.05$ is considered to be statistically significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

B)



B) Representative traces of pressure-diameter relationships for isolated segments of a rat middle cerebral artery in presence and absence of inhibitors and in zero extracellular calcium.

A)

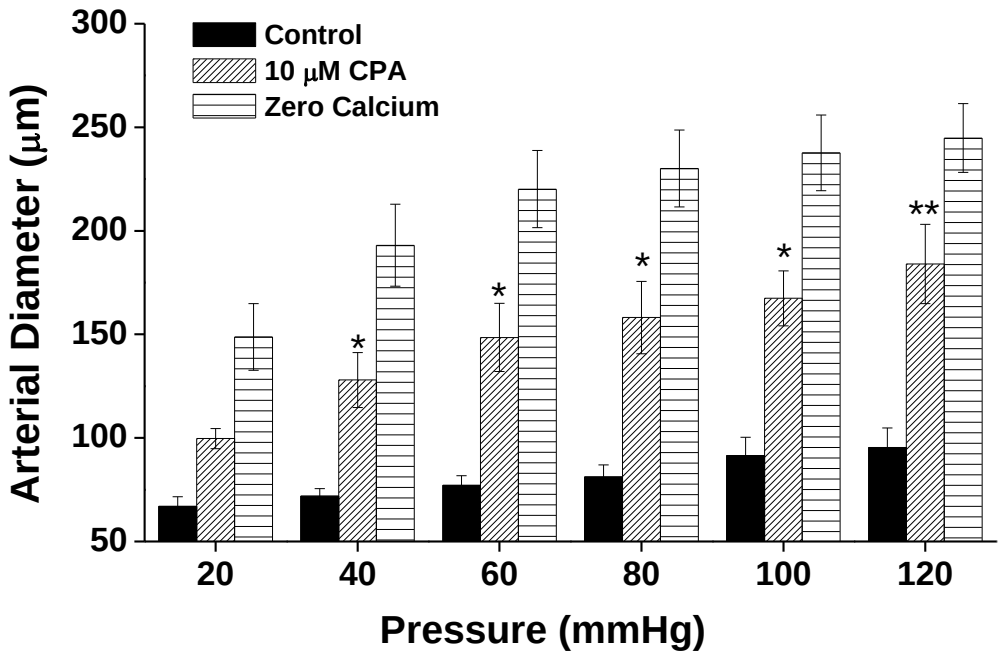
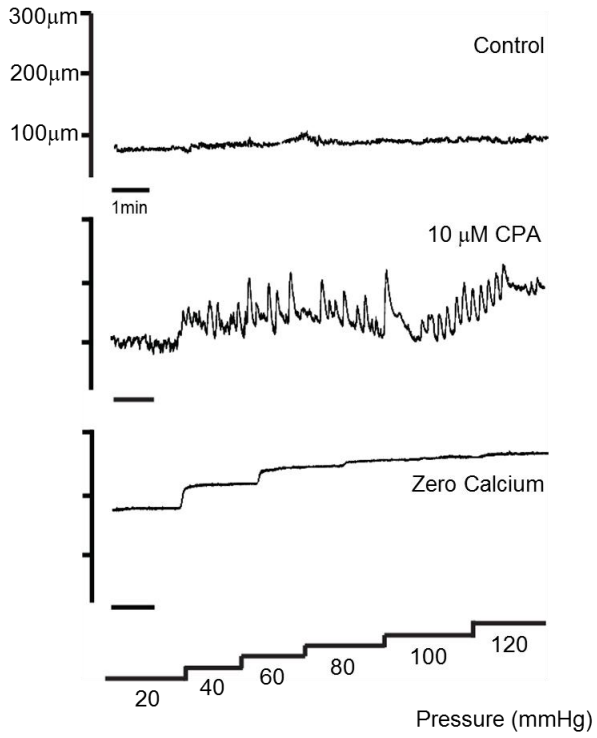


Figure 4.12: Luminal extracellular calcium may not be responsible CPA-induced loss of the myogenic response.

A) Mean pressure-diameter relationships for isolated segments of rat middle cerebral artery mounted in a pressure myograph and subjected to increases in pressure from 20-120 mmHg in 20 mmHg increments. The fluid inside the vessels was been replaced with zero calcium Kreb's solution. Mean responses are shown in the presence and absence of cyclopiazonic acid (CPA; an inhibitor of SERCA; 10 μM) or zero extracellular calcium (passive response). Values are presented as mean ± SEM (n=4). When compared to control, a level of $P < 0.05$ is considered to be statistically significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

B)



B) Representative traces of pressure-diameter relationships for isolated segments of a rat middle cerebral artery in the presence and absence of CPA and zero extracellular calcium.

A)

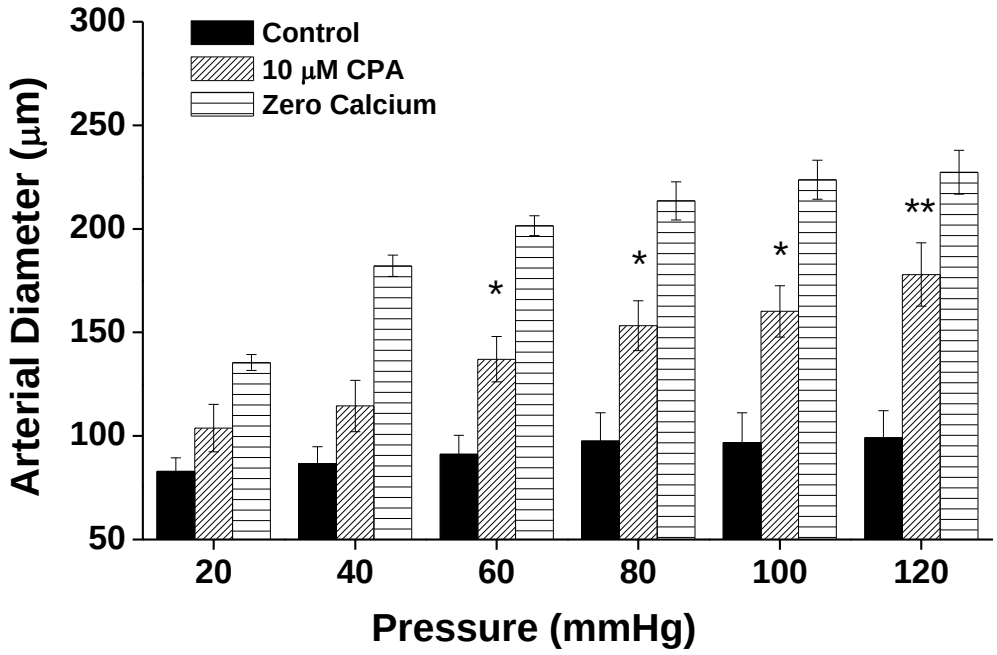
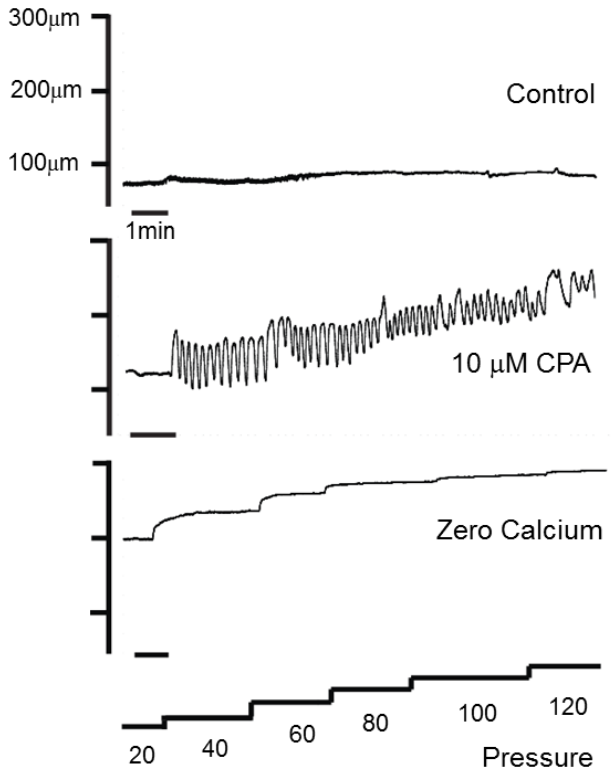


Figure 4.13: Perfusion of Kreb's solution intraluminally had no effect on CPA-evoked inhibition of myogenic response.

A) Mean pressure-diameter relationships for isolated segments of rat middle cerebral artery mounted in a pressure myograph and subjected to increases in pressure from 20-120 mmHg in 20 mmHg increments. The vessels were perfused intraluminally with 300 μl of Kreb's solution. Mean responses are shown in the presence and absence of cyclopiazonic acid (CPA; an inhibitor of SERCA; 10 μM) or zero extracellular calcium (passive response). Values are presented as mean ± SEM (n=4). When compared to control, a level of $P < 0.05$ is considered to be statistically significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

B)



B) Representative traces of pressure-diameter relationships for isolated segments of a rat middle cerebral artery in the presence and absence of CPA and zero extracellular calcium.

Chapter 5: General Discussion

The myogenic response is a key autoregulatory mechanism in the cerebral circulation and other parts of the vascular system. My data demonstrate that either disruption of the smooth muscle SR or the endothelial endoplasmic reticulum results in loss of myogenic tone in middle cerebral arteries.

5.1 Limitations

Investigation of the role of intracellular calcium in vascular function is limited by the fact that removal of extracellular calcium results in the loss of all active tone. Thus, as in other studies, my experimental design is reliant on the use of pharmacological inhibitors to disrupt calcium handling in the vascular cells. The three main limitations of this approach are: **1.** the selectivity of these agents at the concentrations used, **2.** if no effect is observed, whether the inhibitors are active or can access their site of action, and **3.** we cannot determine that they are indeed altering calcium handling.

1. The concentrations of the inhibitors used in my experiments were carefully selected based on the published literature. However, the possibility that they interacted with other targets cannot be ruled out. The same batch of CPA that caused endothelium-dependent loss of myogenic response also stimulated EDH- and NO-dependent relaxation of mesenteric and aortic tissues,

respectively but had no effect on phenylephrine-induced tone in endothelium-denuded vessels (Kale and Dane McMillan, personal communication). This strongly supports the observed endothelium-dependence of the effect of CPA on myogenic response. However, it does not preclude the fact that CPA could be causing the effect by an action on a target other than SERCA.

2. For inhibitors which had no effect in my experiments, positive controls were carried out. For example, the same batch of xestospongin used in my experiments was shown to inhibit agonist-induced vasoconstriction in mesenteric arteries. A positive result obtained in a different assay using the same batch of inhibitor provides confidence that the negative result obtained in my experiments was valid.

3. Simultaneous measurement of changes in intracellular calcium within endothelial or smooth muscle cells and vessel diameter of myogenically active arteries is technically difficult. This is due to the need for a microscope with a long working distance specifically set up for the purpose and the possibility of movement artefacts. This is reflected in the fact that there are very few reports in the literature of such an approach being applied to smooth muscle cells in intact arteries (Zacharia *et al.,* 2007; Mufti *et al*., 2010; Westcott and Jackson, 2011) and even less in which changes in endothelial cell calcium were analysed (Marrelli, 2001; 2002; Kansui *et al.,* 2008).

Thus, in order to address this issue, we have initiated a collaboration with Dr. Jonathan Ledoux at the Montreal Heart Institute. Dr. Ledoux has experience in measuring changes in endothelial and smooth muscle calcium levels in small arteries (Ledoux *et al.*, 2008). As shown in Appendix 1, preliminary experiments have been carried out to look at the effect of CPA on calcium in the endothelium of isolated middle cerebral arteries cut open longitudinally, and pinned to a Sylgard block with the endothelium facing up. The data from these experiments is shown in Appendix 1. Although limited, so far this data confirms that CPA does increase endothelial calcium levels.

5.2 Future work

Further work will be carried out to confirm the actions of CPA, caffeine and ryanodine on endothelial and smooth muscle cell calcium levels within rat isolated middle cerebral arteries. These measurements will continue to be made in pinned out arteries as this will allow us to investigate the effects of manipulations such as calcium removal. However, it does not provide a direct link between calcium changes and tone and so the eventual aim will be to make such measurements in myogenically active vessels.

As described in Chapter 4, disruption of endothelial calcium stores had a dramatic effect on the myogenic response. Thus, examination of endothelial calcium handling in pathological conditions associated with loss of myogenic

autoregulation may provide new insights into the underlying mechanism and possible new therapeutic approaches.

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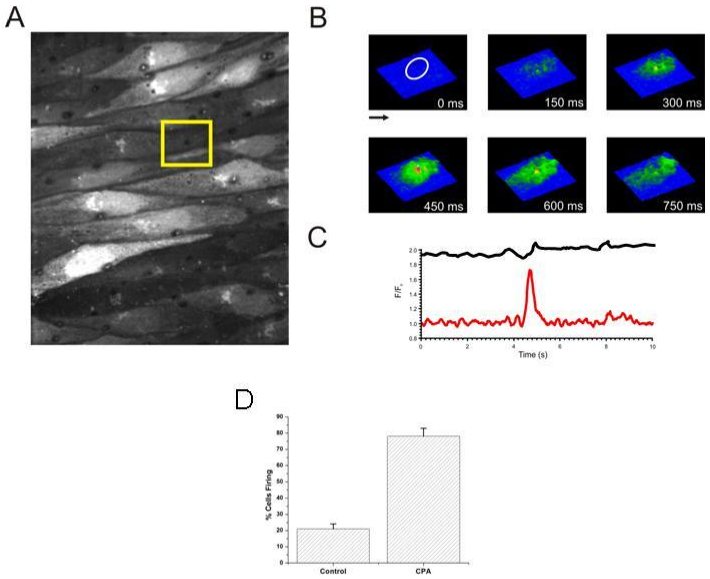
Appendix 1: Calcium imaging

Methods

Rat middle cerebral arteries were cleaned of connective tissue, cut longitudinally, and pinned to a Sylgard block with the endothelium facing up.

Calcium imaging was performed with a Revolution Andor confocal system (Andor Technology) with an electron-multiplying CCD camera on an upright Nikon microscope with a ×60, water-dipping objective (NA 1.0). Images were acquired at 15–30 frames per second with Andor Revolution TL acquisition software (Andor Technology). Bound calcium was detected by exciting at 488 nm with a solid-state laser and collecting emitted fluorescence above 510 nm. Fractional fluorescence (F/Fo) was evaluated by dividing the fluorescence of a region of interest (ROI) in the collected image by an average fluorescence of 50 images without activity from the same ROI using custom-designed software. The endothelial cell surface was measured by automated analysis of the area enclosed by a freehand ROI drawn around the outline of individual endothelial cells. Global calcium levels were measured over the entire area of a cell, defined by the freehand ROI outline. Individual calcium events (pulsars) were analyzed by using an ROI defined by a 5 × 5 pixel box positioned at a point corresponding to peak pulsar amplitude. Line-scan analysis was performed offline. Endothelial cells were preferentially loaded with Fluo-4 (10 μM) for 45 min at 30°C in the presence of pluronic acid (2.5 μg/ml) before imaging.

CPA (10 μM) was applied for 15 minutes during which time all measurements were made.



Calcium events in endothelium of rat middle cerebral artery: A. Fluo-4 loaded endothelial cells in a cerebral artery showing the characteristic spindle shape of endothelial cells. **B.** Time course of a localized calcium signal in endothelium, (yellow square). **C.** Representative trace illustrating kinetics of a calcium event shown in B (redline) and bulk calcium (black line) in the presence of CPA (10 μM). **D.** Effect of CPA on the number of cells firing calcium events (n=3).

Results

Endothelial cells of middle cerebral arteries showed spontaneous localized calcium events (pulsars). Application of CPA caused an initial significant increase in the number of cells showing calcium pulsars (firing) and also an increase in bulk calcium. However, after 15 minutes the occurrence of pulsars was decreased below baseline although bulk calcium remained elevated.

Conclusion

These preliminary data indicate that endothelial cells of rat middle cerebral arteries spontaneous calcium release events similar to those previously described in the endothelium of mouse and rat mesenteric arteries (Ledoux *et al*., 2008; Kansui *et al*., 2008). It is not clear why CPA initially increased the firing of pulsars but inhibition of these events on prolonged application supports the contention that CPA leads to depletion of SR calcium stores. This is also supported by the observation that CPA caused an immediate and prolonged increase in bulk endothelial calcium.

Appendix 2: Publications

Abstracts

2012 **Tam R,** Kerr PM, Plane F. Endothelium-dependent modulation of cerebral artery myogenic tone by cyclopiazonic acid. *Faseb J, In press.*

2011 Kerr PM, **Tam R**, Tran CHT, Sandow S, Welsh DG, Plane F. Smooth Muscle to Endothelial Communication in Mesenteric Arteries. *Faseb J,* 25, 819.17.

2010 **Tam R,** Kerr P, Tran A, Plane F. Intracellular calcium stores contribute to the myogenic response of rat cerebral arteries. *Faseb J.*, 24, 985.

2009 **Tam R**, Kerr P, Tran A, Plane F. Intracellular calcium stores contribute to the myogenic response of rat cerebral arteries. *Can. J. Cardiol.,* 25 (suppl. B), 80B.

Book Chapter

2011 Kerr PM, **Tam R,** Plane F. Endothelium. In "Mechanisms of Vascular Disease: A Reference for Vascular Specialists". Pages 1-20, Eds. R Fitridge and M Thompson, University of Adelaide Press.

Invited review

2012 Kerr PM, **Tam R**, Mittal R, Ondrusova K, Plane F. Endothelial feedback and the myoendothelial projection. *Microcirculation. In press.*

In revision

Narang D, Kerr PM, **Tam R**, Baserman J, Searle G, Light P, Holt A, Plane F. Inhibition of voltage-operated calcium channels by Triton-X 100. *J. Pharmacol. Exp. Thera.*

Submitted

Kerr PM, **Tam R**, Baserman J, Sandow S, Plane F. Myoendothelial communication in mesenteric resistance arteries. *Am. J. Physiol.*

In preparation

Tam R, Kerr PM, Ledoux J, Plane F. Intracellular calcium in the regulation of myogenic tone.