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Regulation of Myogenic Tone in Cerebral and Mesenteric Resistance Arteries by Metabolic Agents and Second Messenger Systems

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ABSTRACT

1. The pressure-perfusion myograph, permeabilisation techniques and also intracellular membrane potential recordings were used to examine the regulation of myogenic tone in cerebral and mesenteric resistance arteries by metabolic agents and second messenger systems.

2. After pressurisation to 60 mmHg, rat isolated mesenteric and cerebral resistance arteries developed spontaneous myogenic tone, resulting in a 26 ± 1% (n = 42) and 30 ± 2% (n = 14) reduction in diameter respectively.

3. The metabolic vasodilator adenosine and the K_{ATP} channel opener cromakalim each produced a dose-dependent dilatation of pressurised mesenteric arteries. The cromakalim-evoked dilatation was inhibited by glibenclamide (1 μM), demonstrating the presence of the K_{ATP} channel in the mesenteric artery and their activation as the mechanism for cromakalim-evoked dilatation. In contrast neither adenosine nor cromakalim produced a dilatation of pressurised cerebral arteries.

4. Adenosine-evoked dilatation of mesenteric arteries was unaffected by the nitric oxide synthase inhibitor L-NAME (100 μM), antagonists of the K_{ATP} channel (glibenclamide; 1 μM), the small conductance Ca^{2+} activated K^+ channel (apamin; 0.3 μM) and the large conductance, Ca^{2+} activated K^+ channel (TEA; 1 mM). Further to this, cromakalim (10 μM) but not adenosine (100 μM) produced a hyperpolarisation of the pressurised mesenteric artery. This suggests that neither nitric oxide synthesis nor K^+ channel activation contributed to the adenosine-evoked dilatation.

5. Adenosine evoked a dose-dependent dilatation of β-escin permeabilised mesenteric arteries, where the intracellular Ca^{2+} concentration was

clamped to ≈600 nM. The mechanism of adenosine-evoked dilatation may involve a decreased myofilament Ca^{2+} sensitivity.

6. An increase in extracellular potassium ion concentration ($[K^+]_o$) may link increased neuronal activity and regional cerebral blood flow. Elevation of $[K^+]_o$ from 4.7 to 10 mM evoked a sustained dilatation of isolated pressurised thalamo-perforating cerebral arterioles.

7. The K^+-evoked dilatation was inhibited by the inward rectifier K^+ channel (K_{IR}) inhibitor Ba^{2+} (50μM), and the K^+ channel inhibitor cesium (20mM) but was not blocked by inhibitors of the ATP-sensitive (K_{ATP}) and the Ca^{2+}-activated K^+ channel (K_{Ca}), glibenclamide (1μM) and TEA (1mM) respectively. Nor was the dilatation altered with the neurotoxin tetrodotoxin (TTX, 0.3μM). The K^+-evoked dilatation was associated with a membrane hyperpolarisation to -58 ± 1 mV (n = 5), from a control value of -42 ± 1 mV (n = 10).

8. It is proposed that increased $[K^+]_o$ evokes a dilatation of thalamo-perforating cerebral arteries via an activation of K_{IR} channels and smooth muscle cell hyperpolarisation.

9. An increase in $[Ca^{2+}]_o$ to approximately 700 nM evoked a 30 ± 3 % (n = 28) constriction of isolated β-escin permeabilised cerebral resistance arteries.

10. Under $[Ca^{2+}]_i$ clamped conditions the putative PKC activator indolactam evoked a 20 ± 2% constriction of the artery. The PKC inhibitor ($PKC_{(19-36)}$; 1 μM) produced a near maximal (85 ± 4 %) reversal of the indolactam-evoked constriction of the artery, while $PKC_{(19-36)}$ (1 μM) produced only a minor (12 ± 3 %) reversal of the Ca^{2+}-induced constriction, thus confirming that the indolactam-evoked constriction was due to an activation of PKC.

11. The MLCK antagonist SM-1 (100 μM) reversed both the Ca^{2+}- and the indolactam-evoked constriction of the artery. The calmodulin antagonist RS-20 (0.1 - 100 μM) dose-dependently reversed the Ca^{2+}-evoked constriction but, even up to a concentration of 300 μM, did not reverse the indolactam evoked-constriction of the artery.

12. It is proposed that MLCK but not calmodulin plays a role in the PKC-evoked smooth muscle contraction.

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For my Father.....

Digging *by Seamus Heaney*

Between my finger and my thumb
The squat pen rests; snug as a gun.

Under my window, a clean rasping sound
When the spade sinks into gravelly ground;
My father, digging. I look down.

Till his straining rump among the flowerbeds
Bends low, comes up twenty yeas away
Stooping in rhythm through potato drills
Where he was digging.

The coarse boot nestled on the lug, the shaft
Against the inside knee was levered firmly.
He rooted out tall tops, buried bright edge deep
To scatter new potatoes that we picked
Loving their cool hardness in our hands.

By God, the old man could handle a spade.
Just like his old man.

My grandfather cut more turf in a day
Than any other man on Toner's bog.
Once I carried him milk in a bottle
Corked sloppily with paper. He straightened up
To drink it then fell right away

Nicking and slicing neatly, heaving sods
Over his shoulder, going down and down
For the good turf. Digging.

The cold smell of potato mould, the squelch and slap
Of soggy peat, the curt cuts of an edge
Through living roots awaken in my head.
But I have no spade to follow men like them.

Between my finger and my thumb
The squat pen rests.
I'll dig with it.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AA	arachadonic acid
ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
bas	basilar artery
$[Ca^{2+}]_o$	extracellular Ca^{2+} concentration
$[Ca^{2+}]_i$	intracellular Ca^{2+} concentration
CaM	calmodulin
cAMP	adenosine 3',5'-cyclic monophosphate
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CTX	charybdotoxin
DAG	1,2-diacylglycerol
DHET	vic-dihydroxyeicosatetraenoic acid
DIDS	diisothiocyanatostilbene-2,2'-disulphonic acid
DMSO	dimethylsulphoxide
EET	epoxyeicosatetraenoic acid
EGTA	ethylene glycol-bis (β-aminoethylether)-N,N,N′,N′-tetraacetic acid
H-7	1-(5-isoquinolinylsulfonyl)-2-methyl piperazine

HEPES	N-2-Hydroxyethylpiperazine-N′-2-ethanesulphonic acid
HETE	hydroxyeicosatetraenoic acid
IAA-94	indanyloxyacetic acid
IP_3	inositol 1,4,5-triphosphate
$[K^+]_o$	extracellular K^+ concentration
K_{ATP}	ATP-sensitive potassium channel
KCa	Ca^{2+}-activated K^+ channel
K_{IR}	inward rectifier K^+ channel
K_v	voltage-dependent K^+ channel
L-NAME	L-nitro methylester
MLC	myosin light chain
MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
pcer	posterior cerebral artery
pcom	posterior communicating artery
pH_i	intracellular pH
pH_o	extracellular pH
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase
PLC	phospholipase C
PSS	physiological saline solution
SAC	stretch activated channel

scba	superior cerebellar artery
Ser	serine
SEM	standard error of the mean
SR	sarcoplasmic reticulum
TEA	tetraethylammonium
thp	thalamo-perforating artery
Thr	threonine
TTX	tetrodotoxin
VDCC	voltage dependent Ca^{2+} channel
U-73122	(1-[6-([(17β)-3-methoxyestra-1,3,5 (10-trien-17-yl]amino) hexyl]-1-pyrrole-2,5-dione)
4-AP	4-aminopyridine
3,4-DAP	3,4-diaminopyridine

PUBLICATIONS

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DECLARATION

The investigations presented in this thesis were conducted by its author. No part of the has been previously been presented in fulfilment of the regulations for any degree or diploma, either at this University or any other institution.

H.S.Routledge

Chapter 1

General Introduction

1.1 Introduction

Resistance arteries are of physiological significance in the regulation of both total peripheral resistance and tissue blood flow. These blood vessels develop basal spontaneous contraction that is modulated by alterations in tissue metabolic requirements as well as changes in blood pressure. The resistance offered by these small arteries, to blood flow, as well as the high level of modulation of their contraction, by physiological events, ensures that resistance arteries play a central role in normal circulatory homeostasis.

Until relatively recently it had been assumed that resistance vessels consisted of arterioles alone, i.e. precapillary vessels having diameters of less than 100 μm (Duling *et al.,* 1981). However, pressure profile studies examining the relationship between the systemic blood pressure and intravascular blood pressure, in varying sized blood vessels, found that the precapillary resistance is shared between the small arteries (100 - 500 μm) and the arterioles (<100μm). Up to 50% of the peripheral resistance occurred upstream of arterioles (Davis *et al.,* 1986). It is now accepted that small arteries as well as the arterioles, play an important role in maintaining the vascular peripheral resistance (Mulvany & Aalkjaer, 1990).

As well as differing in size, the resistance arteries differ from arterioles in the number of smooth muscle cell layers present within the *tunica media.* Arterioles are characterised by a single smooth muscle cell layer (Walmsley *et*

al., 1982) while resistance arteries have up to six smooth muscle cell layers (Lee *et al.*, 1983). The smooth muscle cells within the *tunica media* are arranged circumferentially and connected via collagen fibres. It is the contraction of the smooth muscle cells within the *tunica media* that regulates the vessel diameter in response to various stimuli. Apart from the difference in the number of smooth muscle cell layers within the *tunica media* both resistance arteries and arterioles share the common features of the *tunica adventitia* and a *tunica intima.* The *tunica adventitia* contains all of the nerve endings in the vessel and the connective tissue (elastin and collagen) by which the vessel is held to the surrounding tissue, while the *tunica intima,* consists of a continuous layer of squamous endothelial cells. The endothelial cells can affect vascular contractility by releasing both contracting and relaxing factors in response to mechanical and chemical stimuli (Furchgott, 1983; Rubanyi *et al.*, 1986).

A central theme in resistance artery research has developed around the phenomenon of "blood flow autoregulation". This is the tendency for blood flow to remain constant despite changes in arterial perfusion pressure. Two main mechanisms of blood flow autoregulation have been recognised. These are, the *extrinsic control* mechanisms which includes metabolic vasoactive agents as well as neurogenic influences and the *intrinsic* or "*myogenic*" regulation of blood vessel diameter which enables the resistance artery to both sense and respond to changes in local tissue blood flow demands.

In addition to its accepted importance in normal physiological blood flow control, alterations in autoregulation are associated with several cardiovascular disease conditions. For example, in essential hypertension the blood flow autoregulatory pressure range is extended - an adaptation which may be protective (Osol & Halpern, 1985). Part of the autoregulatory adaptation that occurs in hypertension is achieved through a remodelling of the blood vessel wall due to smooth muscle hypertrophy and/or hyperplasia bringing about a thickening of the media. The remodelling reduces wall stress (Osol & Halpern, 1985). In other disease states however, such as diabetes mellitus, the blood flow autoregulatory response may be impaired (Hayashi *et al.,* 1992b; Hill & Meininger, 1993; Forster *et al.,* 1994). This reduction in blood flow regulation and the subsequent decrease in afferent arteriolar resistance results in hyperperfusion and an increased glomerular capillary pressure. The resultant glomerular hyperfiltration increases the development of glomerulosclerosis (Hostetter *et al.,* 1982; Zatz *et al.,* 1985).

Blood flow autoregulation is clearly important in normal physiological blood flow control yet the contribution of extrinsic (metabolic) and intrinsic (myogenic) influences on blood flow autoregulation remains a debated and contentious issue. Early studies held that autoregulation arose principally from metabolic control but more recently myogenic systems are proposed as the preeminent mechanism. These are reviewed below.

1.2 Metabolic Regulation of Blood Flow

Increased metabolic activity associated with for example, increased skeletal or cardiac muscle contraction, results in an increased O_2 consumption and production of vasodilator metabolites e.g. CO_2, adenosine, K^+. The reduction in oxygen tension (PO_2) and increase in vasodilator metabolite concentration evokes an autoregulatory vasodilatation producing an increase in blood flow to match the increased metabolic rate (Olsson, 1981; Johnson, 1986). A second mechanism of autoregulatory vasodilatation involves a decrease in blood flow due to, for example, a reduction in systemic blood pressure, leading to a reduced washout of vasodilator metabolites. The subsequent build up of metabolites again evokes a vasodilatation and an increase in blood flow (Olsson, 1981; Johnson, 1986). It is this local metabolic regulation that is thought to act as the link between increased metabolic activity and oxygen consumption and the associated increased local blood flow (Muller *et al.*, 1996).

One theory for the local regulation of blood flow is based on the suggestion that decreased blood pressure (reducing blood flow), or increased tissue metabolism each decrease tissue PO_2 (Johnson, 1964). This tissue hypoxia may then act either directly on the vascular smooth muscle, evoking relaxation (Guyton *et al.*, 1964), or indirectly, via the production of vasodilator metabolites from the hypoxic cells (Berne, 1964). Conversely, if metabolism is reduced or there is an increase in blood flow, tissue PO_2 would increase and

vasoconstriction results (Morff & Granger, 1982; Jackson & Duling, 1983). Resistance artery dilatation due to the direct action of reduced PO_2 has been demonstrated in several studies including pial arteries of the cat (Kontos *et al.*, 1978) and skeletal muscle arteries of the rat (Fredricks *et al.*, 1994). In one particular study, cat middle cerebral artery segments dilated in response to a decrease in PO_2 from 150 mmHg to 40 mmHg (Lombard *et al.*, 1986). It was also demonstrated that reduced PO_2 decreased the depolarisation of vascular smooth muscle in response to increased transmural pressure and reduced the associated myogenic contraction. It was concluded that the decrease in membrane depolarisation reduced transmembrane Ca^{2+} influx and thus inhibited vascular smooth muscle tone (Lombard *et al.*, 1986). It has since been demonstrated, in isolated rat renal arterioles, that the inhibition of myogenic vasoconstriction, evoked by reduced PO_2, is produced by K_{ATP} channel activation, leading to membrane hyperpolarisation subsequent inhibition of voltage dependent Ca^{2+} channels and a decreased Ca^{2+} entry (Loutenhiser & Parker, 1994).

Reduced tissue PO_2 can also indirectly regulate local blood flow, via an alteration in tissue metabolism and the production of vasodilator metabolites (Johnson, 1986). Some of the possible vasodilator metabolites including adenosine, CO_2, pH and K^+ are discussed below.

Adenosine

Adenosine has been recognised as a potential mediator of metabolic vasodilatation for a number of years (Berne, 1963; Berne, 1964) and an adenosine-evoked vasodilatation has been demonstrated in a number of vascular beds including the coronary (Berne, 1980), cerebral (Winn *et al.*, 1981) and the mesenteric (Jacobson & Pawlik, 1994) circulation. It is suggested that decreased PO_2 leads to the dephosphorylation of adenosine 5'-triphosphate (ATP) to adenosine 5'-diphosphate (ADP). ADP, in turn is dephosphorylated producing 5'-adenosine monophosphate (AMP; via myokinase) and then to adenosine by 5'-nucleotidase, an ectoenzyme located on the cell membrane (Rubio *et al.*, 1973). Adenosine is released into the extracellular space where it is suggested to bind to the adenosine A_2 receptor on vascular smooth muscle (Olsson & Pearson, 1990), leading to the activation of adenylate cyclase (Hori & Kitakaze, 1991) and production of adenosine 3',5'-cyclic monophosphate (cAMP) (Herlihy *et al.*, 1976; Kukovetz *et al.*, 1978). One mechanism of action for adenosine-evoked vasodilatation may involve cAMP-dependent protein kinase A (PKA) activation of the ATP-sensitive potassium channels (K_{ATP}) in smooth muscle (Quayle & Standen, 1994; Zhang *et al.*, 1994; Kleppisch & Nelson, 1995). Supporting this, several studies have demonstrated an adenosine evoked glibenclamide-sensitive vasodilatation and hyperpolarisation of coronary (Daut *et al.*, 1990; Akatsuka *et al.*, 1994), skeletal muscle (Jackson, 1993) and large (>400μm) cerebral arteries (Nagao *et al.*, 1996). In addition to the activation of K_{ATP} channels, PKA has been

suggested to phosphorylate myosin light chain kinase (de Lanerolle *et al.*, 1984), decreasing its Ca^{2+} sensitivity and relaxing the muscle (Kamm & Stull, 1985; Stull *et al.*, 1990). Furthermore, cAMP may also regulate intracellular Ca^{2+} homeostasis. For example, cAMP may inhibit the sarcolemmal Ca^{2+} channels or increase the activity of the Ca^{2+}-ATPase, each of which would result in a decrease in intracellular Ca^{2+} concentration and a resultant vasodilatation (see reviews Jacobson & Pawlik, 1994; Lincoln *et al.*, 1996). The role and possible mechanisms of action of adenosine-evoked dilatation has been discussed further in chapter 3 of this thesis.

CO_2 and pH

CO_2 production is directly coupled to oxygen consumption and ATP production giving rise to the suggestion that CO_2 may act as a mediator in the metabolic regulation of blood flow (Olsson, 1981). Vasodilatation in response to increased CO_2 (hypercapnia) has been demonstrated in a number of vascular beds, including cerebral (Kontos *et al.*, 1977; Faraci *et al.*, 1994), skeletal muscle (Deal & Green, 1954) and coronary circulation's (Ledingham *et al.*, 1970). It has been suggested that in the cerebral circulation, hypercapnic vasodilatation is exerted via an associated reduction in extracellular pH and not molecular CO_2 alone (Kontos *et al.*, 1977; Toda *et al.*, 1989). In support of this, a more recent study examined the role of both intracellular (pH_i) and extracellular pH (pH_o) in hypercapnic relaxation of isolated segments of rat cerebral arteries (Tian *et al.*, 1995). It was concluded that the smooth muscle relaxation due to hypercapnic acidosis was indeed exerted via a reduction of

pH_o, not a reduction of pH_i or molecular CO_2 *per se* (Tian *et al.*, 1995). The mechanism by which hypercapnic acidosis evokes vasodilatation is unclear but several studies have suggested a role for the activation of K_{ATP} channels (Faraci *et al.*, 1994; Ishizaka & Kuo, 1996; Kinoshita & Katusic 1997). These studies demonstrated that glibenclamide, a K_{ATP} channel antagonist, inhibited arterial dilatation induced by both hypercapnia, in rabbit cerebral arterioles (Faraci *et al.*, 1994), and acidosis, in porcine coronary arterioles (Ishizaka & Kuo, 1996) and canine cerebral arteries (Kinoshita & Katusic 1997). K_{ATP} channel activation is known to produce vasodilatation via vascular smooth muscle membrane hyperpolarisation (Nelson & Quayle, 1995), therefore, it is plausible to suggest that hypercapnic acidosis evokes a dilatation of vascular smooth muscle via a reduced pH_o, K_{ATP} channel activation and a subsequent membrane hyperpolarisation.

Extracellular K^+

As well as hyercapnic acidosis, extracellular K^+ concentration ($[K^+]_o$) serves as a metabolic vasodilator. For example, in the cerebral circulation $[K^+]_o$ can vary widely during periods of increased neuronal activity, resulting in an increase of $[K^+]_o$ from the resting level of approximately 3 mM up to 10 mM or higher (Somjen, 1979). Astrocytes are suggested to regulate increased $[K^+]_o$ via a process known as "K^+ syphoning" in which excess K^+ enters the astrocytes and a directed flow of K^+ current leaves the cell through the endfoot processes - regions of high K^+ permeability (Newman, 1986; Paulson & Newman, 1987). The endfeet terminate on the pial arterioles (Varon & Somjen, 1979). The net

effect is a flow of K^+ current from areas of high $[K^+]_o$ to areas of low $[K^+]_o$. Cerebral arteries and arterioles are each sensitive to $[K^+]_o$, and an increase in $[K^+]_o$ from 6 mM to 15 mM produces a dilatation of cerebral arteries (McCarron & Halpern, 1990; Knot *et al.*, 1996). Taken together these results suggest that $[K^+]_o$ may act as a mediator in the metabolic regulation of cerebral blood flow. Whereby a combination of increased $[K^+]_o$ during neuronal activity, and K^+-syphoning, results in a K^+-evoked dilatation of cerebral arteries and arterioles, the concurrent increase in regional cerebral blood flow would increase the supply of oxygen and nutrients to the exact area of increased activity. The mechanism of K^+-evoked dilatation in the cerebral circulation is discussed further in chapter 4 of this thesis.

As well as metabolic regulation of blood flow, autoregulation arises from the intrinsic or myogenic mechanisms operating in the blood vessel. In recent years the myogenic response has been accepted as a central blood flow control mechanism which serves to maintain constant tissue blood flow irrespective of changes in blood pressure (Johnson, 1981). Indeed the blood flow autoregulatory response linked to the myogenic mechanism ensures that blood flow will not change over a wide blood pressure range.

1.3 Historical Background of the Myogenic Response

The hypothesis of the myogenic response was first proposed by Bayliss in 1902. The hypothesis was proposed to explain the observation that a sudden

increase in blood pressure, within the hindlimbs of cats and dogs, produced a large initial increase in the blood volume, followed by a decrease in the hindlimb blood volume. A decrease in pressure produced an increase in the hindlimb blood volume (Bayliss, 1902). The response to blood pressure change was not due to nervous influence, since it remained even after the nerve fibers in the hindlimb were destroyed. The accumulation of metabolic products also was considered unlikely due to the speed of the response. It was proposed that the blood flow volume changes occurred by a mechanism that was intrinsic to the blood vessels themselves and "myogenic" in nature. Support for the proposal was drawn from experiments on excised canine carotid arteries, where increased intraluminal pressure resulted in artery contraction, while decreased pressure relaxed the artery (Bayliss, 1902). Since the artery was isolated from the tissue, metabolic products could not accumulate. The innervation was also severed and the myogenic mechanism seemed the most plausible mechanism (Bayliss, 1902).

The "myogenic response" as proposed by Bayliss was not accepted initially and the hypothesis was subject to criticism. For example, in studies repeating the work of Bayliss, in the canine hindlimb preparation, it was found that the removal of both the adrenal glands abolished the active constriction to a blood pressure increase. It was concluded that the changes in blood volume were due to adrenaline release and were not myogenic in nature (Anrep, 1912).

Very little work in this field was performed over the subsequent years and it was not until Folkow in 1949, that interest in the myogenic response was renewed. In the denervated hindquarters of dogs, cats and rabbits a pressure-dependent vascular tone developed even after the adrenal glands had been removed (Folkow, 1949). It is now accepted that the myogenic response plays an integral role in the regulation of blood flow (Johnson, 1981). The principal site of control it is proposed is at the resistance artery.

1.4 The Myogenic Response

A typical pressure-induced myogenic response can be demonstrated in vitro by exposing a cannulated vessel to a step increase in pressure. This response consists of two distinct phases, (1) an initial passive distention due to the increased pressure followed by, (2) an active constriction of the vessel which reaches equilibrium within a short time and is maintained over the same time course as the stimulus (figure 1.1; Mellander, 1989; Davis & Sikes, 1990).

The myogenic response is now accepted as being important in the maintenance of constant blood flow regardless of changes in blood pressure. However, many aspects of the development of the response remain unclear. Even some of the most basic questions remain unanswered. For example, an increased blood pressure will produce a resistance artery contraction matched to the change in blood pressure so that blood flow remains constant. Yet the nature of the stimulus for the contraction is unknown. Several possibilities exist. **Stretch -**

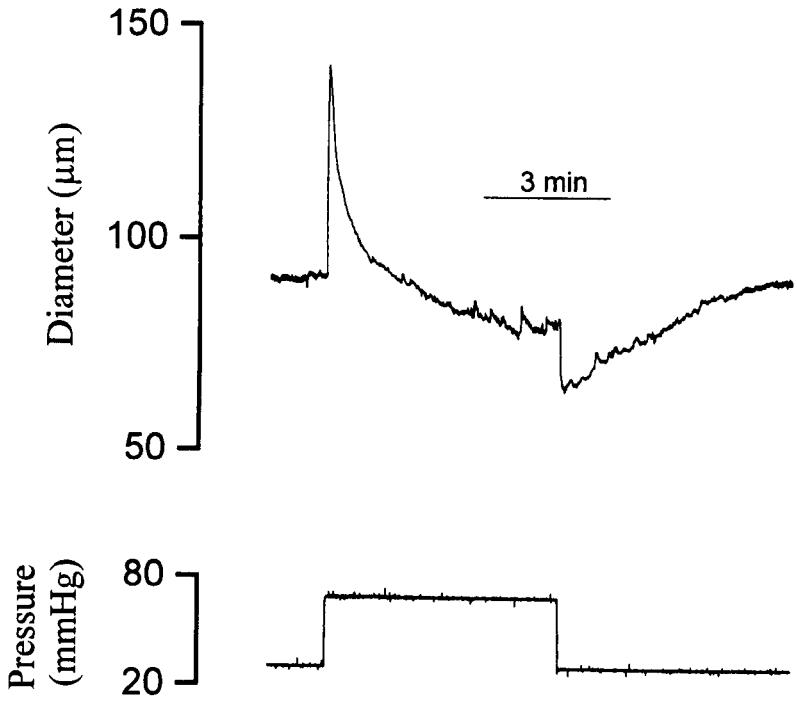


Figure 1.1. Illustration of a pressure-induced myogenic response of the rat posterior cerebral artery.
After a rapid pressure increment there was an initial passive distension of the artery followed by an active constriction of the artery.

an increase in blood pressure will stretch the resistance artery wall and thus stretch has been proposed as the stimulus for myogenic contraction. Yet since the artery contracts to a diameter smaller than the initial artery diameter the response removes the stimulus and yet the artery remains contracted. Stretch therefore seems an unlikely candidate. **Pressure** - if pressure alone was the stimulus then the response would increase the stimulus and cause a positive feedback evoking yet further constriction. **Tension** - wall tension and not intraluminal pressure may be the sensing system. According to the Laplace relationship arterial constriction would reduce tension (Johnson, 1981). Maintenance of a constant wall tension has been considered a mechanism in the development of myogenic contraction. However wall tension does not correlate well with myogenic contraction suggesting wall tension may not be an important contributor (Halpern *et al.,* 1984).

To enable a better understanding of the control of the myogenic response several experimental approaches have been used. These include varied techniques including *in vivo* (hamster cheek pouch arteriole), *in situ* (cremaster muscle arteriole), and *in vitro* (isolated artery myograph) methods.

In vivo or *in situ* sympathetic nerve stimulation (Liu *et al.,* 1994) or metabolic mediators e.g. oxygen, carbon dioxide or pH (Johnson, 1986; DeFily & Chilian, 1995) affect myogenic contraction. *In vitro* methods allow the investigation of each of these factors individually. The wire myograph and the

pressure myograph are the two key methods of *in vitro* analysis of the myogenic response.

Wire Myograph

The wire myograph has been used extensively in the investigation of mechanical and pharmacological properties of stretch induced myogenic tone in isolated blood vessels (Mulvany & Aalkjaer, 1990). Using the wire myograph, stretch-induced myogenic tone has been demonstrated in resistance blood vessels from a variety of vascular beds in a number of different species including the rabbit facial (Henrion *et al.,* 1994) and portal vein (Bevan, 1982), rabbit ear resistance artery (Hwa & Bevan, 1986c) and cerebral arteries from the rabbit (Bevan, 1982; Nakayama, 1982), dog (Laher & Bevan, 1989a), cat (Harder, 1987) and rat (Osol & Halpern, 1985).

Pressure Myograph

The pressure myograph system allows the cannulation and subsequent pressurisation of small blood vessels to any desired value. This methodology provides a major advantage over the wire myograph technique in that it allows blood vessels to maintain their physiological configuration, experience a true transmural pressure, diameter is allowed to change and the endothelium remains untouched (Halpern & Kelly, 1991; Falloon *et al.,* 1995). The pressure-induced myogenic response has been demonstrated in a number of blood vessel types from various vascular beds including the human cerebral (Wallis *et al.,* 1996) and coronary (Miller *et al.,* 1997) arteries and the rat

cerebral (McCarron *et al.*, 1997), mesenteric (Sun *et al.*, 1992) and skeletal muscle (Sun *et al.*, 1994) arteries.

1.5 Factors Regulating the Myogenic Response

1.5.1 Ca^{2+} Dependence of Myogenic Tone

Studies on myogenic contraction emphasize the importance of extracellular Ca^{2+} entry into smooth muscle as part of the response and that the removal of extracellular Ca^{2+} inhibits myogenic tone (Harder *et al.*, 1987; Jackson & Duling, 1989; Bulow, 1996). The mechanism(s) of Ca^{2+} entry however, remains uncertain. Early studies proposed the existence of a unique stretch-activated Ca^{2+} entry pathway. The conclusion was based on the resistance of the myogenic response to specific inhibitors of the voltage-dependent Ca^{2+} channel (VDCC). For example, the development and maintenance of stretch-induced myogenic tone in the rabbit ear resistance artery was only partially reduced by the dihydropyridine VDCC antagonists nimodipine, nifedipine and (-)-PN 200-110 (Hwa & Bevan, 1986a). Other studies also demonstrated a stretch-dependent myogenic tone resistant to the inhibitory effect of (-)-PN 200-110 (Laher *et al.*, 1988b). A stretch activated Ca^{2+} channel was proposed to account for Ca^{2+} entry, however, in most, if not all, of the studies proposing, a stretch-activated Ca^{2+} channel, the myogenic response had been evoked by stretching the artery on a wire myograph. In these experiments the normal configuration of the artery had been completely lost and often the experiments had been

carried out at higher than normal temperature (42°C). Together these considerations raise questions about the relevance of the stretch-induced Ca^{2+} entry pathway to the pressure-evoked contraction observed under normal physiological temperatures and artery configurations.

The voltage dependent Ca^{2+} channel (VDCC) is also proposed as the major Ca^{2+} entry pathway for myogenic contraction. This conclusion arises from the observation that myogenic tone is blocked by inhibitors of the VDCC as well as the response of the artery to hyperpolarising vasodilators.

VDCC in vascular smooth muscle have been characterised as high voltage activated channels similar to the L-type Ca^{2+} channel (Bean *et al.*, 1986; Benham *et al.*, 1987; Ganitkevich & Isenberg, 1990) and low voltage activated channels similar to the T-type Ca^{2+} channel (Bean *et al.*, 1986; Ganitkevich & Isenberg, 1990).

In several independent studies, myogenic contraction has been abolished by the dihydropyridine (nimodipine), phenylalkamine (verapamil) and benzothiazepine (diltiazem) classes of L-type Ca^{2+} channel antagonists (Gustafsson *et al.*, 1988; Langton, 1993; McCarron et al., 1997). These results raise the possibility that the L-type Ca^{2+} channel may be the significant Ca^{2+} entry pathway for myogenic contraction. For example, in the autoperfused cat hind limb preparation, the dihydropyridine felodipine, upon intra-arterial infusion, produces a dose-dependent decrease in basal vascular resistance and

perfusion pressure (Nordlander & Thalen, 1987; Gustafsson *et al.*, 1988). Similarly in the pressurised cerebral artery it was demonstrated that myogenic tone is dependent on extracellular Ca^{2+} and was abolished by the dihydropyridine nimodipine (Quayle *et al.*, 1990; McCarron *et al.*, 1997).

The question arises as to how VDCC are activated during the myogenic contraction. The channel itself may be activated by stretch (McCarron *et al.*, 1997) or alternatively stretch may activate other conductance's such as the stretch-activated non-selective cation channel which would depolarise the smooth muscle membrane and activate the VDCC (Davis *et al.*, 1992b).

1.5.2 Membrane Potential

Alteration in membrane potential significantly change myogenic contraction i.e. hyperpolarisation decreases while depolarisation increases myogenic contraction (Harder, 1984; Knot & Nelson, 1998). The myogenic contraction accompanying an increased intravascular pressure is associated with a membrane depolarisation of the smooth muscle cell (Harder, 1984; Harder *et al.*, 1987; Knot & Nelson, 1998). The depolarisation itself was suggested to be of a myogenic nature since tetrodotoxin (TTX) and alpha-adrenergic block with phentolamine produced no change in the vascular smooth muscle cell depolarisation (Harder, 1984). When membrane potential was plotted against pressure the relationship has a positive slope as expected if the depolarisation underlay the myogenic contraction. Reduction of the extracellular Ca^{2+}

concentration ($[Ca^{2+}]_o$) reduced the slope of the relationship and conversely an increase in $[Ca^{2+}]_o$ increased the slope (Harder, 1984). These results suggest that an increased Ca^{2+} conductance is the main ionic mechanism responsible for myogenic activation. Since this early study a number of other ion conductances have been proposed to account for the depolarisation associated with myogenic contraction including stretch-activated non-selective cation channels and the anion channels.

Mechanosensitive or stretch-activated channels (SAC) were first identified in cultured embryonic chick skeletal muscle (Guharay & Sachs, 1984). Since their discovery, SAC's have been characterised in a variety of cell types including toad stomach smooth muscle (Kirber *et al.*, 1988), pig coronary artery smooth muscle (Davis *et al.*, 1992a) and rat intact cerebral artery (Welsh *et al.*, 2000). SAC's distribution in both mammalian and non-mammalian systems, suggest their involvement in a number of cellular functions including myogenic contraction (Kirber *et al.*, 1988; Hisada *et al.*, 1991; Meininger & Davis, 1992).

In vascular smooth muscle cells, suction applied to a membrane patch activates nonselective cation channels permeable to K^+, Na^+ and Ca^{2+}, while whole cell stretch resulted in a depolarisation similar to that seen in isolated pressurised arteries (Davis *et al.*, 1992a). In other studies, on vascular smooth muscle, although Ca^{2+} entry via a stretch-activated nonselective cation channel was observed (Davis *et al.*, 1992b), the low Ca^{2+} permeability at physiological Ca^{2+} concentrations (Kirber *et al.*, 1988), together with its relatively low density

would exclude a major contribution in the maintenance of the Ca^{2+} entry necessary for myogenic tone (Nelson *et al.*, 1990; Davis *et al.*, 1992a).

In a recent study examining the hypothesis that non-selective cation channels contribute to the depolarisation of vascular smooth muscle, both swelling- (produced by hyposmotic challenge) and pressure-evoked depolarisation of smooth muscle cells in isolated cerebral arteries were blocked by Gd^{2+}, a cation channel antagonist (Welsh *et al.*, 2000). This and other studies has led to the suggestion that both swelling- and pressure-evoked depolarisation and contraction of isolated cerebral arteries is produced by the activation of a nonspecific cation channel and that nonspecific cation channels play a role in the development of myogenic tone (Setoguchi *et al.*, 1997; Bae *et al.*, 1999; Welsh *et al.*, 2000).

Other studies have suggested that stretch-activated chloride (Cl^-) conductance rather than non-selective cation currents may be of significance in myogenic depolarisation. Several studies have demonstrated the presence of chloride channels in smooth muscle (Pacaud, *et al.*, 1991; Van Renterghem & Lazdunski, 1993; Large & Wang, 1996), some of which are activated by cell swelling (Yamakazaki *et al.*, 1998; Greenwood & Large, 1998). The chloride channel blockers, indanyloxyacetic acid (IAA-94) and 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS), hyperpolarised and dilated cerebral arteries i.e. abolished myogenic tone (Nelson *et al.*, 1997). The hyperpolarisation and dilatation was not due to inhibition of the calcium-

activated chloride channel since niflumic acid, a specific inhibitor of these channels, did not affect arterial tone. Furthermore, both IAA-94 and DIDS did not affect membrane potential or arterial tone, under conditions of low intraluminal pressure, where myogenic tone would be absent. Together these results suggested a chloride channel sensitive to intraluminal pressure or stretch played a role in regulating myogenic depolarisation and constriction (Nelson *et al.*, 1997).

In contrast, in renal afferent arterioles myogenic contraction was not altered by either the chloride channel blocker IAA-94 or a reduction in the extracellular chloride concentration (Takenaka *et al.*, 1996) raising the possibility that chloride channels do not contribute to myogenic contraction in this preparation. A recent study demonstrated that although a depolarisation and constriction of intact rat cerebral arteries, produced by a hyposmotic challenge (produces cell swelling), was inhibited by the Cl^- channel antagonists DIDS and tamoxifen (Welsh *et al.*, 2000), the swelling-activated current, associated with the cell depolarisation, was carried by cations not anions and the current could be inhibited by Gd^{2+} (Welsh *et al.*, 2000), a cation channel antagonist. Gd^{2+} was also shown to block both swelling- and pressure-induced depolarisation of smooth muscle cells in isolated cerebral arteries. It was concluded that both swelling- and pressure-induced depolarisation and constriction of rat cerebral arteries was due to the activation of a nonselective cation channel and not the activation of a swelling- or pressure-sensitive Cl^- channel (Welsh *et al.*, 2000),

strengthening the suggestion that there is no role for Cl^- channel activation in the development of myogenic tone.

As well as the depolarising inward currents accompanying myogenic contraction outward (K^+) hyperpolarising currents have been reported. These outward K^+ currents act as a negative feedback pathway, serving to limit the depolarising influences activated during myogenic contraction. The large conductance Ca^{2+}-activated K^+ channel (K_{Ca}), found in virtually every type of smooth muscle examined (Nelson & Brayden, 1993), has been suggested as an important candidate for this negative feedback pathway. K_{Ca} is activated by both membrane depolarisation and increased intracellular Ca^{2+} (Benham *et al.*, 1986; Berczi *et al.*, 1992b; Brayden & Nelson, 1992; Nelson & Quayle, 1995; Gokina *et al.*, 1996). The smooth muscle cell hyperpolarisation by K_{Ca} would close VDCC reducing Ca^{2+} influx and therefore inhibit cell contraction.

K_{Ca} channel block (e.g. with tetraethylammonium; TEA) resulted in greater diameter reductions to increased intravascular pressure compared to control values in the perfused rat saphenous artery (Berczi *et al.*, 1992b). In other studies TEA and charybdotoxin (CTX), also produced dose-dependent depolarisation and constriction of pressurised cerebral arteries. However, when intracellular Ca^{2+} was reduced by lowering intravascular pressure or the inhibition of VDCC with nimodipine, neither channel blocker had any significant effect upon arterial tone, demonstrating the dependence of activation of K_{Ca} channels on intracellular free Ca^{2+} (Brayden & Nelson, 1992).

Similar results have also been demonstrated in the human cerebral artery (Gokina *et al.*, 1996). Together these results support the proposal that pressure-induced contraction of arterial muscle is attenuated by activation of a TEA and CTX sensitive K_{Ca} channels and their importance as a possible negative feedback system in the regulation of smooth muscle membrane potential and hence myogenic tone (Berczi *et al.*, 1992b; Brayden & Nelson, 1992; Gokina *et al.*, 1996). A proposed mechanism of action for K_{Ca} channels in combination with the other ion channels discussed is presented in figure 1.2.

A role has also been proposed for other K^+ channels in the control of myogenic tone. Inhibitors of voltage-dependent K^+ channels (K_v), 4-aminopyridine (4-AP) and 3,4-diaminopyridine (3,4-DAP), both produced a depolarisation and constriction of rabbit pressurised cerebral arteries (Knot & Nelson, 1995). The depolarisation and constriction were not affected by inhibitors of Ca^{2+} channels, Ca^{2+}-activated K^+ channels, ATP-sensitive K^+ channels or inward rectifier K^+ channels. It was concluded that K_v channels may play some role in the regulation of membrane potential and myogenic tone in rabbit small cerebral arteries (Knot & Nelson, 1995).

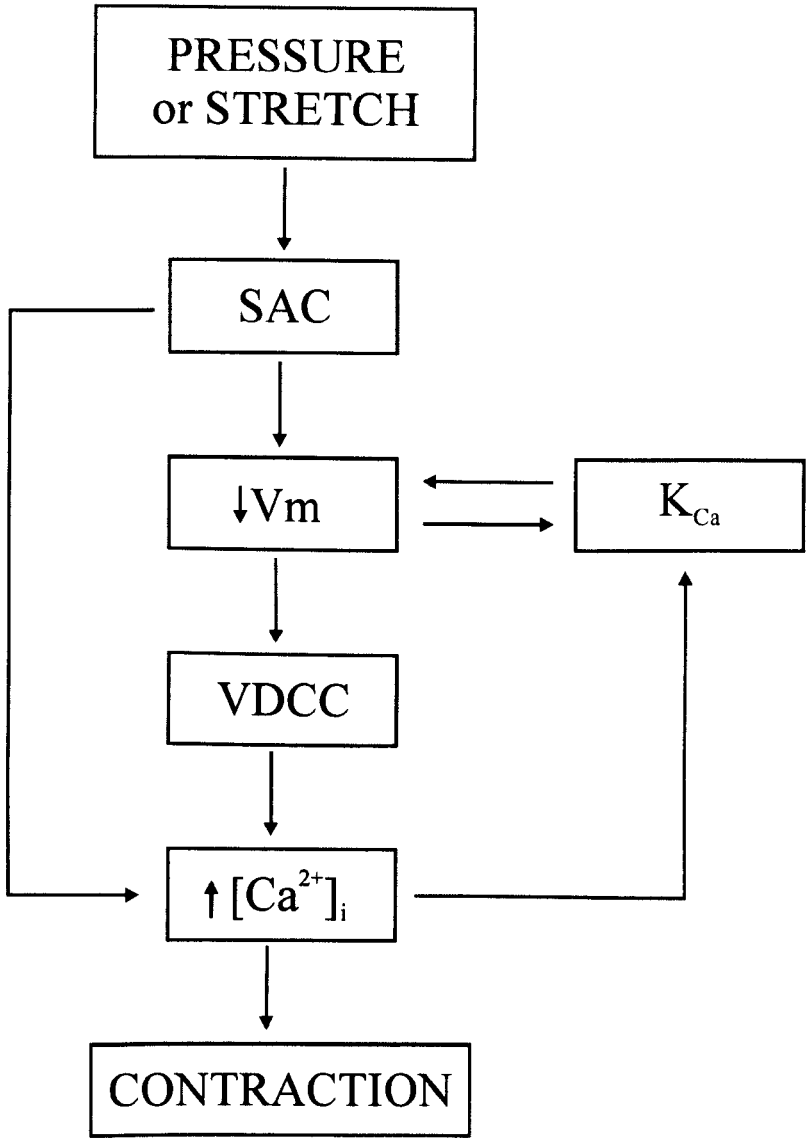


Figure 1.2 Schematic Diagram illustrating the role of K_{Ca} channels in the regulation of myogenic tone (adapted from D'Angelo and Meininger, 1994; Nelson and Quayle, 1995).

1.5.3 Endothelium

The role of the endothelium in the regulation of myogenic tone and the myogenic response has been debated. In the late eighties Harder and colleagues suggested that the myogenic response in pressurised feline cerebral (Harder *et al.*, 1987; Harder *et al.*, 1989a; Harder *et al.*, 1989b) and canine renal (Harder *et al.*, 1989a) arteries was dependent upon an intact endothelium. Chemical denudation of the endothelium, by perfusing the arterial lumen with either a collagenase and elastase enzyme mixture or the detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), abolished the pressure-induced myogenic tone. Since the work of Harder and colleagues, a number of studies on a variety of vessel types has been performed in an attempt to clarify the role of the endothelium in the regulation of myogenic tone.

Subsequently it has been suggested that it was the method of endothelium removal rather than the removal of the endothelium which abolished the myogenic tone (Kuo *et al.*, 1990a). In studies performed on porcine coronary arterioles different methods of endothelium removal were employed. For example, intraluminal passage of an air bolus or the chemical removal endothelium by intraluminal perfusion with CHAPS resulted in vessels that reacted passively to increases in pressure and were devoid of any myogenic response (Kuo *et al.*, 1990a). However, in the same preparation myogenic responses were preserved after mechanical removal of the endothelium with an abrasive micropipette. Studies in the canine renal artery, also established that

the myogenic response was attenuated after endothelium removal due the intraluminal passage of an air bolus but remained unaffected after mechanical removal (Liu *et al.*, 1994). Although still debated current views suggest that an intact endothelium is not necessary for the myogenic response of blood vessels (table 1.1).

1.5.4 Role of Second Messengers

A number of intracellular second messengers including phospholipase C (PLC), 1,2-diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) are activated by an increased transmural pressure or stretch of small arteries (Harder *et al.*, 1991; Meininger & Davis, 1992; Osol *et al.*, 1993). Activation of PLC would result in the hydrolysis of its substrate, the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2), producing equimolar amounts of DAG and IP_3 (Berridge, 1987; Lee & Severson, 1994). IP_3 produces a rise in intracellular Ca^{2+} by causing a release of Ca^{2+} from the sarcoplasmic reticulum (SR). DAG on the other hand, activates protein kinase C (PKC), to evoke a number of cellular actions such as an alteration of the Ca^{2+} sensitivity of the contractile apparatus (Nishimura & Van Breemen, 1989). IP_3 and DAG have been proposed as a possible link between stretch and myogenic tone (Berridge, 1987). In support, U-73122, an inhibitor of PLC, produced a concentration-dependent dilatation of cannulated rat cerebral arteries (Osol *et al.*, 1993). Furthermore, significant increases in endogenous IP_3 and DAG concentrations were found upon pressurisation of isolated perfused canine renal

Table 1.1 The Endothelial Dependence of the Myogenic Response

Species	Tissue	Vessel	Diameter (μm)	Denudation process	Endothelium dependent	Reference
Cat	Brain	Left middle cerebral a.	200-1000	Chemical	Yes	Harder, 1987.
Dog	Kidney	Interlobular arcuate a.	200-500	Air bolus	Yes	Harder *et al.*, 1989a.
Dog	Brain	Basilar a.	>1000	Rubbing	Yes	Katusic, Sheperd and Vanhoutte, 1987.
Dog	Kidney	Renal arteries	200-500	Air bolus Rubbing	Yes No	Liu, Harder and Lombard, 1994.
Pig	Heart	Coronary arteriole	40-70	Chemical Air bolus Rubbing	Yes Yes No	Kuo, Chilian and Davis, 1990.
Rat	Heart	Coronary arteriole	75-120	Rubbing	No	Park *et al*, 1996.
Rat	Mesentery	Mesenteric arteriole	30-400	Air bolus	No	Sun *et al.*, 1992.
Rat	Muscle	Gracilis arteriole	20-80	Air bolus	No	Sun, Kaley and Koller, 1994.
Rat	Muscle	Cremaster arteriole	100-200	Rubbing	No	Falcone, Davis and Meininger, 1991.
Rat		Femoral a.	200	Rubbing	No	Bulow and Nilsson, 1991.
Rat	Brain	Posterior cerebral a.	100-200	Rubbing	No	Osol, Cipolla and Knutson, 1989.
Rat	Brain	Posterior cerebral a.	100-200	Rubbing	No	McCarron, Osol and Halpern, 1989.
Cat	Lung	Pulmonary a.	250-800	Rubbing	No	Kulik, Evans and Gamble, 1988.
Rabbit	Skin	Ear artery	900-1100	Gas bolus	No	Macpherson, McLeod and Rasiah, 1991.

arteries (Narayanan *et al.*, 1994), Together these results suggest that an increase in transmural pressure of canine renal arteries results in the activation of PLC.

In other studies IP_3 itself has been proposed as a central component in the generation of myogenic tone. Stretch-induced contraction of rabbit cerebral arteries was only progressively inhibited during repeated stretch in Ca^{2+}-free PSS, suggesting stretch-induced myogenic responses are dependent on the release of Ca^{2+} from intracellular stores, at least in part (Nakayama, 1982). Similarly, it has been demonstrated that stretch of individual cells isolated from porcine coronary arteries resulted in release of Ca^{2+} from the SR as well as causing an influx of Ca^{2+} across the cell membrane (Davis *et al.*, 1992b). In contrast other studies found inhibition of SR Ca^{2+} release with ryanodine did not affect the myogenic response in rat pressurised cerebral arteries, suggesting that the response is independent of Ca^{2+} release from the SR in rat cerebral arteries (Watanabe *et al.*, 1994; McCarron *et al.*, 1997).

Numerous studies have proposed a role for PKC in the myogenic response, an action mainly attributed to its ability to alter the Ca^{2+} sensitivity of the contractile apparatus (Laher & Bevan, 1987; Hill *et al.*, 1990b; Osol *et al.*, 1991). For example, in rat skeletal muscle arterioles, activation of PKC with indolactam produced a dose-dependent contraction without a measurable increase in intracellular Ca^{2+}. Inhibition of PKC with either H-7 or staurosporine produced a dose-dependent inhibition of the pressure-induced myogenic response in the same preparation (Hill *et al.*, 1990b). In rat

pressurised cerebral arteries, the PKC inhibitor staurosporine produced a gradual loss of basal myogenic tone while the PKC activator indolacatam, produced a dose-dependent increase in basal myogenic tone (Osol *et al.*, 1991). PKC activation with phorbol esters also increased (Laher & Bevan, 1987) while an inhibition of PKC with staurosporine dose-dependently decreased the stretch-induced tone (Laher & Bevan, 1989b). These studies suggest some role for PKC in the regulation of myogenic tone. However, concern over the specificity of PKC inhibitors H-7 and staurosporine for PKC has led to the use of other antagonists with greater selectivity for the kinase with different results. Both H-7 and staurosporine inhibit other kinases such as myosin light chain kinase (MLCK), at concentrations used to block PKC (Ruegg & Burgess, 1989). Inhibition of MLCK rather than PKC may account for staurosporine and H-7's effect on myogenic tone. The specific PKC inhibitor RO318425, which has a much greater separation in its IC_{50} for PKC over that of either H-7 or staurosporine, did not inhibit myogenic reactivity and pressure increase failed to alter the myofilament sensitivity (McCarron *et al.*, 1997). The precise role of PKC in the myogenic response remains unclear but more specific inhibitors of the kinase will allow a better understanding of its role.

1.5.5 Arachadonic Acid Metabolites

Arachadonic acid (AA) is produced by the enzymatic degradation of 1,2-diacylglycerol (DAG) by DAG lipase. AA itself then undergoes oxidative metabolism by one of three enzyme systems, cyclooxygenase, lipoxygenase

and cytochrome P-450 group of enzymes, to form a number of metabolites (McGiff, 1991; Capdevila *et al.*, 1992). These AA metabolites possess vasoactive properties (Harder *et al.*, 1995).

Several investigators have suggested that cytochrome P-450 metabolites may be involved in the regulation of myogenic tone (Kauser *et al.*, 1991; Ma *et al.*, 1993; Harder *et al.*, 1994; Imig *et al.*, 1994; Chlopicki *et al.*, 1996). The metabolism of AA by cytochrome P-450 enzymes can be divided into two categories, the epoxygenases that catalyze the formation of epoxyeicosatrienoic acids (EET's) and their corresponding vic-dihydroxyeicosatetraenoic acids (DHET's) (Karara *et al.*, 1990; Capdevila *et al.*, 1992), and the ω-1 and ω-hydroxylases that produce 19- and 20-hydroxyeicosatetraenoic acids (19- and 20-HETE's) (McGiff, 1991). Both EET's and HETE's possess vasoactive properties. For example it has been demonstrated that an AA metabolite that coeluted with 20-HETE, during reverse phase chromatography, produced a constriction of isolated perfused canine renal arteries, and that this constriction was completely reversed by P-450 inhibitors (Kauser *et al.*, 1991). Similarly, exogenous 20-HETE produced a constriction of canine renal artery arteries. This constriction was associated with a depolarisation and sustained increase in intracellular calcium concentration (Ma *et al.*, 1993). Using cat cerebral arteries, exogenous 20-HETE produced a concentration-dependent constriction of small arteries (Harder *et al.*, 1994). A plausible mechanism for the depolarisation and constriction is an inhibition of the large conductance Ca^{2+}-activated K^+ channel by 20-HETE (Harder *et al.*, 1995). Collectively these

results suggest that 20-HETE could play a role in the regulation of myogenic tone via the control of large conductance Ca^{2+}-activated K^+ channels (Harder *et al.*, 1995; Gebremedhin *et al.*, 2000).

In other studies EET metabolites dilated small renal and cerebral arteries via the activation of large conductance Ca^{2+}-activated K^+ channels (K_{Ca}) (Gebrehmedhin *et al.*, 1992; Zou *et al.*, 1994). Dilatation of isolated renal arterioles evoked by 11,12-EET was attenuated by the K_{Ca} channel inhibitor, TEA (Zou *et al.*, 1994). Further to this, 11,12-EET evoked a TEA-sensitive dilatation of cat cerebral arteries (Gebrehmedhin *et al.*, 1992). Cell attached patch clamp experiments, using isolated cat cerebral artery myocytes, found that both 8,9-, and 11,12-EET evoked a TEA-sensitive increase in the frequency of opening, mean open time and open-state probability of the K_{Ca} channel (Gebrehmedhin *et al.*, 1992). Similarly, EET's activation of K_{Ca} channels has been demonstrated in isolated cells from the rabbit portal vein, rat caudal artery, guinea pig aorta and porcine coronary artery (Hu & Kim, 1993).

In summary, local production of cytochrome P-450 metabolites of AA (EET's and HETE's) may play a role in metabolic regulation of arteriolar blood flow by modulating cerebral vascular tone through the regulation of K_{Ca} channels (Gebremedhin *et al.*, 2000).

Chapter 2

Materials and Methods

2.1 Dissection of Arteries

2.1.1 Dissection of posterior cerebral artery

Male, Wistar-Kyoto rats, aged between 11 and 20 weeks were killed by a sodium pentobarbitone overdose (150 $mgkg^{-1}$ Inter-peritoneal). The rat was decapitated using a guillotine. The brain was carefully removed from the skull and transferred to a Sylgard-coated Petri dish containing cold (≈ 4°C) physiological saline solution (PSS). PSS had the following ionic composition (mM): 119 NaCl, 24 $NaHCO_3$, 4.7 KCl, 1.18 KH_2PO_4, 1.17 $MgSO_4.7H_2O$, 1.6 $CaCl_2$ and 5.5 glucose. The posterior cerebral artery was located from the underside of the brain (figure 2.1a) with the aid of a dissection microscope (Nikon SMZ 2T) and a light source (Schott KL 1500 electronic). Second order branches of the posterior cerebral artery were carefully dissected from the pial membrane and an artery segment (2-3 mm) was removed and transferred for cannulation to a 10 ml vessel chamber of a Halpern pressure-perfusion myograph containing PSS (see section 2.2).

2.1.2 Dissection of thalamo-perforating cerebral artery

From the posterior cerebral artery a variable number of thalamo-perforating arteries, usually three from each side, course rostally and dorsally to reach the ventral posterior region of the thalamus (figure 2.1b). The overlying hypophysis (pituit) and any adherent brain tissue was carefully removed until a

Figure 2.1a

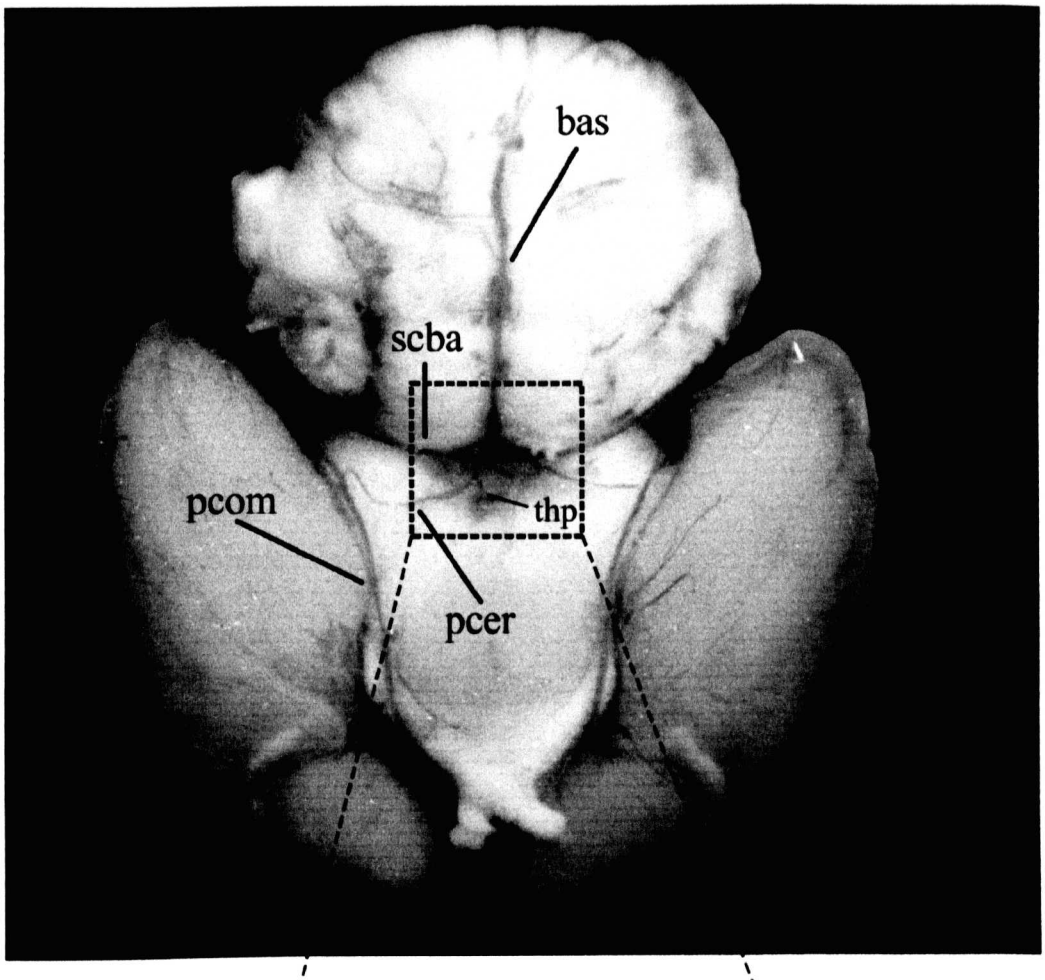
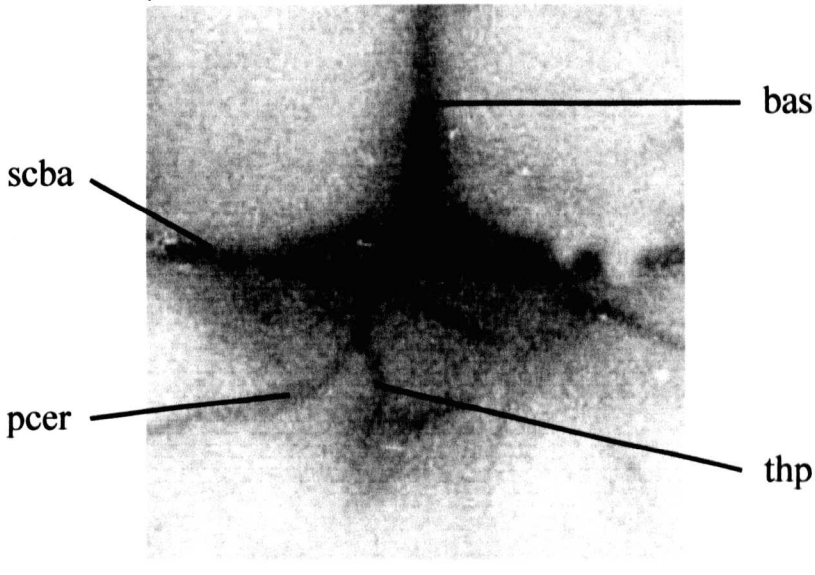


Figure 2.1b



section of the thalamo-perforating artery, large enough to be dissected, could be seen. An artery segment (0.5-1 mm) was removed and transferred for cannulation to a 10 ml vessel chamber of a Halpern pressure-perfusion myograph, containing PSS (see section 2.2).

2.1.3 Dissection of mesenteric artery

Following a mid-line incision, the peritoneal cavity was opened and the intestinal tract, with the mesenteric bed, was quickly removed and transferred to a Sylgard-coated Petri dish containing cold PSS. A 2-3 cm segment of the small intestine, 5-6 cm distal to the pylorus, was removed. Using the terminology described by Sun *et al.,* (1992), a fourth order branch was carefully dissected from any adherent connective and adipose tissue (figure 2.2). An artery segment (0.5-1 mm) was removed and transferred for cannulation to a 10 ml vessel chamber of a Halpern pressure-perfusion myograph containing PSS (see section 2.2).

2.2 The Halpern Pressure-Perfusion Myograph

Resistance artery function was examined using the Halpern pressure-perfusion myograph (figure 2.3; Living Systems Instrumentation (LSI), Burlington, Vermont, USA). Two different vessel chambers were used during the course of this study. First, a standard 10 ml volume arteriograph with separate inflow and outflow tubes for circulating physiological solutions around the vessels.

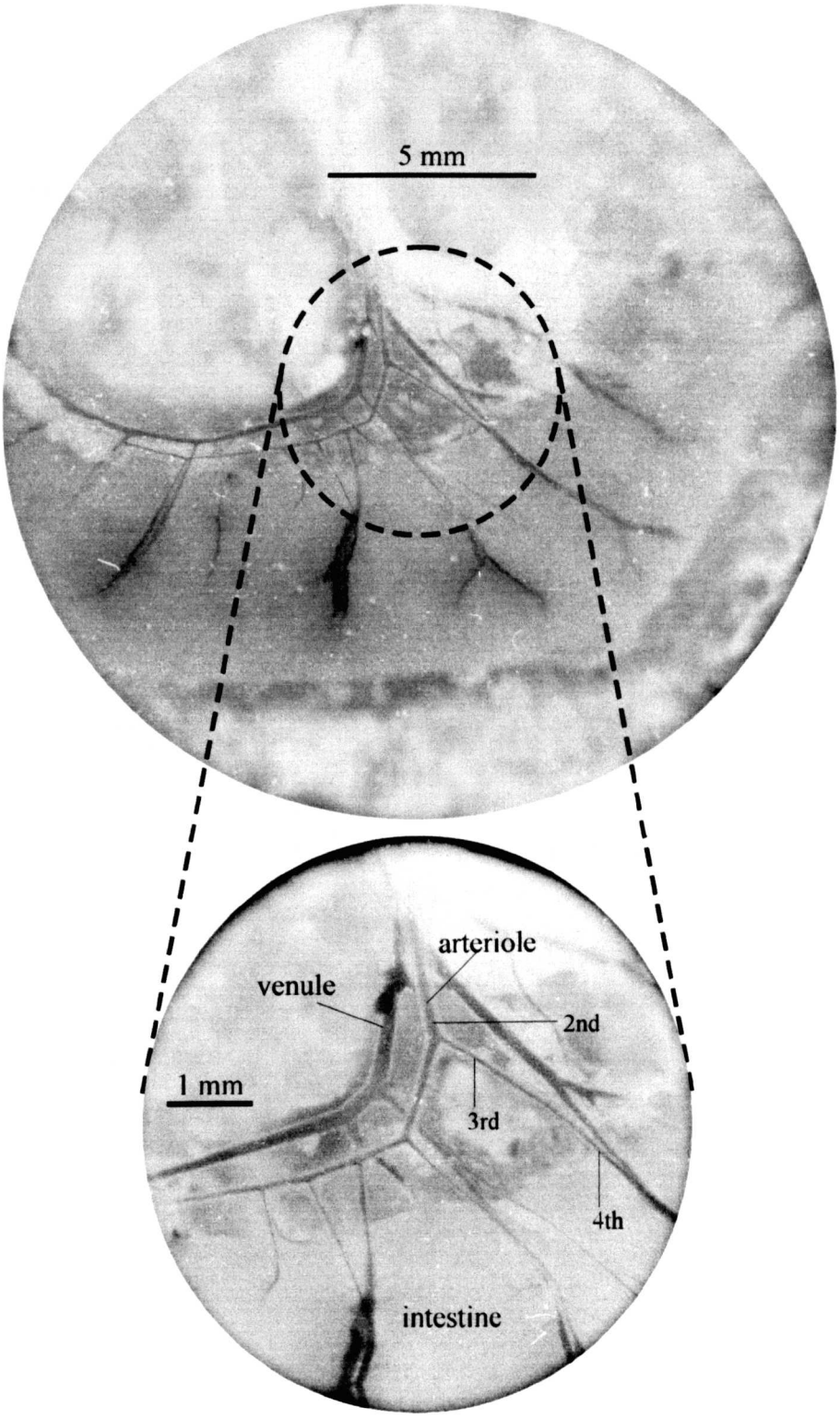


Figure 2.2 The arteriolar network of the rat mesentry. 4th order mesenteric arteries were used.

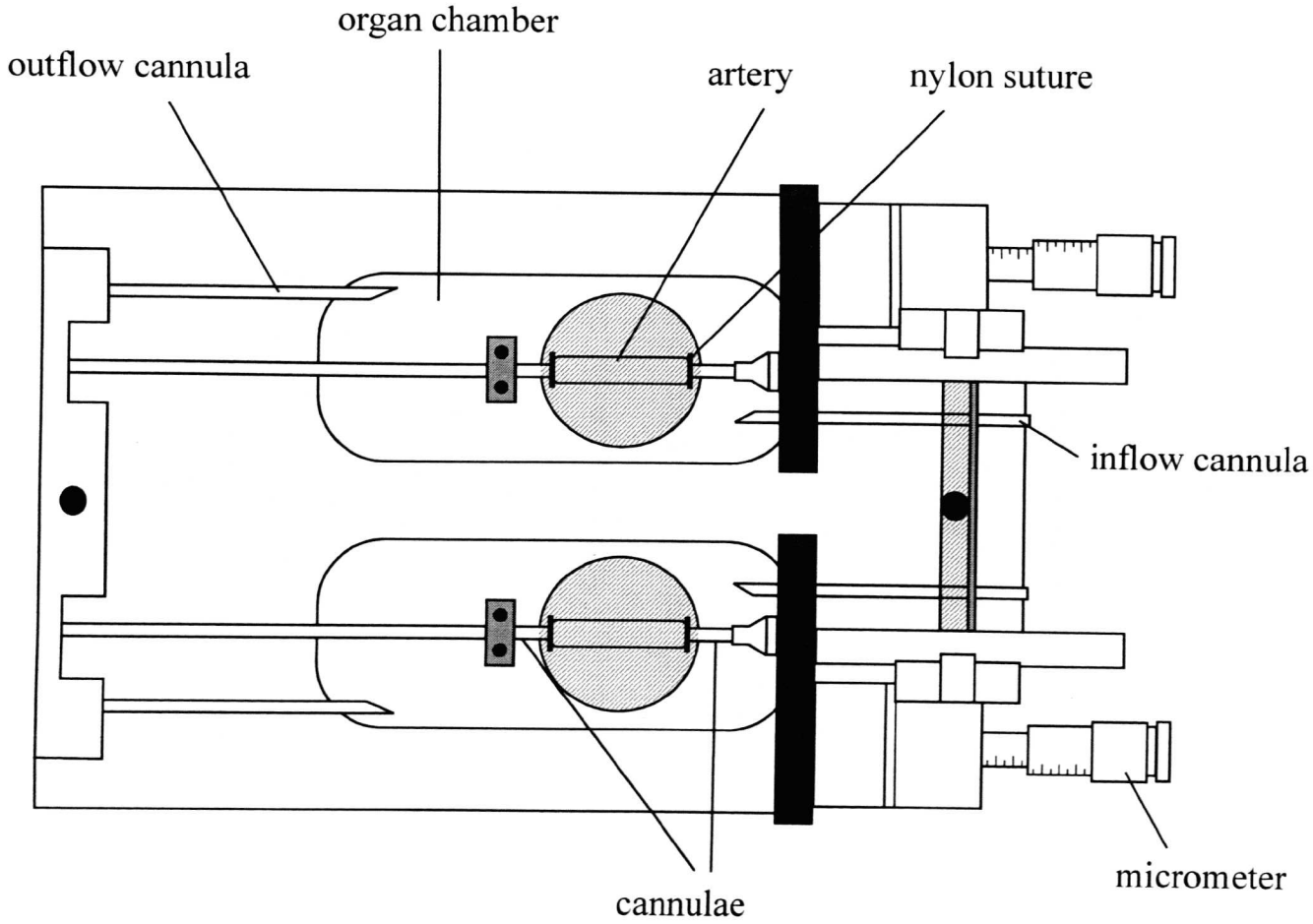


Figure 2.3 The Halpern Pressure-Perfusion Myograph

Secondly, a small volume (3 ml) arteriograph. The solution exchange in the chamber of this arteriograph was achieved with the aid of a syringe or suction system. The small volume arteriograph was used during experimental protocols involving permeabilized arteries.

2.2.1 Resistance Artery Cannulation

All studies described in this thesis were performed on pressurised resistance arteries. To enable pressurisation of the artery it was first cannulated. Prior to artery cannulation the vessel chamber was superfused with gassed (95% O_2, 5% CO_2) PSS (pH 7.4). Both cannulae and tubing connections were filled with PSS. Cannulae were pulled from borosilicate glass capillaries (≈1.2 mm in diameter, World precision instruments, Florida, USA.) on a horizontal puller (P-87, Sutter Instruments, Novato, California, USA) to produce an external tip diameter of ≈10 μm.

Extra fine point No. 5 microforceps were used to grasp the vessel walls at one end and the vessel was opened to display the lumen. The vessel was gently pulled onto the tip of the cannula; care was taken not to pierce the walls or damage the endothelium. The vessel was secured with ties produced from single strands of polyester thread, 18 μm in diameter (AI inc. Mount Holly, N.C., USA). The lumen was gently flushed with gassed (95% O_2, 5% CO_2,) PSS to remove any remaining blood cells and the distal end of the artery was then cannulated and secured. The length of the vessel was altered using a

micrometer attached to one of the cannulae to remove any buckling of the artery (figure 2.4).

The arteriograph was moved to the stage of a microscope (Nikon, Labphot-2). One of the cannulae was closed off and the other connected in parallel to a pressure transducer, and a pressure peristaltic pump. A pressure servo unit (model PS/200/Q, LSI) maintained pressure by activating the peristaltic pump to inject PSS until the artery was pressurised to the desired experimental value. This system allowed any selected pressure between 0 and 200 mmHg to be established and controlled.

Leaks within the system were detected by raising the pressure to 60 mmHg and switching the pressure servo unit from automatic to manual. Any reduction in pressure was indicative of a leak in the vessel itself, from a side branch, the ties or from the connecting tubing. If the leak could not be corrected the vessel was excluded from the study.

The artery was imaged using a CCD video camera (Watec, WAT-902A) which was attached to a viewing tube on the microscope. The artery was observed on a television monitor (Panasonic, WV-5410) and analysed using a video dimension analyser (VDA), (model V91, LSI). The VDA automatically records changes in artery diameter at video rates (approximately every 20 ms). The VDA detected optical density changes of the image at any chosen scan line, which perpendicularly intersected the artery. Two windows of the scan line are

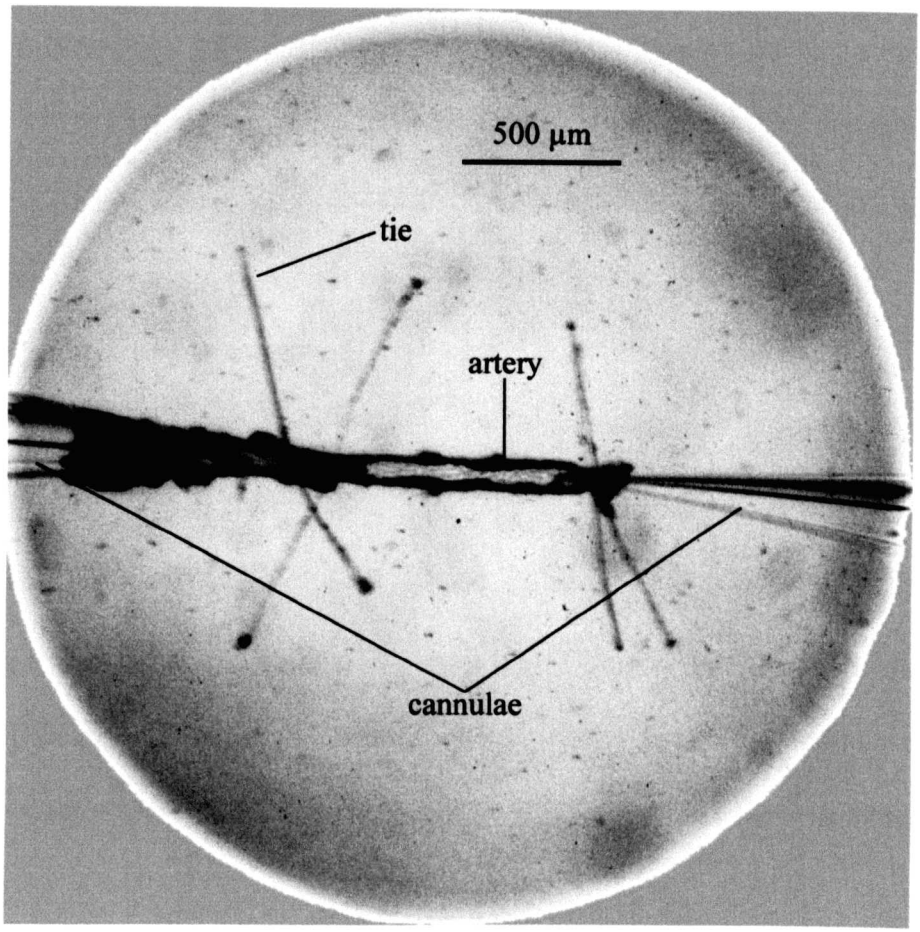


Figure 2.4 Rat cannulated thalamo-perforating cerebral artery. The artery is cannulated at each end, secured with strands of polyester thread and pressurised to 60 mmHg.

fixed on the artery walls. Due to the greater optical density of the walls than either the lumen or the field outside the vessel, the wall thickness and diameter can be detected and was displayed in microns on the digital panel of the VDA. Data (diameter, pressure and wall thickness) were digitised (10 Hz) and stored on a personal computer (Dell, 466 DL) for later analysis using a labmaster A/D converter and acquisition software (Newtape, developed by Dr. Francis Burton) (figure 2.5).

The arteriograph was connected to a 500 ml reservoir of PSS which was bubbled vigorously with 95% O_2 , 5% CO_2 gas mixture, and circulated using a Masterflex pump (Cole-Palmer, Chicago, Illinois, USA) at a rate of ~100 $mlmin^{-1}$ (pH 7.4). A constant temperature (37 °C) was maintained by passing the PSS through a heat exchanger connected to a heating pump (M12, Lauda). The artery was equilibrated for approximately 60-90 minutes at a transmural pressure of 60 mmHg before any experimental procedure started. At the end of each experimental protocol the fully relaxed diameter of the artery was determined by the addition of Ca^{2+}-free PSS. Ca^{2+}-free PSS was identical to PSS, however, $CaCl_2$ was omitted and 1mM ethylene glycol-bis (β-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) was added to chelate any free Ca^{2+} ions.

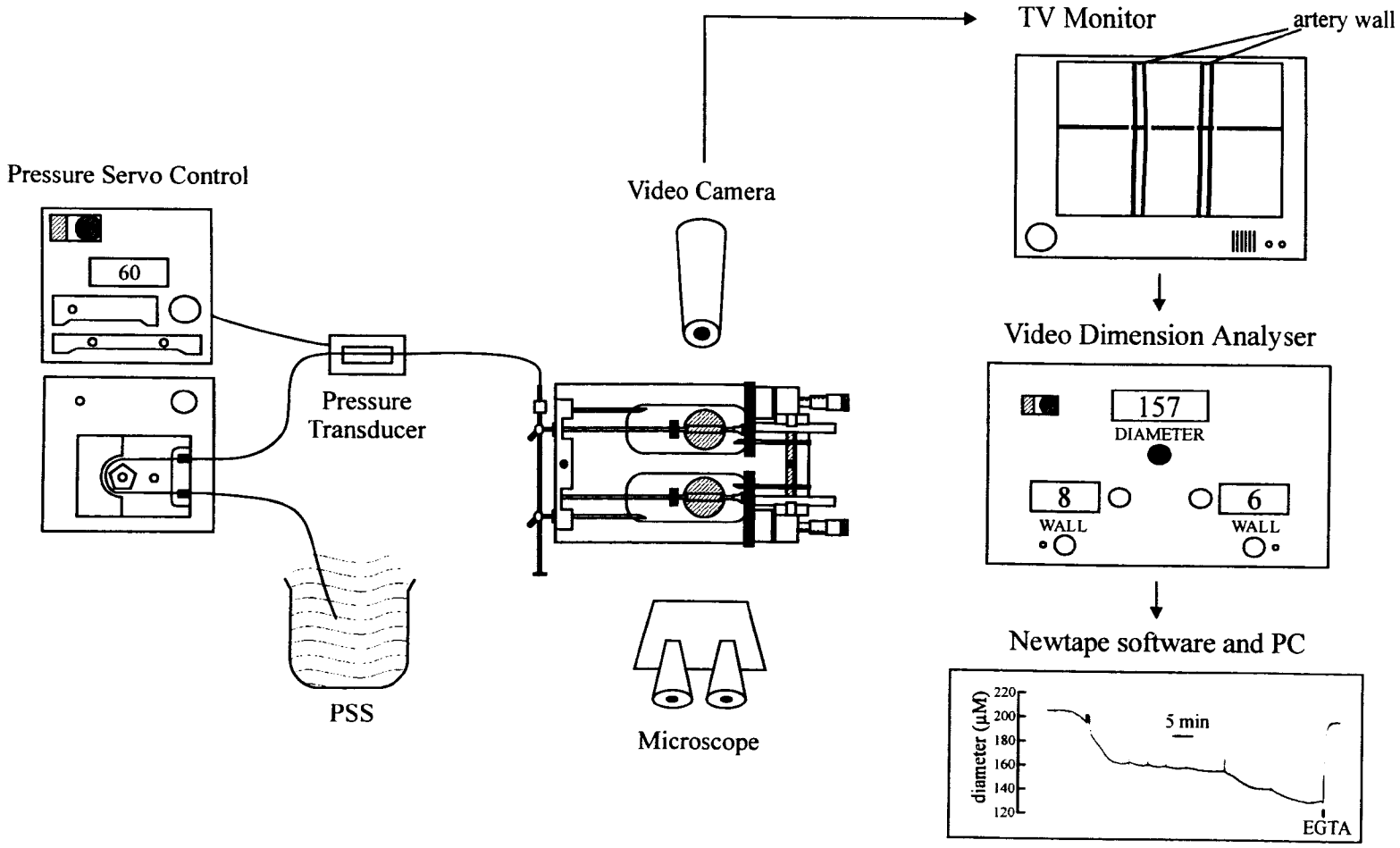


Figure 2.5 Schematic diagram of the Halpern pressure-perfusion myograph system

2.3 Permeabilized arteries

2.3.1 Solutions

The solutions used daily were made from stock solutions. The stocks were made at regular intervals and stored at 4°C. These were 1M KCl and $MgCl_2$, 100 mM CaEGTA, EGTA and KH_2PO_4, and 500 mM HEPES. The amount of EGTA added to the stock solution was calculated with reference to the purity of EGTA obtained from Sigma (Bers, 1982, Miller & Smith, 1985). From the stock solutions and together with Na_2ATP (5 mM) and Na_2CrP (15 mM) three basic solutions were prepared. These were "10 activating" (which contains 10 mM CaEGTA, 60 μM free Ca^{2+}), "10 relaxing" (which contains 10 mM EGTA, <1 nM free Ca^{2+}) and "0.2 relaxing" (which contains 0.2 mM EGTA). The full composition of these solutions are shown in Table 2.1. To change free [Ca^{2+}] in the bath over the course of an experiment the strongly calcium-buffered solutions ("10 activating" and "10 relaxing"), were mixed together in varying ratios. The free [Ca^{2+}] was calculated using a computer programme (REACT) written by G. L. Smith and D. J. Miller. The affinities of EGTA and other ligands for Ca^{2+} and the other metal ions have been incorporated. REACT can provide a complete profile of the free metal ion concentrations and ligand-metal concentrations. Details of the binding constants, correction for pH, ionic strength and temperature are provided elsewhere (Smith & Miller, 1985).

Table 2.1 Composition of Permeabilisation Solutions

SOLUTION	10 ACTIVATING (mM)	10 RELAXING (mM)	0.2 RELAXING (10mM)
KCl	100	100	120
HEPES	25	25	25
Na_2CrP	15	15	15
EGTA	-	10	0.2
CaEGTA	10	-	-
$MgCl_2$	5.35	5.35	5.35
Na_2ATP	5.0	5.0	5.0
KH_2PO_4	1.0	1.0	1.0

2.3.2 Permeabilization

The arteries were mounted in the small volume chamber. In a "10 Relaxing" bathing solution (room temperature 20 ± 2°C, pH 7.2) at a pressure of 60 mmHg, arteries were permeabilized using β-Escin (100 μM) for ~2 minutes. From preliminary work this was determined to be the optimum time course and concentration of β-Escin to produce full permeabilization. Free $[Ca^{2+}]$ in the bathing solution was controlled by mixing together, in varying ratios, the "10 activating" and "10 relaxing" solutions. The "0.2 relaxing" solution was used as the luminal solution. The lower buffering capacity of this solution relative to the bath solution ensured the saline bathing the outside of the artery dictated the $[Ca^{2+}]$ within the wall and lumen of the permeabilized artery.

2.4 Membrane potential recording

Intracellular electrical recordings were made using conventional capillary glass microelectrodes (external diameter 1.5 mm, internal diameter 0.8 mm, GC 150F-10, Clark Electromedical, Pangbourne, Reading, RG8 7HU, U.K.). The microelectrodes were pulled to a tip resistance of 40-100 MΩ when filled with filtered 3 M KCl. The microelectrodes were connected to a probe with an Ag-AgCl half-cell and the indifferent electrode was an Ag-AgCl pellet. Electrical signals were amplified (NL102, Neurolog, Digitimer), displayed on a digital storage oscilloscope and recorded using a data acquisition system (Newtape) running on a personal computer (Dell, 466 DL).

Criteria for acceptance of membrane potential recordings were 1) an abrupt change in potential upon impalement of cells, 2) stable membrane potential for at least 1 minute, 3) Unchanged tip resistance before and after impalements.

2.5 Data Analyses

Summarised data were expressed in two ways: 1) Diameters were expressed as a mean diameter ± SEM (μm), 2) Diameter responses were expressed as a percentage of the initial level of myogenic tone (i.e. before any drug treatment - the smallest diameter) and the diameter when fully relaxed (i.e. in calcium-free PSS containing EGTA - the largest diameter) as follows:

$$\frac{\text{[Drug concentration diameter] - [diameter in absence of drugs]}}{\text{[EGTA diameter - diameter in absence of drugs]}} \text{ X100}$$

This expression shows the initial level of tone as 0% and the artery completely dilated as 100%. Unless otherwise stated, the statistical test was a two tailed, paired, student's t-test with the null hypothesis rejected at $P<0.05$, n = number of animals.

2.6 Chemicals

Adenosine 5′-triphosphate disodium salt (ATP), barium chloride ($BaCl_2$), cadmium chloride ($CdCl_2$), caesium chloride ($CsCl_2$), cromakalim, ethylene glycol-bis (β-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), ouabain and potassium phosphate (KH_2PO_4) tetrodotoxin (TTX) were obtained from Sigma (Fancy Road, Poole, Dorset, BH12 4QH, England). Glucose, N-2-Hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES), sodium chloride ($NaCl_2$), sodium hydrogen carbonate ($NaHCO_3$) and magnesium sulphate ($MgSO_4$) were obtained from Fischer Scientific UK Ltd. (Bishop Meadow Road, Loughborough, Leicestershire, LE11 5RG, UK). Calcium chloride ($CaCl_2$), magnesium chloride ($MgCl_2$) and potassium chloride (KCl) were obtained from BDH Laboratory Supplies (Poole, Dorset, BH15 1TD, England).

Phosphocreatine (PCr) was obtained from Fluka Chemicals (The Old Brickyard-New Road, Gillingham, Dorset, SP8 4XT, England). Protein kinase C inhibitor (PKC 19-36), RS-20 and SM-1 were obtained from Alexis Corporation UK Ltd. (3 Moorbridge Court, Moorbridge Road East, Bingham, Nottingham, NG13 8QG, England). All the above chemicals were dissolved in distilled water. The following chemicals were dissolved in dimethylsulphoxide (DMSO) with the final bath concentration of DMSO <0.01%. Adenosine (Sigma), 4-aminopyridine (4-AP, Sigma), apamin (Sigma), β-escin (Sigma), calmidazolium (Sigma), glibenclamide (Sigma), indolactam (Sigma), L-nitro methylester (L-NAME, Sigma), N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7, Sigma), nimodipine (Research Biochemicals International, One Strathmore Road, Natick, MA, USA), α-toxin (GibcoBRL, Life Technologies Inc. Gaithersberg, MD, USA).

Chapter 3

Adenosine's Modulation of Myogenic Tone

3.1 Summary

1. Upon pressurisation to 60 mmHg, rat isolated mesenteric and cerebral resistance arteries developed spontaneous myogenic tone, resulting in a 26 ± 1% (n = 42) and 30 ± 2% (n = 14) reduction in diameter respectively.

2. The metabolic vasodilator adenosine and the K_{ATP} channel opener cromakalim each produced a dose-dependent dilatation of pressurised mesenteric arteries.

3. In contrast neither adenosine nor cromakalim produced a dilatation of pressurised cerebral arteries.

4. Adenosine-evoked dilatation of mesenteric arteries was independent of the endothelium, since it was unaffected by the nitric oxide synthase inhibitor L-NAME (100 μM).

5. The adenosine-evoked dilatation was not blocked by antagonists of the K_{ATP} channel (glibenclamide; 1 μM), the small conductance Ca^{2+} activated K^+ channel (apamin; 0.3 μM) or the large conductance, Ca^{2+} activated K^+ channel (TEA; 1 mM). This suggests no role for K^+ channel activation in the adenosine-evoked dilatation.

6. The cromakalim-evoked dilatation was inhibited by glibenclamide (1 μM), demonstrating the presence of the K_{ATP} channel in the mesenteric artery and their activation as the mechanism for cromakalim-evoked dilatation.

7. Cromakalim (10 μM) but not adenosine (100 μM) produced a hyperpolarisation of the pressurised mesenteric artery. Supporting the suggestion that adenosine-evoked dilatation is independent of K^+ channel activation.

8. Adenosine evoked a dose-dependent dilatation of β-escin permeabilised mesenteric arteries, where the intracellular Ca^{2+} concentration would be effectively clamped.

9. Adenosine-evoked dilatation of pressurised mesenteric arteries is independent of nitric oxide synthesis, K^+ channel activation and membrane hyperpolarisation. The mechanism of adenosine-evoked dilatation may involve a decreased myofilament Ca^{2+} sensitivity.

3.2 Introduction

Blood flow is linked to tissue metabolic activity. The precise agent coupling metabolic activity to blood flow is unclear but adenosine is one possibility. During periods of increased tissue metabolism adenosine triphosphate (ATP) is dephosphorylated to adenosine diphosphate (ADP). Some of the ADP, in turn is dephosphorylated to 5'-adenosine monophosphate (AMP; via myokinase) and then to adenosine by enzyme 5'-nucleotidase. The primary site of dephosphorylation of 5'-AMP is the cell membrane, the site of 5'-neucleotidase. Adenosine is released into the interstitial space where it may dilate small arteries and arterioles.

A number of studies linked adenosine-mediated relaxation of vascular smooth muscle to the activation of ATP-sensitive potassium channels (K_{ATP}) (Daut, *et al.*, 1990; Belloni & Hintze, 1991; von Beckerath *et al.*, 1991; Dart & Standen, 1993). The first evidence, for this proposal, comes from the observation that adenosine-induced decreases in coronary perfusion pressure were attenuated by the K_{ATP} channel blocker glibenclamide. Since this finding, adenosine has been shown to produce glibenclamide-sensitive vasodilatation and hyperpolarisation in coronary arteries (Daut *et al.*, 1990; Clayton *et al.*, 1992; Nakhostine & Lamontagne, 1993; Akatsuka *et al.*, 1994), skeletal muscle (Jackson, 1993) and large (>400μm) cerebral arteries (Nagao *et al.*, 1996).

Adenosine stimulation of A_2 receptors activates adenylate cyclase (Hori & Kitakaze, 1991), so producing adenosine 3',5'-cyclic monophosphate (cAMP) (Herlihy *et al.*, 1976). Since K_{ATP} channels in smooth muscle are activated by cAMP-dependent protein kinase A (PKA) (Quayle & Standen, 1994; Zhang *et al.*, 1994; Kleppisch & Nelson, 1995), adenosine-induced dilatation may be mediated by the following sequence of events: (1) Adenosine stimulates A_2 receptors, which activates adenylate cyclase; (2) there is a consequent increase in intracellular cAMP; (3) this stimulates protein kinase A, which (4) through a phosphorylation step activates K_{ATP} channels; (5) the outward current flowing through the open K_{ATP} channels results in membrane hyperpolarisation; (6) the hyperpolarisation reduces the open state probability of voltage dependent calcium channels (Dunne *et al.*, 1988); (7) this leads to a decrease in calcium influx therefore a reduction in the intracellular free calcium and thus relaxation of vascular smooth muscle (Noma, 1983; Standen *et al.*, 1989; Nichols & Lederer, 1991).

In contrast to the proposal that K_{ATP} channels underlie adenosine-induced dilatation, others have found glibenclamide-insensitive dilatations in rat mesenteric arteries (Tabrizchi & Lupichuk, 1995), and rabbit and cat small cerebral arteries (Taguci *et al.*, 1994; Nagao *et al.*, 1996; Wei *et al.*, 1996). The question arises therefore as to whether or not dilatation of arteries is dependent on K_{ATP} channel activation and hyperpolarisation. In this study therefore we have examined the action of adenosine on two vascular preparations. The first was resistance arteries from the rat mesentery. This preparation has been

selected, as the smooth muscle cells isolated from this preparation are known to possess K_{ATP} channels (Standen *et al.*, 1989). The second was rat cerebral resistance arteries a preparation lacking K_{ATP} channels (McCarron *et al.*, 1991, McPherson & Stork, 1992).

3.3 Methods

3.3.1 Dissection of posterior cerebral artery

Male, Wistar-Kyoto rats, aged between 11 and 20 weeks were killed by a sodium pentobarbitone overdose (150 $mgkg^{-1}$ Inter-peritoneal). The rat was decapitated using a guillotine. The brain was carefully removed from the skull and second order branches of the posterior cerebral artery were carefully dissected from the pial membrane (figure 2.1a). An artery segment (2-3 mm) was removed and transferred for cannulation (see section 2.2.1).

3.3.2 Dissection of mesenteric artery

Following a mid-line incision, the peritoneal cavity was opened and the intestinal tract, with the mesenteric bed, was quickly removed and transferred to a Sylgard-coated Petri dish containing cold PSS. A 2-3 cm segment of the small intestine, 5-6 cm distal to the pylorus, was removed. A fourth order branch (Sun *et al.*, 1992) was carefully dissected from any adherent connective and adipose tissue (figure 2.2). An artery segment (0.5-1 mm) was removed and transferred for cannulation (see section 2.2.1).

3.3.3 Cannulation and Pressure-Perfusion Myograph

Second order cerebral and fourth order mesenteric arteries were cannulated and set up in a Halpern pressure-perfusion myograph, as previously described (Halpern *et al.*, 1984, section 2.2, figure 2.3).

3.3.4 Permeabilised arteries

Second order posterior cerebral arteries were mounted in a small volume chamber (~3 ml). In a "10 Relaxing" bathing solution (room temperature 20 ± 2°C, pH 7.2) at a pressure of 60 mmHg, arteries were permeabilised using β-Escin (100 μM) for ~2 minutes. Free $[Ca^{2+}]$ in the bathing solution was controlled by mixing together, in varying ratios, the "10 activating" and "10 relaxing" solutions. The "0.2 relaxing" solution was used as the luminal solution. The lower buffering capacity of this solution relative to the bath solution ensured the saline bathing the outside of the artery dictated the $[Ca^{2+}]$ within the wall and lumen of the permeabilised artery (permeabilisation procedure and all solutions used have been described previously in section 2.3)

3.3.5 Membrane potential recording

Intracellular electrical recordings were made using conventional capillary glass microelectrodes (external diameter 1.5 mm, internal diameter 0.8 mm). The microelectrodes were pulled to a tip resistance of 40-100 MΩ when filled with

filtered 3 M KCl. The microelectrodes were connected to a probe with an Ag-AgCl half-cell and the indifferent electrode was an Ag-AgCl pellet. Electrical signals were amplified, displayed on a digital storage oscilloscope and recorded using a data acquisition system (Newtape) running on a personal computer (further details have been described previously in section 2.4). Criteria for acceptance of membrane potential recordings were 1) an abrupt change in potential upon impalement of cells, 2) stable membrane potential for at least 1 minute, 3) Unchanged tip resistance before and after impalements.

3.4 Results

Rat isolated mesenteric and cerebral resistance arteries set up in the pressure myograph, at a transmural pressure of 60 mmHg and in Ca^{2+}-free PSS had fully relaxed diameters of 146 ± 4 μm (n=42) and 174 ± 3 μm (n=14) respectively. Upon equilibration at 37°C and in the presence of 1.6 mM Ca^{2+}-PSS, the arteries developed spontaneous tone, resulting in a 26 ± 1% (n = 42) reduction in mesenteric artery diameter and a 30 ± 2% (n = 14) reduction in cerebral artery diameter.

The metabolic vasodilator adenosine (0.1 μM to 300 μM) produced a dose dependent dilatation of pressurised mesenteric resistance arteries with an EC_{50} value of 7 ± 1 μM, n=23 (figure 3.1A). Cromakalim (0.03 μM to 10 μM) the ATP-sensitive K^+ channel opener, produced a dose dependent dilatation of pressurised mesenteric resistance arteries with an EC_{50} value of 0.1 ± 0.4 μM, n=9 (figure 3.1B) suggesting the presence of K_{ATP} channels. Summarised adenosine and cromakalim concentration response relations are presented in figure 3.1C.

In contrast to their effects on pressurised mesenteric resistance arteries, neither adenosine, (0.1 μM to 300 μM) n=7, (figure 3.2A) nor cromakalim, (0.03 μM to 10 μM) n=6, (figure 3.2B) produced a dilatation of pressurised posterior

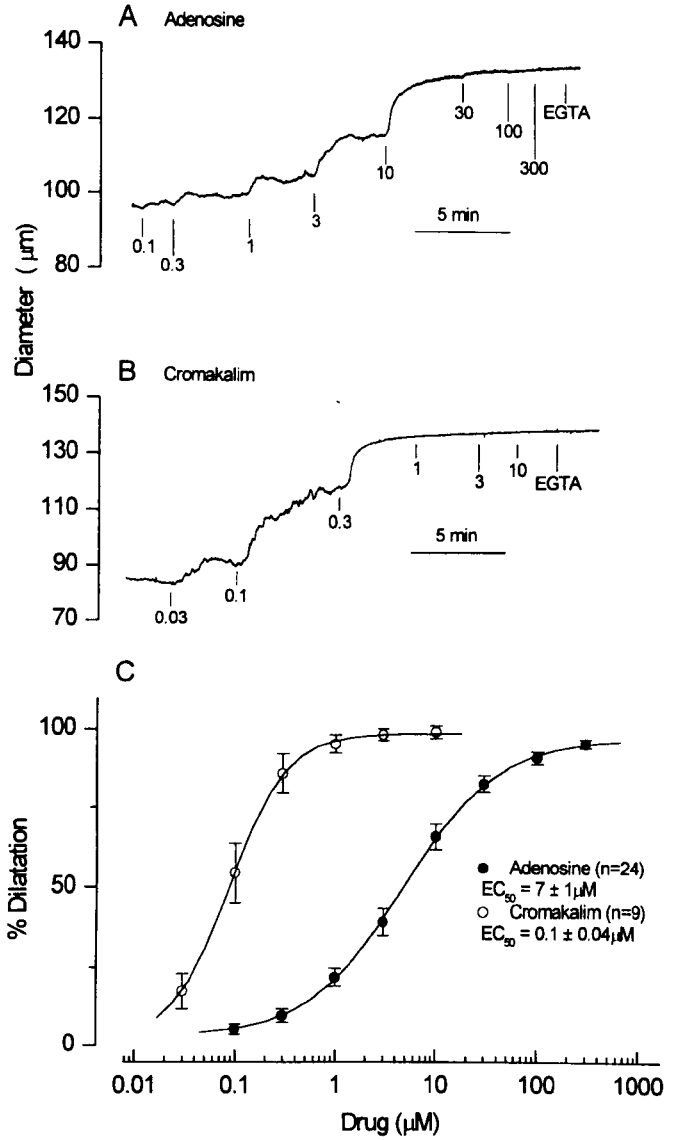


Figure 3.1 (A) Concentration dependent dilatation of rat mesenteric resistance arteries to increasing doses of adenosine (0.1 µM to 300 µM), and (B) cromakalim (0.03 µM to 10 µM). (C) Summarised concentration response relations to adenosine (•, EC_{50} = 7 ± 1 µM, n=24) and to cromakalim (o, EC_{50} = 0.1 ± 0.04 µM, n=9). In this and subsequent figures dilatation is related to initial level of myogenic tone (0%) and fully relaxed diameter (100%).

cerebral resistance arteries. Summarised results are presented in figure 3.2C. Therefore cerebral arteries which lack K_{ATP} channels do not dilate to adenosine.

The mechanism of action of adenosine on mesenteric arteries was next examined. As a first step the role of nitric oxide production from the endothelium in mediating adenosine-induced dilatations was assessed. The nitric oxide synthase inhibitor, L-NAME, however, had no significant ($P>0.05$) effect on the adenosine-induced dilatation. Thus the EC_{50} was 4 ± 8 μM (n=3), before (figure 3.3A) and 8 ± 3 μM (n=3), after (figure 3.3B) the addition of L-NAME (100 μM). Summarised data is shown in figure 3.3C. These results suggest there is no contribution of nitric oxide in the adenosine-induced dilatation in rat mesenteric arteries.

The role of K_{ATP} and other K^+ channels was next investigated by examining the effect of a number of K^+ channel antagonists upon the adenosine-induced dilatation. The K^+ channel antagonists used were (1) the small conductance, Ca^{2+} activated, K^+ channel antagonist apamin, (2) the large conductance, Ca^{2+} activated, K^+ channel antagonist tetraethylammonium (TEA) and, (3) the K_{ATP} channel antagonist gilbenclamide. Each of the K^+ channel antagonists however, failed to produce any significant inhibition of the adenosine-induced

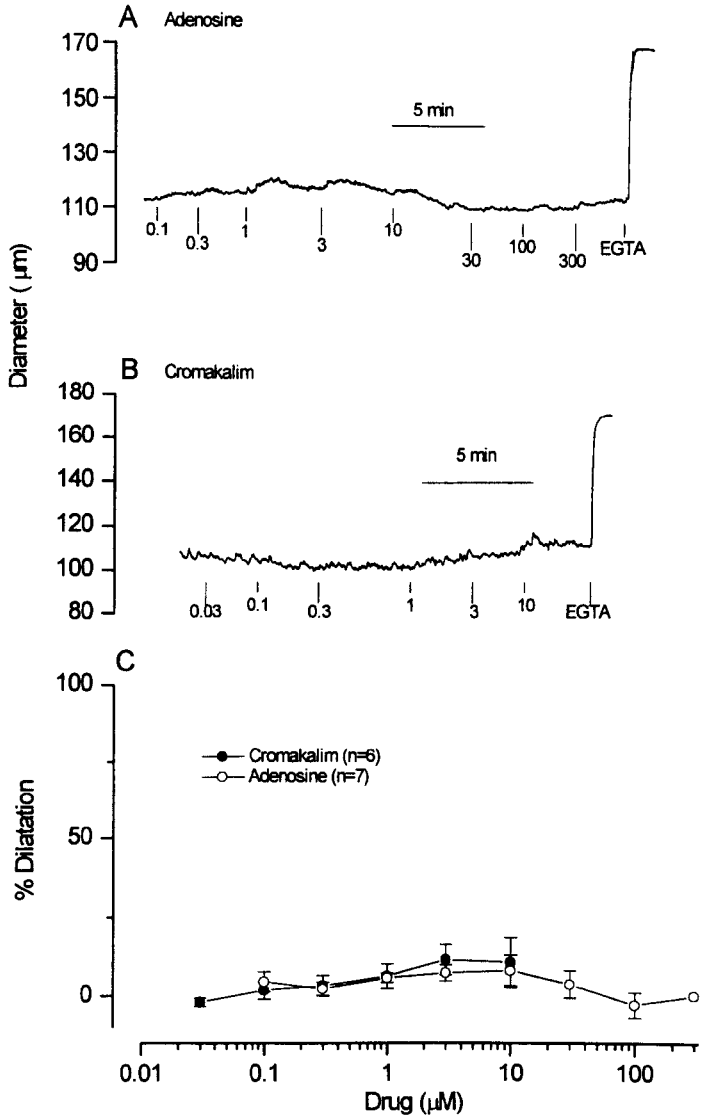


Figure 3.2 (A) Absence of effect of adenosine (0.1 μM to 300 μM), and (B) cromakalim (0.03 μM to 10 μM) on the diameter of the rat cerebral resistance artery. (C) Summarised concentration response relations to adenosine (o, n=7) and cromakalim (•, n=6).

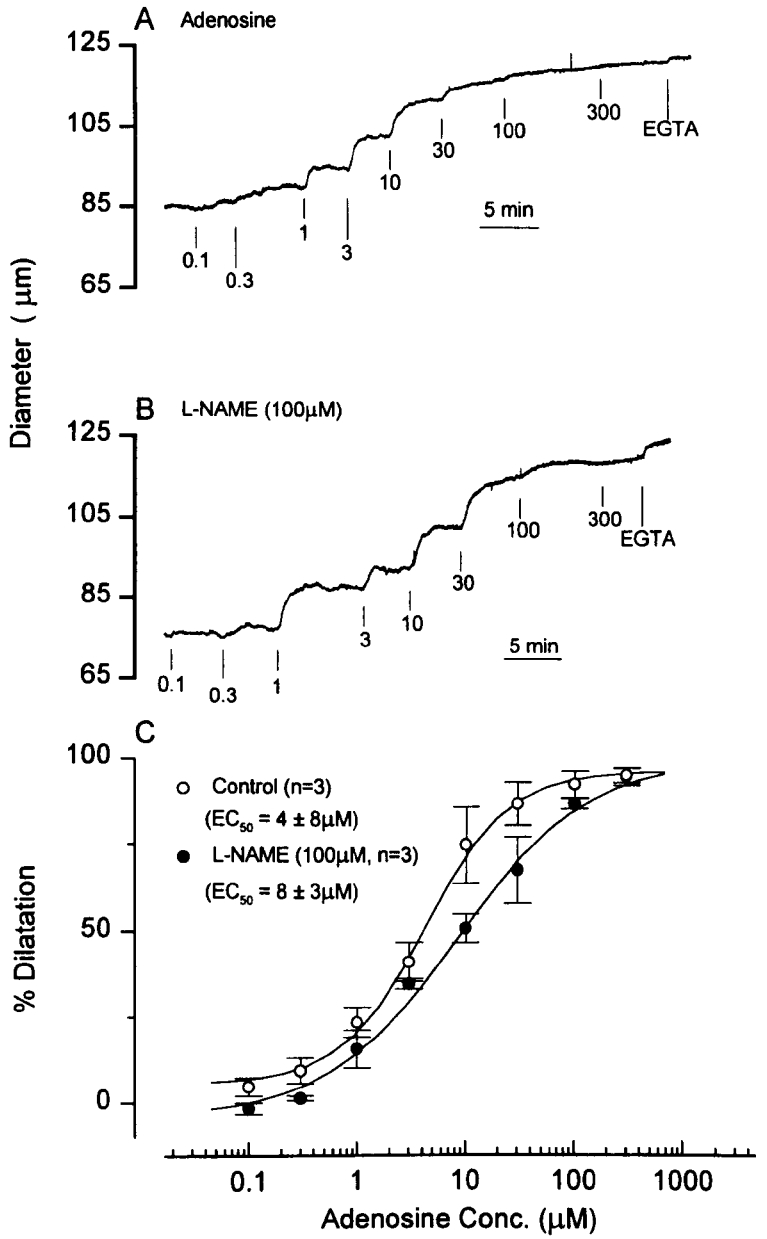


Figure 3.3 (A) Concentration dependent dilatation of rat mesenteric resistance artery to increasing doses of adenosine (0.1 µM to 300 µM). (B) The nitric oxide synthase inhibitor L-NAME (100 µM) had no effect on the adenosine induced dilatation of rat mesenteric resistance arteries. (C) Summarised adenosine concentration response relations with (•, EC_{50} = 8 ± 3 µM, n=3) and without (o, EC_{50} = 4 ± 8 µM n=3) the presence of L-NAME (100 µM).

dilatation. Thus in the presence of apamin (0.3 μM) the adenosine-induced dilatation had an EC_{50} value of 3 ± 1 μM (n=3; P>0.05), not significantly different to the paired control dilatation in the absence of apamin 4 ± 1 μM (n=3), (figure 3.4). TEA (1 mM) also had no significant change in the adenosine-induced dilatation, with EC_{50} values of 6 ± 3 μM (n=3; P>0.05) and 5 ± 3 μM (n=3) in the presence and absence of TEA respectively (figure 3.5). Finally, and unexpectedly, glibenclamide (1 μM) produced no significant inhibition of the adenosine-induced dilatation with EC_{50} values of 4 ± 0.7 μM (n=8) before and 6 ± 2 μM (n=8; P>0.05) after the addition of glibenclamide (figure 3.6).

The failure of glibenclamide to reverse the adenosine-induced dilatation may occur because adenosine does not activate K_{ATP} channels in this tissue or, alternatively, because glibenclamide does not inhibit K_{ATP} channels in rat mesenteric arteries. To distinguish between these possibilities the effect of glibenclamide was examined on the K_{ATP} channel opener cromakalim. Cromakalim dilated the arteries with an EC_{50} value of 0.2 ± 0.1 μM (n=3). After glibenclamide (1 μM) the EC_{50} value was markedly reduced to 3 ± 2 μM (n=3). This result agrees with a number of studies where cromakalim produced a glibenclamide-sensitive dilatation of mesenteric arteries (Standen *et al.*, 1989, McCarron *et al.*, 1991). Summarised results are presented in figure 3.7C.

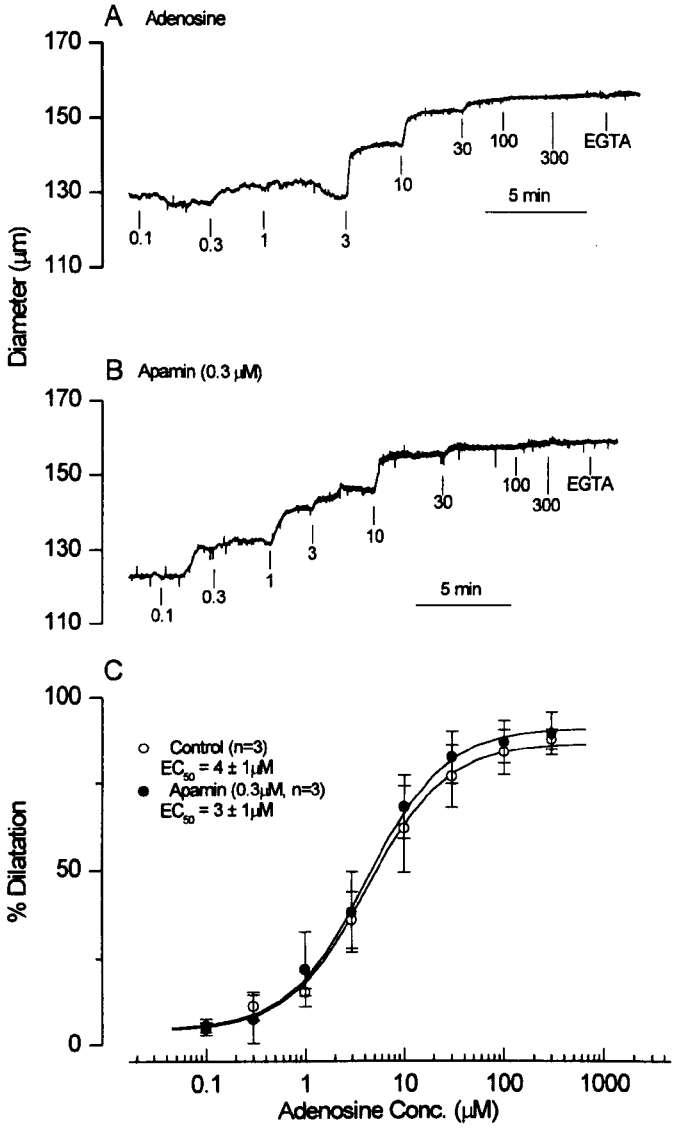


Figure 3.4 (A) Concentration dependent dilatation of rat mesenteric resistance artery to increasing doses of adenosine (0.1 µM to 300 µM). (B) The small conductance, Ca^{2+} activated, K^+ channel antagonist apamin (0.3 µM) had no effect on the adenosine induced dilatation of rat mesenteric resistance arteries. (C) Summarised adenosine concentration response relations with (•, EC_{50} = 3 ± 1 µM, n=3) and without (o, EC_{50} = 4 ± 1 µM n=3) the presence of apamin (0.3 µM).

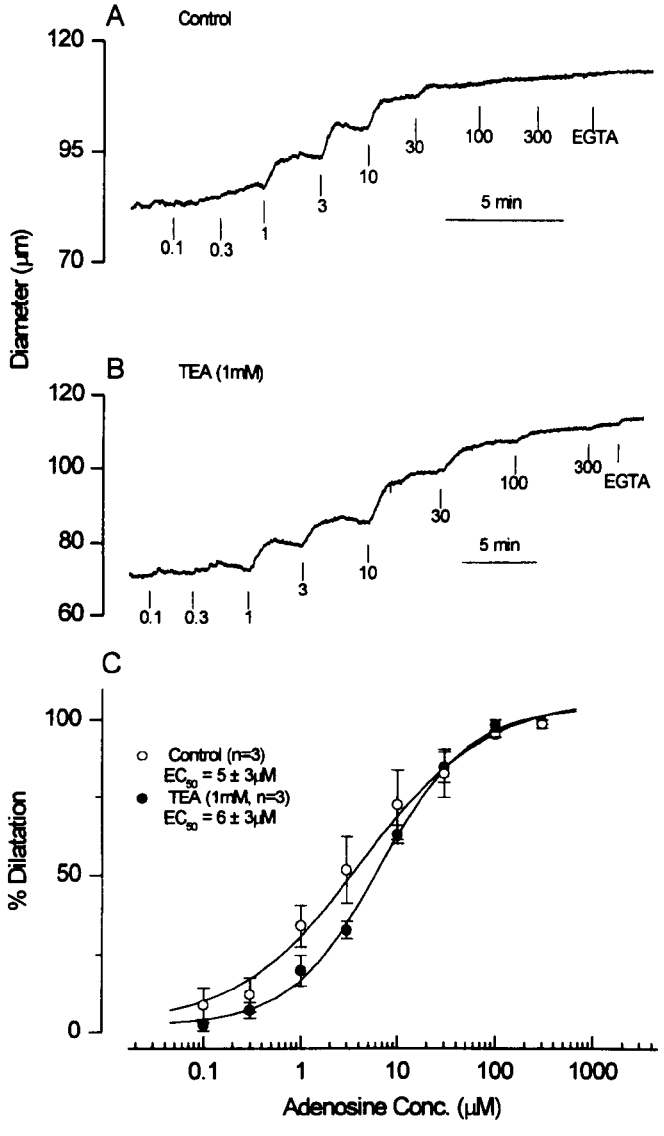


Figure 3.5 (A) Concentration dependent dilatation of rat mesenteric resistance artery to increasing doses of adenosine (0.1 µM to 300 µM). (B) The large conductance, Ca^{2+} activated, K^+ channel antagonist tetraethylammonium (TEA, 1 mM) had no effect on the adenosine induced dilatation of rat mesenteric resistance arteries. (C) Summarised adenosine concentration response relations with (•, EC_{50} = 6 ± 3 µM, n=3) and without (o, EC_{50} = 5 ± 3 µM n=3) the presence of TEA (1 mM).

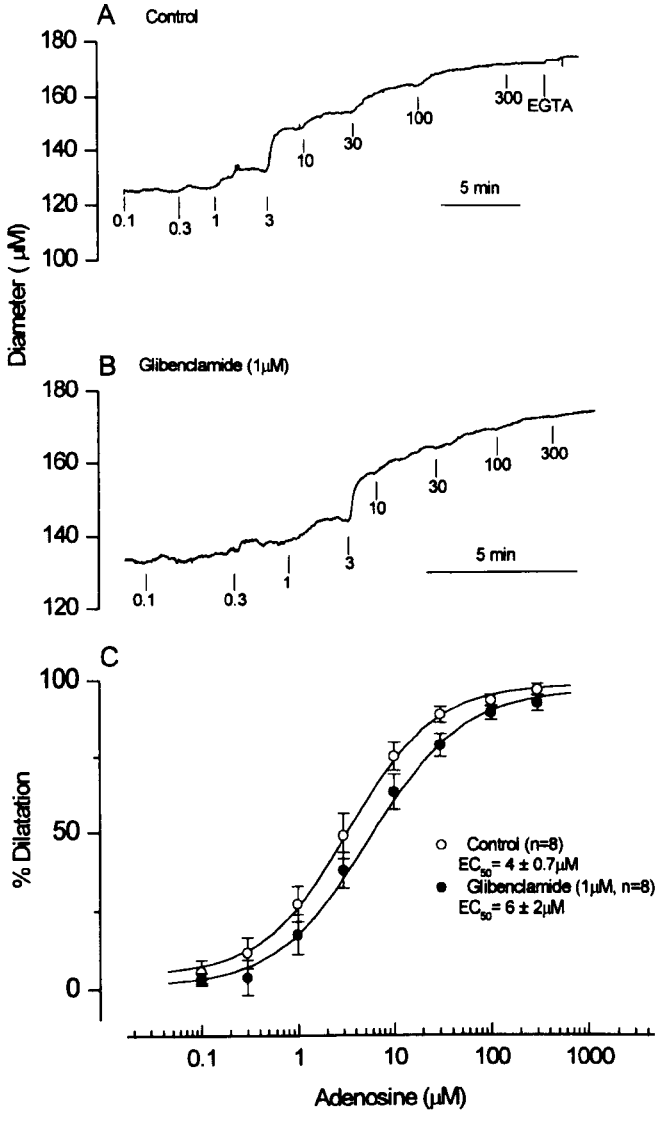


Figure 3.6 (A) Concentration dependent dilatation of rat mesenteric resistance artery to increasing doses of adenosine (0.1 μM to 300 μM). (B) Glibenclamide (1 μM) had no effect on the adenosine induced dilatation of rat mesenteric resistance arteries. (C) Summarised adenosine concentration response relations with (•, EC_{50} = 6 ± 2 μM, n=8) and without (o, EC_{50} = 4 ± 0.7 μM, n=8) the presence of glibenclamide (1 μM).

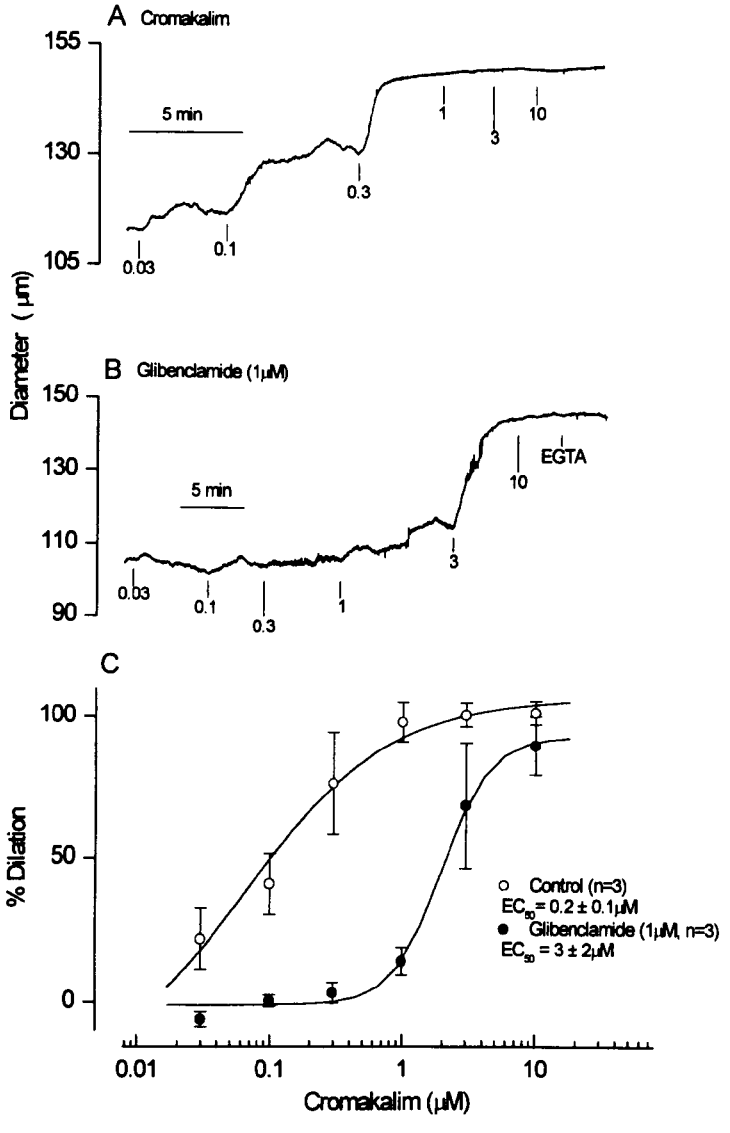


Figure 3.7 (A) Concentration dependent dilatation of rat mesenteric resistance artery to increasing concentrations of cromakalim (0.03 μM to 10 μM). (B) Glibenclamide (1 μM) significantly attenuates cromakalim-induced dilatation of rat mesenteric resistance arteries. (C) Summarised cromakalim concentration response relations with (•, EC_{50} = 3 ± 2 μM, n=3) and without (o, EC_{50} = 0.2 ± 0.1 μM, n=3) the presence of glibenclamide (1 μM).

To verify further the effect of glibenclamide upon both adenosine- and cromakalim-induced dilatations a different experimental protocol was adopted, where upon a near maximal dilatation to adenosine or cromakalim, 0.5 μM then 1 μM of glibenclamide was added. Glibenclamide again had no effect upon the adenosine-induced dilatation (figure 3.8A), although it produced a substantial inhibition of cromakalim-induced dilatation (figure 3.8B), consistent with the previous findings. Collectively these results suggest that adenosine-induced dilatation of rat pressurised mesenteric resistance arteries may be independent of K^+ channel activity.

As an additional test of the possible contribution of K^+ channels in adenosine dilatation, the effect of adenosine upon membrane potential was examined. Adenosine-induced dilatation has been shown to involve an associated membrane hyperpolarisation in a number of studies. The mean resting membrane potential of pressurised (60 mmHg) mesenteric resistance arteries was -42 ± 1 mV (n=21) a value similar to that reported for other arterial preparations (Brayden & Wellman, 1989; Nelson et al., 1997). After cromakalim (10 μM) the membrane potential significantly hyperpolarised to -55 ± 2 mV (n=8, $P<0.01$) and the arteries maximally dilated. Adenosine (100 μM) however, while fully dilating the artery, did not significantly ($P>0.05$) alter the resting membrane potential (-41 ± 1 mV, n=6). These results suggest that adenosine-induced dilatation was independent of any change in membrane

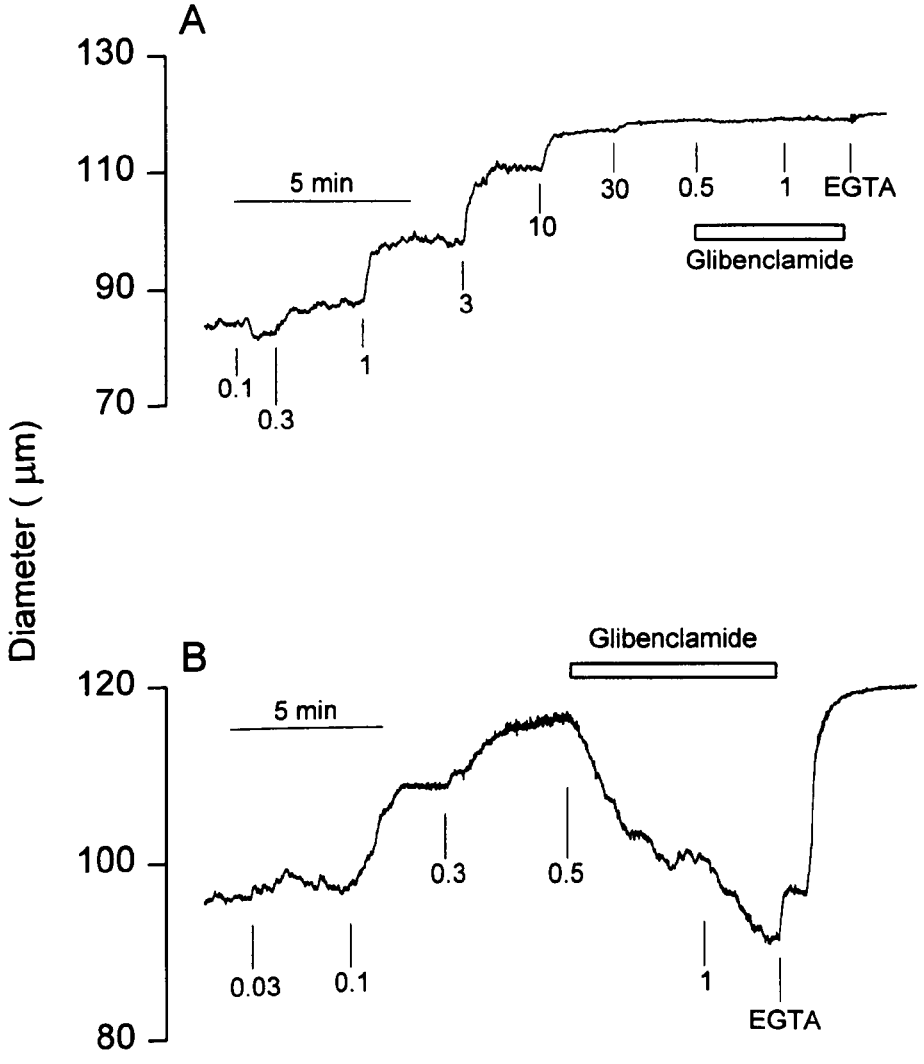


Figure 3.8 (A) Concentration dependent dilatation of rat mesenteric resistance artery to increasing concentrations of adenosine (0.1 μM to 30 μM). The addition glibenclamide (0.5 μM and 1 μM) had no effect upon the adenosine-induced dilatation. (B) Concentration dependent dilatation of rat mesenteric resistance artery to increasing concentrations of cromakalim (0.03 μM to 0.3 μM). The addition of glibenclamide (0.5 μM and 1 μM) completely abolished the cromakalim-induced dilatation.

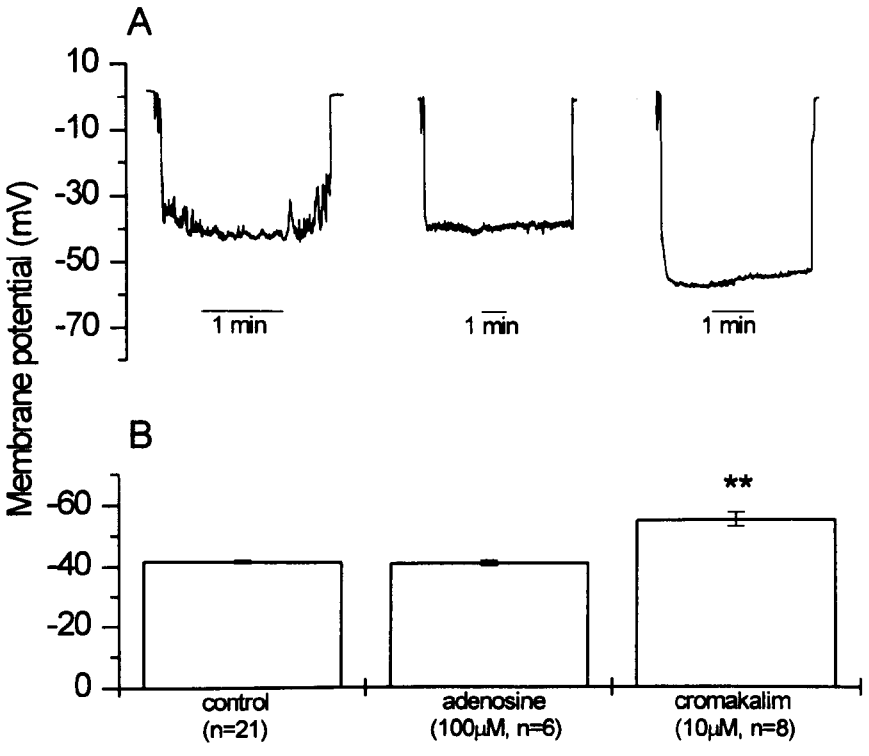


Figure 3.9 (A) Typical membrane potential recordings from the rat mesenteric resistance artery under control conditions and in the presence of either adenosine (100 μM) or cromakalim (10 μM). Adenosine (100 μM) produced a maximal dilatation of the artery (with reference to a maximal dilatation produced by bathing in Ca^{2+}-free PSS containing 1 mM EGTA, not shown) but had no significant effect upon the membrane potential. Cromakalim (10 μM) however produced a maximal dilatation accompanied with a significant hyperpolarisation of the membrane potential. (B) Summarised data showing the mean membrane potential of rat mesenteric resistance artery under control conditions (-42 ± 1 mV, n=21) and in the presence of either adenosine (-41 ± 1 mV, n=6) or cromakalim (-55 ± 2 mV, **P<0.01, n=8).

potential. Membrane potential recordings under control, adenosine or cromakalim treated conditions, with summarised results are presented in figure 3.9.

Adenosine although not altering the membrane potential did evoke a maximal dilatation. One possible mechanism by which adenosine may evoke vasodilatation is by modulating myofilament Ca^{2+} sensitivity. To explore this possibility arteries were permeabilised using ß-escin (100 μM) so allowing the Ca^{2+} present in the extra-cellular bathing solution to dictate the intra-cellular Ca^{2+} concentration. Arteries were permeabilised in Ca^{2+}-free PSS containing 1 mM EGTA, then as shown in figure 3.10A, extracellular Ca^{2+} was subsequently increased in steps up to ~600 nM. At this [Ca^{2+}] the artery had contracted to a level approximately equal to that of myogenic tone. Under Ca^{2+} clamped conditions adenosine (0.1 μM to 3 mM) still produced a concentration dependent dilatation of the mesenteric artery although with a reduced EC_{50} value of 355 ± 98 μM, n=7, compared to that of 7 ± 1 μM, n=24, in non-permeabilised control arteries. Summarised results are presented in figure 3.10B.

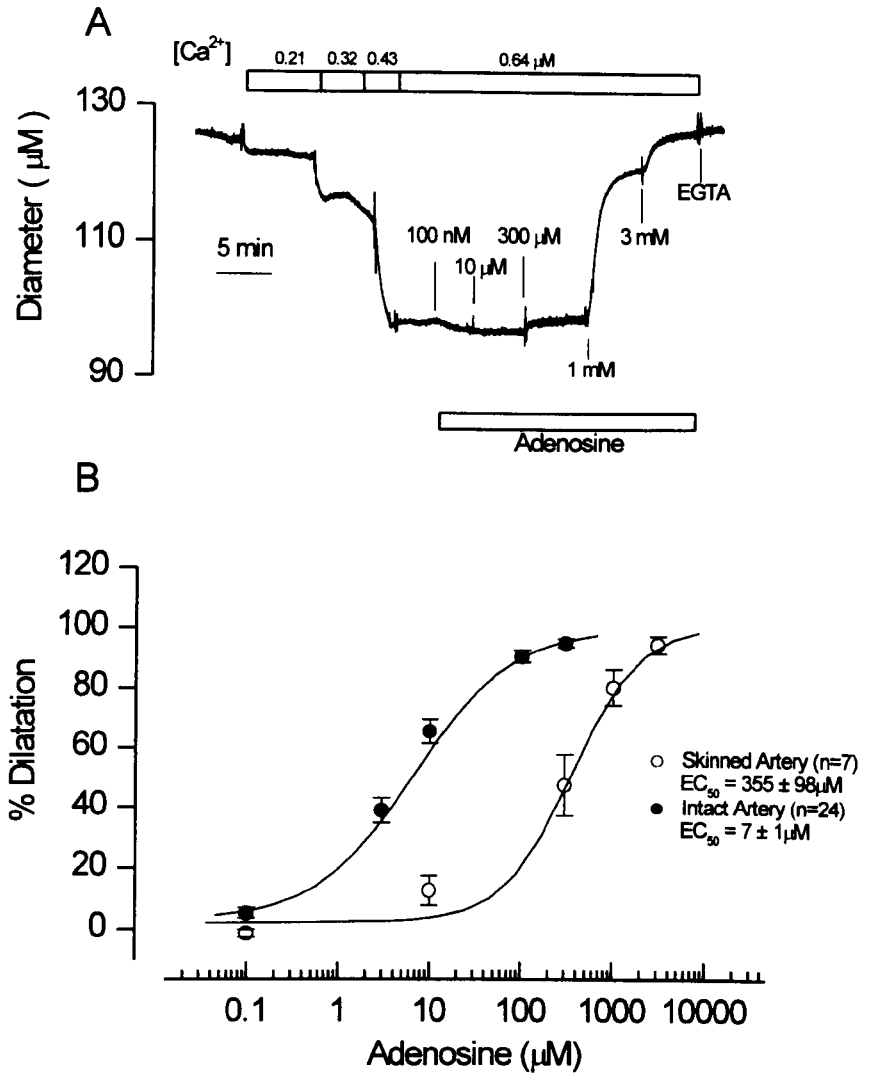


Figure 3.10 (A) Typical recording showing a rat mesenteric resistance artery permeabilised in a zero Ca^{2+} bathing solution. When the Ca^{2+} concentration was increased stepwise up to 0.64 µM (shown by the upper bar), the artery contracted to a level approximate of that seen in spontaneous myogenic tone. Under these $[Ca^{2+}]_i$-clamped conditions adenosine (0.1 µM to 3 mM) produced a concentration dependent dilatation of the mesenteric artery. (B) Summarised adenosine concentration response relations on intact (•, EC_{50} = 7 ± 1 µM, n=24) and permeabilised (o, EC_{50} = 355 ± 98 µM, n=7) rat mesenteric resistance arteries.

3.5 Discussion

In this study the effect of adenosine on rat cerebral and mesenteric arteries has been examined. Adenosine dilated mesenteric arteries but it did not dilate cerebral arteries. The finding that adenosine does not dilate cerebral arteries raises the possibility that it may not be a metabolic vasodilator in the cerebral circulation. In mesenteric arteries, adenosine-evoked dilatation was independent of nitric oxide synthesis, K^+ channel activation and membrane hyperpolarisation. A possible mechanism of adenosine-induced dilatation may be a decreased myofilament Ca^{2+} sensitivity.

Adenosine-mediated relaxation of vascular smooth muscle has been linked to the activation of ATP-sensitive potassium channels (K_{ATP}) (Daut, *et al.*, 1990; Belloni & Hintze, 1991; von Beckerath *et al.*, 1991; Dart & Standen, 1993). K_{ATP} channel activation would hyperpolarise the smooth muscle membrane and reduce the open state probability of VDCC so decreasing intracellular Ca^{2+} concentration (Nelson *et al.*, 1988; Nelson *et al.*, 1990). Thus, adenosine evoked a glibenclamide-sensitive (a K_{ATP} channel antagonist) hyperpolarisation or vasodilatation, in coronary arteries isolated from the dog (Akatsuka *et al.*, 1994), guinea pig (Daut *et al.*, 1990) and rabbit (Nakhostine & Lamontagne, 1993), rat skeletal muscle arteries (Marshall *et al.*, 1993) and rabbit large cerebral arteries (Nagao *et al.*, 1996).

However, in the present study, on rat mesenteric arteries, glibenclamide did not alter the adenosine-evoked dilatation. Similarly the K^+ channel antagonists apamin (small conductance, Ca^{2+} activated K^+ channel antagonist) and TEA (large conductance, Ca^{2+} activated K^+ channel antagonist) also did not alter the adenosine-evoked dilatation. Furthermore, while producing full dilatation of the artery, adenosine did not hyperpolarise the arterial smooth muscle cell membrane potential. In contrast, the K_{ATP} channel activator, and vasodilator, cromakalim did evoke a significant hyperpolarisation of the arterial smooth muscle cell and a maximal dilatation of the artery. K_{ATP} channels are therefore present in the mesenteric artery and their activation evokes a relaxation of the artery (Nelson *et al.,* 1990; Nelson & Quayle, 1995). However, activation of K_{ATP} channels, or the large or small conductance, Ca^{2+} activated K^+ channels seems an unlikely mechanism for adenosine-induced dilatation.

In agreement with these findings, other studies also report that adenosine-induced dilatation of rat mesenteric arteries was not attenuated by glibenclamide (Tabrizchi & Lupichuk, 1995). Similarly, glibenclamide-insensitive adenosine-induced dilatations, have been observed in small pial arteries of the cat (Wei *et. al.* 1996) and rabbit (Nagao *et. al.* 1996). In the rabbit pial artery, while aprakalim (a K_{ATP} channel activator) evoked a glibenclamide-sensitive dilatation, adenosine evoked a dilatation which was insensitive to gibenclamide (Taguchi *et. al.,* 1994). Furthermore, a recent study demonstrated a glibenclamide-insensitive, adenosine A_{2A} agonist-evoked dilatation of rabbit renal arteries (Prior *et al.,* 1999).

One possible explanation for the adenosine-evoked dilatation on mesenteric arteries may be a direct modulation of the myofilament Ca^{2+} sensitivity. To investigate this possibility experiments on β-escin permeabilised mesenteric arteries where performed. Under conditions of clamped intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) adenosine evoked a concentration dependent dilatation of the mesenteric artery, indicating that adenosine-evoked dilatation is independent of any change in $[Ca^{2+}]_i$ and suggesting that adenosine may indeed decrease the myofilament Ca^{2+} sensitivity.

In rat mesenteric artery, adenosine-evoked dilatation occurs by activation of adenosine A_2 receptors (Routledge *et al.*, 1998). A_2 receptor stimulation in other tissues activates adenylate cyclase (Hori & Kitakaze, 1991), so increasing intracellular cAMP and activating protein kinase A (PKA; Silver *et al.*, 1983; Pennanen *et al.*, 1994). In other studies, increases in cAMP, produced by forskolin (an activator of adenyl cyclase), resulted in a PKA phosphorylation of myosin light chain kinase (de Lanerolle *et al.*, 1984). The PKA phosphorylation decreased the Ca^{2+} sensitivity of MLCK (Kamm & Stull, 1985; Stull *et al.*, 1990), an effect that would be anticipated to produce relaxation of the muscle.

In contrast to their effects upon the mesenteric artery, neither adenosine nor cromakalim evoked a dilatation of the cerebral artery. Adenosine therefore, may not be a metabolic vasodilator in the cerebral circulation, as had been

suggested by several previous studies (Winn *et al.*, 1981; Morii *et al.*, 1987; Simpson & Phillis, 1991). For example, topical application of adenosine, through a cranial window in anaesthetized rabbits, evoked a dilatation of pial arterioles (Taguchi *et al.*, 1994). Supporting this, a recent study also demonstrated that the topical application of adenosine to the cerebral cortex of the anaesthetized rat, evoked an increase in cerebral blood flow and suggested that adenosine release was the metabolic mediator responsible for hypoxia-induced dilatation in the cerebral cortex (Coney & Marshall, 1998). In agreement with the present findings other studies have demonstrated a lack of cromkalim-evoked dilatation of the rat posterior cerebral artery (McCarron *et al.*, 1991; McPherson & Stork, 1992). In contrast, several studies have reported a cromakalim-induced dilatation of other cerebral arteries, including the rat superior cerebellar (Nagao *et al.*, 1991) and basilar arteries (McPherson & Stork, 1992). A possible explanation for the disparate results may involve a reduced expression of the K_{ATP} channels in the posterior cerebral artery (McCarron *et al.*, 1991). Indeed, a recent study indicated that K_{ATP} channels distribute more densely in proximal rather than distal cerebral arteries of the rabbit (Nagao, *et al.*, 1996).

In summary, the findings of the present study suggest that adenosine-induced dilatation of rat mesenteric arteries is independent of K^+ channel activation, membrane hyperpolarisation and may be accounted for, at least in part, by an alteration in myofilament Ca^{2+} sensitivity. Adenosine, furthermore, seems unlikely to act as a metabolic vasodilator in the cerebral circulation.

Chapter 4

K^+-Evoked Dilatation of Rat Cerebral Arterioles

4.1 Summary

1. An increase in extracellular potassium ion concentration ($[K^+]_o$) may link increased neuronal activity and regional cerebral blood flow.

2. Elevation of $[K^+]_o$ from 4.7 to 10 mM evoked a sustained dilatation of isolated pressurised thalamo-perforating cerebral arterioles.

3. The K^+-evoked dilatation was inhibited by the inward rectifier K^+ channel (K_{IR}) inhibitor Ba^{2+} (50μM), and the K^+ channel inhibitor cesium (20mM).

4. The K^+-evoked dilatation was not blocked by inhibitors of the ATP-sensitive (K_{ATP}) and the Ca^{2+}-activated K^+ channel (K_{Ca}), glibenclamide (1μM) and TEA (1mM) respectively. Nor was the dilatation altered with the neurotoxin tetrodotoxin (TTX, 0.3μM).

5. The K^+-evoked dilatation was associated with a membrane hyperpolarisation.

6. It is proposed that increased $[K^+]_o$ evokes a dilatation of thalamo-perforating cerebral arteries via an activation of K_{IR} channels and smooth muscle cell hyperpolarisation.

4.2 Introduction

In most organs, the metabolic needs of a tissue can be linked to its blood flow. The brain for example, has the remarkable ability to adjust its blood pressure and vascular bed to control blood flow to meet its metabolic demand. During periods of increased metabolic demand, such as during voluntary motor activity, sensory stimulation and also epileptic seizures, there are large increases in regional cerebral blood flow (Plum *et al.*, 1968; Fox & Raichle, 1984). The agent coupling increased brain metabolic activity with increased blood flow still remains unclear, but it has been suggested that potassium ions (K^+) may play a role (Haddy & Scott, 1968). During seizures for example, there are large increases in K^+ levels which coincide with the increased blood flow (Sykova, 1983). Furthermore, K^+ is a potent vasodilator in many vascular beds and particularly that K^+ evokes a hyperpolarisation and vasodilatation of cerebral arteries (Kuschinsky *et al.*, 1972; Toda, 1974; Knot *et al.*, 1996).

Two mechanisms may explain K^+ dilatation of cerebral blood vessels. The first of these is a stimulation of the Na^+, K^+-activated ATPase (Na^+-pump) found in the membrane of the smooth muscle cell. The Na^+-pump, which exchanges 3 Na^+ for 2 K^+ ions is electrogenic and any increases in pump activity, by for example an elevation in external K^+ concentration, would result in a net loss of positive charge from the cell and thus hyperpolarisation. Most of the data describing K^+-evoked dilatation and hyperpolarisation has been attributed to an activation of the Na^+-pump (Thomas, 1972; Chen *et al.*, 1972; Hendrickx &

Casteels, 1974; Toda, 1976). The second proposed mechanism, of K^+-evoked dilatation, involves the inward rectifier K^+ channel (K_{IR}); a channel which allows K^+ to flow out of the cell more readily than into the cell. A small increase in extracellular K^+ produces a rightward shift in the current voltage relationship of the K_{IR} channels leading to an increased outward current, at physiological membrane potentials, and a hyperpolarisation. Studies in small cerebral arteries demonstrate an increase in extracellular K^+ over the 5-15 mM range, activate K_{IR} producing a hyperpolarisation and dilatation (Edwards *et al.*, 1988; McCarron & Halpern, 1990).

The distribution of K_{IR} channels may vary along vascular segments (Nelson *et al.*, 1991). In large cerebral arteries for example the K_{IR} may not be present while it does exist in smaller pial cerebral arteries, a pattern following the disappearance of the sympathetic innervation (Hirst *et al.*, 1986; Hill *et al.*, 1986). More recently it has been proposed that the inward rectifier may again be absent from cortically penetrating cerebral arterioles (Quinn & Beech, 1998). The goal of this study was two fold (1) to determine whether increased extracellular K^+ produced a dilatation of rat isolated cortically penetrating thalamo-perforating cerebral arterioles and (2) if so, what mechanism is responsible, the Na^+, K^+-ATPase or the inward rectifier K^+ channel.

4.3 Methods

4.3.1 Dissection of arteries

Male, Wistar-Kyoto rats, aged between 11 and 20 weeks were killed by a sodium pentobarbitone overdose (150 $mgkg^{-1}$ inter-peritoneal). The rat was decapitated using a guillotine. The brain was carefully removed from the skull and the posterior cerebral artery was located (figure 2.1a). From the posterior cerebral artery a variable number of thalamo-perforating arteries, usually three from each side, course rostally and dorsally to reach the ventral posterior region of the thalamus (figure 2.1b). The overlying hypophysis (pituit) and any adherent brain tissue was carefully removed until a section of the thalamo-perforating artery, large enough to be dissected, could be seen. An artery segment (0.5-1 mm) was removed and transferred for cannulation.

4.3.2 Cannulation and Pressure-perfusion Myograph

Thalamo-perforating arterioles were cannulated and set up in a 10 ml vessel chamber of a Halpern pressure-perfusion myograph containing PSS, as previously described (Halpern *et al.*, 1984, section 2.2, figure 2.3).

4.3.3 Membrane potential recording

Intracellular electrical recordings were measured using microelectrodes fabricated from thin walled borosilicate capillary tubing (external diameter 1.5 mm, internal diameter 0.8 mm). The microelectrodes were pulled to a tip resistance of 40-100 MΩ when filled with filtered 3 M KCl. The microelectrodes were connected to a probe with an Ag-AgCl half-cell and the indifferent electrode was an Ag-AgCl pellet. Electrical signals were amplified using a Neurolog Digitimer NL102, displayed on a digital storage oscilloscope and recorded using a data acquisition system (Newtape) running on a personal computer (further details have been described previously in section 2.4). Criteria for acceptance of membrane potential recordings were 1) an abrupt change in potential upon impalement of cells, 2) stable membrane potential for at least 1 minute, 3) Unchanged tip resistance before and after impalements.

4.4 Results

Rat isolated thalamo-perforating cerebral resistance arteries set up in the pressure myograph, at a transmural pressure of 60 mmHg and in Ca^{2+}-free PSS had fully relaxed diameters of 99 ± 3 μm (n=12). Upon equilibration at 37 °C and in the presence of 1.6 mM Ca^{2+}-PSS, the arteries developed spontaneous tone, resulting in a 26 ± 2% reduction in diameter (n=12).

Increases in external K^+ from 4.7 mM to 10 mM evoked a pronounced and sustained dilatation of the thalamo-perforating resistance artery (Figure 4.1). In three experiments K^+ elevation to 10 mM produced a dilatation of 98 ± 2%, this was reversed fully when K^+ was returned to the control value of 4.7 mM (normal PSS).

To explore the contribution of the inward rectifier K^+ channel in the K^+-evoked dilatation the effects of K^+ channel blockers barium and cesium were examined. In this series of experiments, after the development of myogenic tone, K^+ was again increased from 4.7 mM to 10 mM producing a pronounced and sustained dilatation. After a 5-minute period the K^+ channel blocker barium (50 μM) was introduced and fully reversed the K^+-evoked dilatation. Barium remained for a 10-minute period after which Ca^{2+} free PSS containing 1 mM EGTA was added to determine the maximal lumen diameter. In six experiments K^+ elevation to 10 mM produced a dilatation of 101 ± 1% and barium reversed the dilatation to –77 ± 18% ($P<0.01$, n=6; figure 4.2).

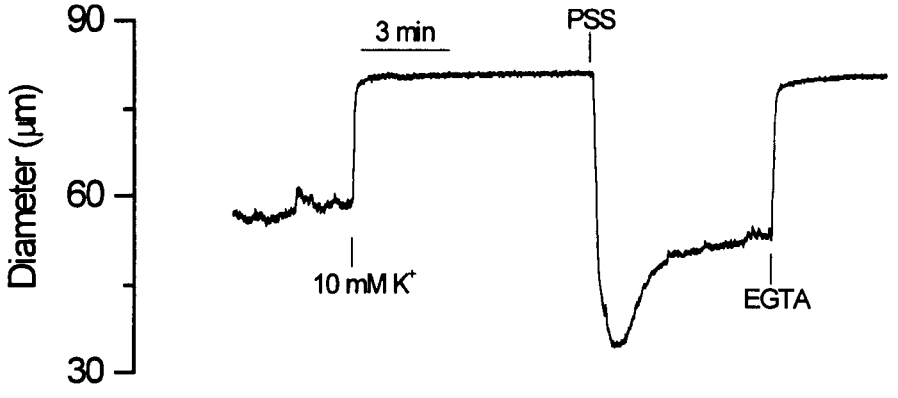


Figure 4.1 Relaxation of a single artery to an increased K^+ from 4.7mM to 10mM. After a 5 minute period normal PSS (4.7mM K^+) was introduced and the artery contracted. In three experiments K^+ elevation to 10mM produced a dilatation of 98 ± 2%. In this and subsequent figures dilatation is related to initial level of myogenic tone, 0% (PSS, 4.7mM K^+), and fully relaxed diameter, 100% (Ca^{2+} free PSS). See section 2.5 for further details.

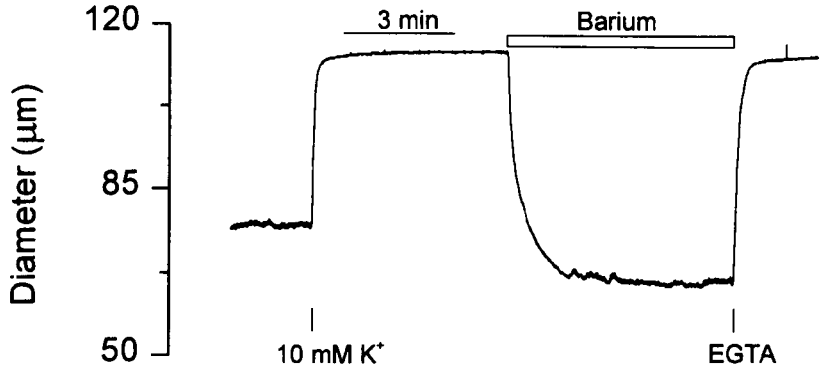


Figure 4.2 Relaxation of a single artery to 10mM K^+. After a 5 minute period barium ($5x10^{-5}M$) was introduced and the artery contracted. Six experiments produced a dilatation of 101 ± 1%, whereas after barium the diameter was –77 ± 18% ($P<0.01$).

Cesium (20 mM) also blocked the 10 mM K^+-evoked dilatation (figure 4.3). After a 5 minute period of the K^+ dilatation cesium (20 mM) was introduced and effectively reversed K^+ dilatation. In five experiments K^+ elevation to 10 mM produced a dilatation of 99 ± 3% of the fully relaxed diameter, and cesium significantly reversed the dilatation (-4 ± 16%, $P<0.01$, n=5; figure 4.3). These results suggest the involvement of K^+ channels in the K^+-evoked dilatation.

To determine whether K^+ channels other than the inward rectifier K^+ channel may underlie K^+-evoked dilatation an additional series of experiments were performed examining the effects other K^+ channel blockers. Barium at the concentration used in this study (50 μM) is known to block the ATP-sensitive K^+ channel. However glibenclamide (1 μM), an inhibitor of ATP-sensitive K^+ channels (Ashcroft, 1988), failed to reverse the 10 mM K^+-evoked dilatation. In five experiments K^+ elevation to 10 mM produced a dilatation of 100 ± 1%, and glibenclamide (1 μM) produced no change in the diameter 102 ± 1% (figure 4.4). Similarly, 1 mM tetraethylammonium (TEA), a concentration known to block Ca^{2+}-activated K^+ channels (Villaroel *et al.*, 1988), did not block the K^+-evoked dilatation. In five experiments K^+ elevation to 10 mM produced a dilatation of 101 ± 2%, and after the addition of TEA (1 mM) diameter remained unchanged at 103 ± 2% (figure 4.5).

Although small cerebral arteries have a scant innervation it is possible that the elevation of K^+ depolarised nerves to release an inhibitory neurotransmitter.

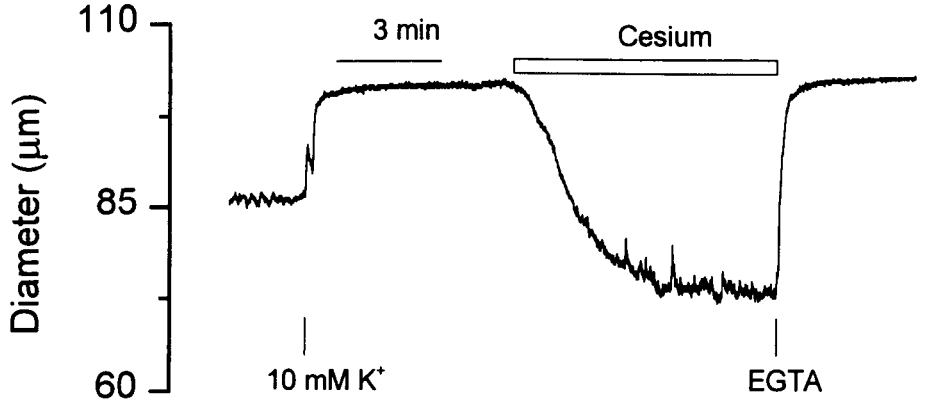


Figure 4.3 Relaxation of a single artery to 10mM K^+. After a 5 minute period cesium ($2x10^{-2}M$) was introduced and the artery contracted. Six experiments produced a dilatation of 99 ± 3%, whereas after cesium the diameter was –4 ± 16% ($P<0.01$).

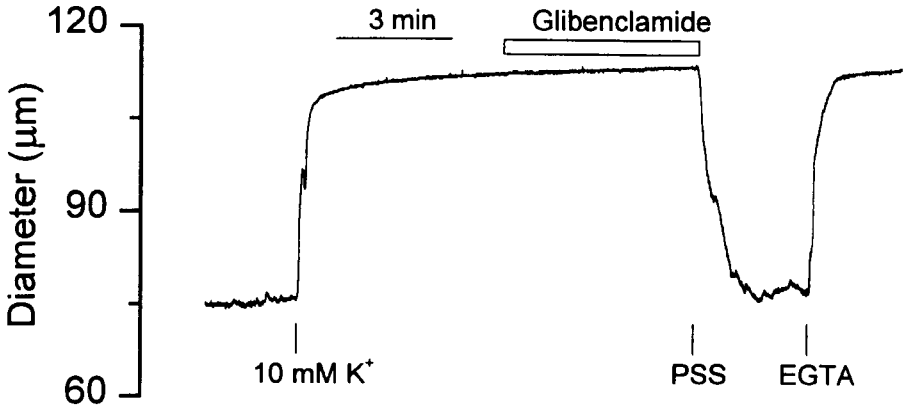


Figure 4.4 Relaxation of a single artery to 10mM K^+. After a 5 minute period glibenclamide ($1x10^{-6}$M) was introduced and had no effect upon the K^+ dilatation. Six experiments produced a dilatation of 100 ± 1%, whereas after glibenclamide the diameter was 102 ± 1%.

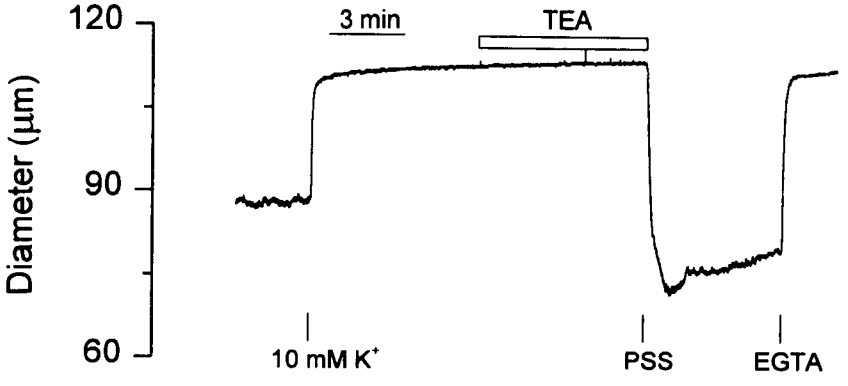


Figure 4.5 Relaxation of a single artery to 10mM K^+. After a 5 minute period tetraethylammonium (TEA) ($1x10^{-3}$M) was introduced and had no effect upon the K^+ dilatation. Six experiments produced a dilatation of 101 ± 2% whereas after TEA the diameter was 103 ± 2%.

Therefore the effect of tetrodotoxin was examined. After a 5-minute period of 10 mM K^+-evoked dilatation tetrodotoxin (0.3 μM) was introduced and did not reverse the dilatation. In five experiments K^+ elevation to 10 mM produced a dilatation of 100 ± 0%, and after the addition of tetrodotoxin diameter was unchanged at 99 ± 1% (figure 4.6).

If the inward rectifier channel did underlie the dilatation then one expectation is that an increase in K^+ should evoke a hyperpolarisation of the artery. The role of the membrane potential in K^+-evoked dilatation in the thalamo-perforating artery was therefore investigated. Under control conditions at 60 mmHg in normal PSS (4.7 mM K^+) and after the development of myogenic tone the artery had a mean membrane potential of -42 ± 1 mV, n=10 (figure 4.7A), a figure comparable to a mean membrane potential of –38 mV, reported for distal segments of the rat middle cerebral artery (Edwards *et al.*, 1988). However in the presence of increased external K^+ (10 mM) which evoked maximal dilatation of the artery there was a hyperpolarisation of the membrane potential to -58 ± 1 mV, ($P<0.01$, n=5; figure 4.7B).

In a final series of experiments the effect of the Na^+-pump inhibitor ouabain was examined. In these experiments, after the development of myogenic tone K^+ was increased from 4.7 mM to 10 mM producing again a pronounced and sustained dilatation. After a 5-minute period ouabain (50 μM) was introduced and effectively reversed K^+ dilatation. Ouabain remained for a 10-minute period after which Ca^{2+} free PSS containing 1 mM EGTA was added to

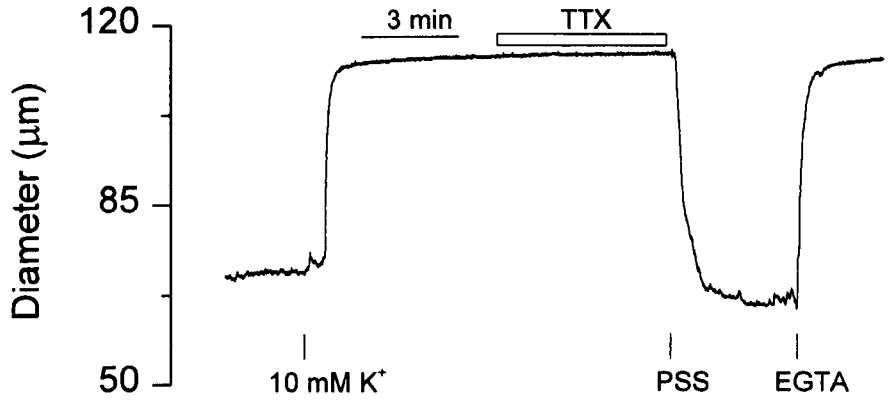


Figure 4.6 Relaxation of a single artery to 10mM K^+. After a 5 minute period tetrodotoxin ($3x10^{-7}M$) was introduced and had no effect upon the K^+ dilatation. Six experiments produced a dilatation of 100 ± 0%, whereas after tetrodotoxin the diameter was 99 ± 1%.

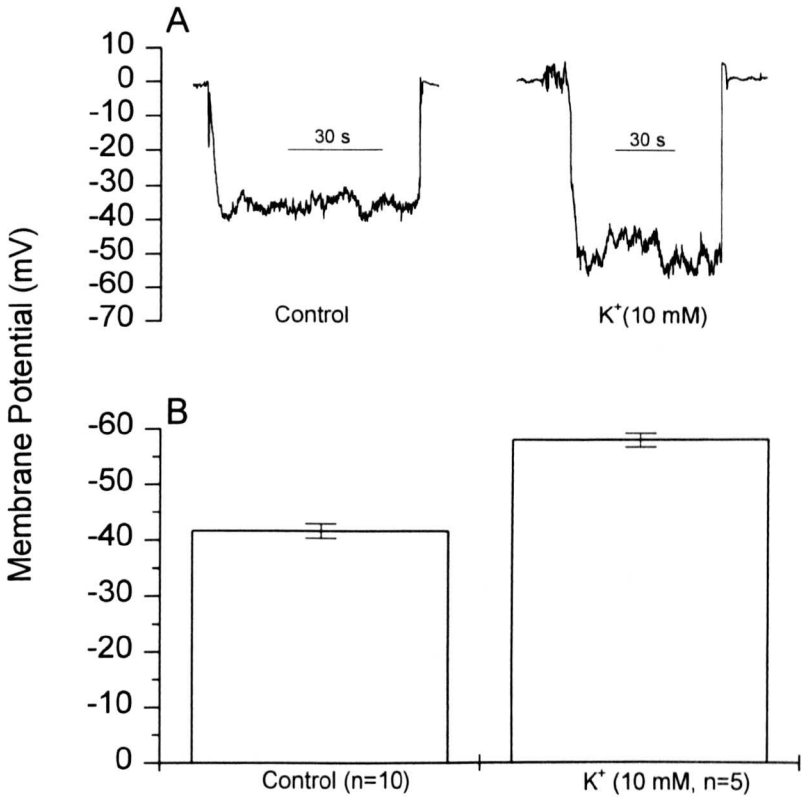


Figure 4.7 (A) Typical membrane potential recordings from the rat thalamo-perforating cerebral artery under control conditions and in the presence of 10mM K^+. In the presence of increased K^+ (10mM) there was a maximal dilatation of the artery (with reference to a maximal dilatation produced by bathing in Ca^{2+}-free PSS containing 1mM EGTA, not shown) and a significant hyperpolarisation of the membrane potential. (B) Summarised data showing the mean membrane potential of rat thalamo-perforating cerebral artery under control conditions (-42 ± 1mV, n=10) and in the presence of 10mM K^+ (-58 ± 1mV, $P<0.01$, n=5).

determine the maximal lumen diameter. In three experiments K^+ elevation to 10 mM produced a dilatation of 101 ± 1%, and the addition of ouabain significantly reversed the dilatation to –28 ± 6% ($P<0.05$, n=3; figure 4.8). This result is consistent with sodium pump involvement in the K^+-evoked dilatation, however, stimulation of the sodium pump predicts a transient dilatation; this was not observed. It is plausible that the reversal of the K^+-evoked dilatation is indirect and arises because of a membrane potential change rather than a direct involvement of the sodium pump.

Table 4.1 displays a summary of the experiments described in figures 4.1 to 4.6 and figure 4.8. Values are means ± SEM; n is number of animals.

Collectively these results demonstrate that an increase in extracellular K^+ concentration evokes a membrane hyperpolarisation and dilatation of isolated thalamo-perforating cerebral arterioles. The most likely explanation for this would be an activation of the inward rectifier K^+ channel.

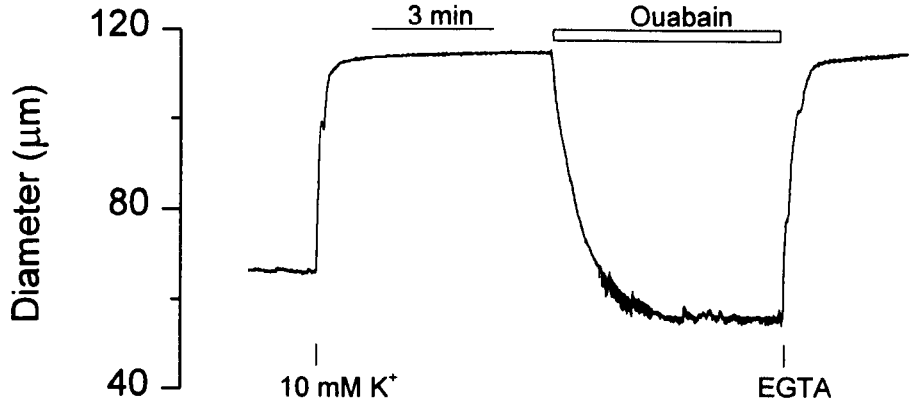


Figure 4.8 Relaxation of a single artery to 10mM K^+. After a 5 minute period ouabain ($5x10^{-5}M$) was introduced and the artery contracted. Six experiments produced a dilatation of 101 ± 1%, whereas after ouabain the diameter was –28 ± 6% ($P<0.05$).

			Change in Diameter (%)	
		n	K+ (10mM)	Drug
Barium	($5x10^{-5}$ M)	6	101 ± 1	-77 ± 18**
Cesium	($2x10^{-2}$ M)	5	99 ± 3	-4 ± 16**
Glibenclamide	($1x10^{-6}$ M)	5	100 ± 1	102 ± 1
Tetraethylammonium	($1x10^{-3}$ M)	5	101 ± 2	103 ± 2
Tetrodotoxin	($3x10^{-7}$ M)	5	100 ± 0.4	99 ± 1
Ouabain	($5x10^{-4}$ M)	3	101 ± 1	-28 ± 6*

Table 4.1 Table showing a summary of experiments described in figures 4.1 to 4.6 and figure 4.8. Values are means ± SEM; n is number of animals; *P<0.05, **P<0.01, using a two tailed paired student t-test.

4.5 Discussion

Increases in $[K^+]_o$ (5-15 mM) evokes a dilatation of blood vessels in several vascular beds. In particular a small elevation in K^+ evokes a hyperpolarisation and dilatation in the cerebral circulation (Kuschinsky *et al.*, 1972; Toda, 1974; Knot *et al.*, 1994). This K^+-evoked dilatation of the cerebral circulation has been suggested as a possible link between brain metabolism and cerebral blood flow. Consistent with this hypothesised schema the results presented here demonstrate that K^+ evokes a dilatation of a cortically penetrating cerebral arterioles (thalamo-perforating cerebral arteriole). To our knowledge this is the first direct demonstration of K^+-evoked dilatation of arterioles within the cerebral cortex.

Two mechanisms of K^+-evoked dilatation of cerebral arteries have been proposed (1) activation of the electrogenic Na^+, K^+-ATPase (Na^+-pump) or (2) activation of inward rectifier K^+ channels (K_{IR}). Early studies attributed K^+-evoked dilatations to the activation of the Na^+-pump (Chen *et al.*, 1972; Hendrickx & Casteels, 1974; Toda, 1976). In one study, small increases in $[K^+]_o$, from 0 to 5 mM, produced transient dilatations of pressurised posterior cerebral arteries, which were abolished by the Na^+ pump inhibitor ouabain but not an inhibitor of the K_{IR} channel (barium) (McCarron & Halpern, 1990). It was suggested that the transient dilatation was due to the increased $[K^+]_o$ activating the Na^+, K^+-ATPase (McCarron & Halpern, 1990). Further increases in $[K^+]_o$ (from 6 to 15 mM) produced a sustained dilatation of the cerebral

artery, however this dilatation was not reversed by ouabain, but was blocked by barium, the dilatation was attributed to the activation of the K_{IR} channel (McCarron & Halpern, 1990). Subsequent studies have supported this proposal (Knot *et al.,* 1996). A recent study examined inwardly rectifying K^+ currents and K^+-evoked dilatation of cerebral arteries from mice that had the Kir2.1 gene deleted (Zaritsky *et al.,* 2000). In the absence of the Kir2.1 gene there was no K_{IR} currents in myocytes isolated from cerebral arteries, and no K^+-evoked dilatation (both K_{IR} currents and K^+-evoked dilatation were demonstrated in myocytes isolated from cerebral arteries of control mice with no Kir2.1 gene deletion) (Zaritsky *et al.,* 2000). These results suggest that the K_{IR} channel is essential for the K^+-evoked dilatation of cerebral arteries.

In the present study elevation of K^+ (5-10 mM) produced a hyperpolarisation and relaxation of cortically penetrating cerebral arteries. Barium reversed the K^+-evoked dilatation. Barium is known not to inhibit the sodium pump (Nelson *et al.,* 1980) but at the concentration used it inhibits the K_{IR} channel (Standen & Stanfield, 1978; Quayle *et al.,* 1993b). This result is consistent with the hypothesis that increased K^+ produces a dilatation of cerebral arterioles due to an activation of K_{IR} channel. The K^+ channel inhibitor cesium also reversed the K^+-evoked dilatation. Cesium inhibits K_{IR} channels (Quayle *et al.,* 1993) providing further support for the involvement of K_{IR} channels. In contrast inhibitors of the ATP sensitive K^+ channel (K_{ATP}) and the Ca^{2+}-activated K^+ channel (K_{Ca}) (glibenclamide and TEA respectively) did not reverse the K^+-evoked dilatation.

It may be possible that increased extracellular K^+ concentration depolarises nerves to release inhibitory neurotransmitters. This seems unlikely because of the scant sympathetic innervation of rat cerebral arterioles (Hirst *et al.*, 1986; Hill *et al.*, 1986) and also because the neurotoxin tetrodotoxin (TTX) failed to reverse the K^+-evoked dilatation. Although the role of the endothelium in the K^+-evoked dilatation was not examined it would seem unlikely to play any part since studies have demonstrated that endothelium removal had no effect upon the K^+-evoked dilatation (McCarron & Halpern, 1990; Knot *et al.*, 1994).

An uneven distribution of ion channels in the cerebral circulation has been described (Nelson *et al.*, 1991). Edwards *et al.*, (1988) demonstrated that the distribution of K_{IR} channels is not uniform along the cerebral circulation, being absent in large cerebral arteries but present in small cerebral arteries (Edwards *et al.*, 1988). In a more recent study it has been suggested that K_{IR} channels may in fact also be absent again from the cortically penetrating cerebral arterioles (Quinn & Beech, 1998). The results presented here suggest that the K_{IR} is present in cortically penetrating arterioles. The differences in findings may be attributed to species (rabbit vs. rat) or the methodology. For example, the cerebral arteriole isolation procedure, used by Quinn & Beech, involved a combination of enzymatic digestion and mechanical agitation, a process that may alter the normal physiology of the arteriole.

The role of the Na^+, K^+-ATPase in the K^+-evoked dilatation was examined by utilizing the Na^+, K^+-ATPase inhibitor ouabain. It was found that high concentrations of ouabain reversed the K^+-evoked dilatation. This result would be consistent with the involvement of the sodium pump in the K^+-evoked dilatation. However, ouabain has been demonstrated to depolarise vascular smooth muscle (Hirst & van Helden, 1982) and since K_{IR} channels are steeply voltage dependent, closing on depolarisation (Edwards et al., 1988), it is possible that ouabain may be producing and a reversal of the K^+-evoked dilatation indirectly via a depolarisation of the smooth muscle cell and a closure of K_{IR} channels.

In conclusion, the results presented demonstrate that increased extracellular K^+ concentration from 4.7 mM to 10 mM produces a dilatation of rat isolated thalamo-penetrating arterioles. In addition, the K^+-evoked dilatation may be due to an activation of K_{IR} channels resulting in a smooth muscle cell hyperpolarisation. It is plausible, therefore, that K^+ acts as a mediator in the regulation of local cerebral blood flow in response to variations in neuronal metabolic activity.

Chapter 5

Protein Kinase C-Evoked Dilatation of Rat Isolated Cerebral Resistance Arteries

5.1 Summary

1. An increase of $[Ca^{2+}]_o$ to approximately 700 nM evoked a concentration-dependent constriction of isolated permeabilised cerebral resistance arteries. With a maximal constriction of 30 ± 3% (n = 28).

2. Under $[Ca^{2+}]_i$ clamped conditions the putative PKC activator indolactam evoked a 20 ± 2% (n = 27) constriction of the artery.

3. The PKC inhibitor ($PKC_{(19-36)}$; 1 μM) produced a near maximal (85 ± 4 %; n = 6) reversal of the indolactam-evoked constriction of the artery, confirming that the indolactam-evoked constriction was due to an activation of PKC.

4. $PKC_{(19-36)}$ (1 μM) produced only a minor (12 ± 3 %; n = 6) reversal of the Ca^{2+}-induced constriction.

5. The MLCK antagonist SM-1 (100 μM) reversed both the Ca^{2+}- and the indolactam- evoked constriction of the artery.

6. The calmodulin antagonist RS-20 (0.1 - 100 μM) dose-dependently reversed the Ca^{2+}-evoked constriction but even up to 300 μM did not reverse the indolactam evoked-constriction of the artery.

7. It is concluded that MLCK but not calmodulin plays a role in the PKC-evoked smooth muscle contraction.

5.2 Introduction

The major mechanism for the regulation of smooth muscle contraction is myosin light chain phosphorylation (Hartshorne, 1987). When intracellular Ca^{2+} is increased, Ca^{2+} binds to calmodulin (CaM) which in turn binds to the enzyme myosin light chain kinase (MLCK), forming a Ca^{2+}-CaM-MLCK complex, the active form of MLCK. The Ca^{2+}-CaM-MLCK complex phosphorylates the 20-kDa regulatory light chain subunit of myosin at serine 19 and threonine 18 (Ikebe, *et al.*, 1986), producing an increase in the actin-activated MgATPase activity of myosin (Ikebe & Hartshorne, 1985). This increase in the MgATPase activity of myosin results in the cyclic interaction of myosin in thick filaments with actin in thin filaments resulting in a contraction of the muscle (Hartshorne, 1987). Upon a reduction in intracellular Ca^{2+}, there is an inactivation of the Ca^{2+}-CaM-MLCK complex through a dissociation of CaM, myosin regulatory light chain is subsequently dephosphorylated by a myosin light chain phosphatase (type 1 protein phosphatase, MLCP) and muscle relaxation occurs.

Although Ca^{2+}-dependent phosphorylation of myosin light chain is well accepted as the main activation pathway of smooth muscle contraction there is accumulating evidence for a secondary pathway (Lee & Severson, 1994). This had previously been suggested due to results demonstrating that agonist-induced contractions do not always correlate well with myosin light chain phosphorylation (Jiang & Morgan, 1987, Wagner & Ruegg, 1986) and with

little or no change in the intracellular Ca^{2+} concentration (Kamm & Stull, 1989, Bradley & Morgan, 1987). The agonist phenylephrine, for example, has been shown to produce smooth muscle contraction at constant and low-intracellular free Ca^{2+} concentrations. This contraction was inhibited by a protein kinase C (PKC) pseudosubstrate inhibitor peptide (corresponding to residues 19-31 of PKC) thus suggesting that the phenylephrine-induced contraction involves PKC activation (Collins *et al.*, 1992). It has since been shown in vascular smooth muscle, that a constitutively active form of PKC produced a Ca^{2+}-independent contraction of a magnitude similar to that produced by phenylephrine (Horowitz *et al.*, 1996a). These results suggest that a Ca^{2+}-independent pathway of smooth muscle contraction involving PKC activation may exist.

However, the mechanism of PKC-evoked regulation of smooth muscle contraction remains unresolved but a number of possibilities have been suggested. First there may be a direct activation of MLCK or an inhibition of MLCP (Stull *et al.*, 1993; Somlyo & Somlyo, 1994) thus affecting the extent of myosin regulatory light chain phosphorylation, and resulting level of contraction. Secondly, there may be a direct phosphorylation of the myosin regulatory light chain, independent of MLCK activation (Singer *et al.*, 1989). Finally there may be a relief of the contractile inhibition by the thin filament-associated regulatory proteins caldesmon and calponin. It has been shown that both caldesmon (Adam *et al.*, 1989) and calponin (Winder & Walsh, 1990) can be phosphorylated *in vitro* by PKC, and once phosphorylated they no longer

inhibit actomyosin ATPase resulting in smooth muscle contraction (Winder & Walsh, 1990; Ikebe & Hornick, 1991).

MLCK and many other protein kinases are autoregulated by an amino acid sequence present within the kinase molecules themselves (see reviews; Hardie, 1988; Lukas *et al.*, 1988). In MLCK the autoinhibtory region connects the catalytic core to the calmodulin-binding domain. This autoinhibitory domain of MLCK folds back onto the catalytic core blocking substrate binding (Kemp *et al.*, 1994). Activation of MLCK, upon Ca^{2+}-calmodulin binding, displaces the regulatory region allowing substrate binding. Synthetic peptides based on the autoinhibitory domain have been used as selective blockers of MLCK and calmodulin. The first, SM-1, corresponds to amino acid residues 480 to 501 of the autoinhibitory domain of MLCK. The second, RS-20, corresponds to amino acid residues 493 to 512 of the Ca^{2+}-calmodulin binding domain of MLCK (Kemp *et al.*, 1987, Lukas *et al.*, 1986).

In the present study SM-1 and RS-20 were used to determine the role that calmodulin and MLCK play in PKC-evoked contraction of vascular smooth muscle. If PKC produced a direct activation of MLCK or an inhibition of MLCP, thus affecting the extent of myosin regulatory light chain phosphorylation. The resulting contraction should be blocked by the peptide SM-1 but not RS-20. If however, PKC-evoked contraction were via a direct phosphorylation of myosin regulatory light chain neither SM-1 nor RS-20 would have any effect upon contraction.

5.3 Methods

5.3.1 Dissection of arteries

Male, Wistar-Kyoto rats, aged between 11 and 20 weeks were killed by a sodium pentobarbitone overdose (150mgkg^{-1} Inter-peritoneal). The rat was decapitated using a guillotine. The brain was carefully removed from the skull and second order branches of the posterior cerebral artery were carefully dissected from the pial membrane (figure 2.1). An artery segment (2-3mm) was removed and transferred for cannulation (see section 2.1.1).

5.3.2 Cannulation and Pressure-Perfusion Myograph

Second order cerebral arteries were cannulated and set up in a small volume (3ml) vessel chamber of a Halpern pressure-perfusion myograph containing a "10 Relaxing" bathing solution (room temperature 20 ± 2°C, pH 7.2).

5.3.3 Permeabilised arteries

In a "10 Relaxing" bathing solution (room temperature 20 ± 2°C, pH 7.2) at a pressure of 60mmHg, arteries were permeabilised using β-Escin (100μM) for ~2 minutes. Free [Ca^{2+}] in the bathing solution was controlled by mixing together, in varying ratios, the "10 activating" and "10 relaxing" solutions. The "0.2 relaxing" solution was used as the luminal solution. The lower buffering

capacity of this solution relative to the bath solution ensured the saline bathing the outside of the artery dictated the $[Ca^{2+}]$ within the wall and lumen of the permeabilised artery (permeabilisation procedure and all solutions used have been described previously in section 2.3)

5.4 Results

Rat isolated cerebral resistance arteries were permeabilised using ß-escin (100μM), allowing the Ca^{2+} present in the extracellular bathing solution to dictate the intracellular Ca^{2+} concentration. In a Ca^{2+} free solution, and pressurised to 60mmHg using the pressure myograph system, the permeabilised arteries had a fully relaxed diameter of 203 ± 3μm (n=55). When external Ca^{2+} was raised to approximately 700nM a concentration dependent constriction of the artery was produced resulting in a 30 ± 3% (n=28) reduction in diameter (figure 5.1A). Under $[Ca^{2+}]_i$-clamped conditions the addition of the putative protein kinase C (PKC) activator indolactam (10μM), evoked a 20 ± 2% (n=27) constriction of the artery (figure 5.1B). This constriction presumably occurs via an increase in myofilament Ca^{2+} sensitivity.

To ensure that the indolactam-induced constriction of the cerebral resistance artery was produced via activation of PKC the effect the selective inhibitor, $PKC_{(19-36)}$, was examined. $PKC_{(19-36)}$ corresponds to residues 19-36 of the regulatory domain of the PKC family and so overlies and inhibits the catalytic

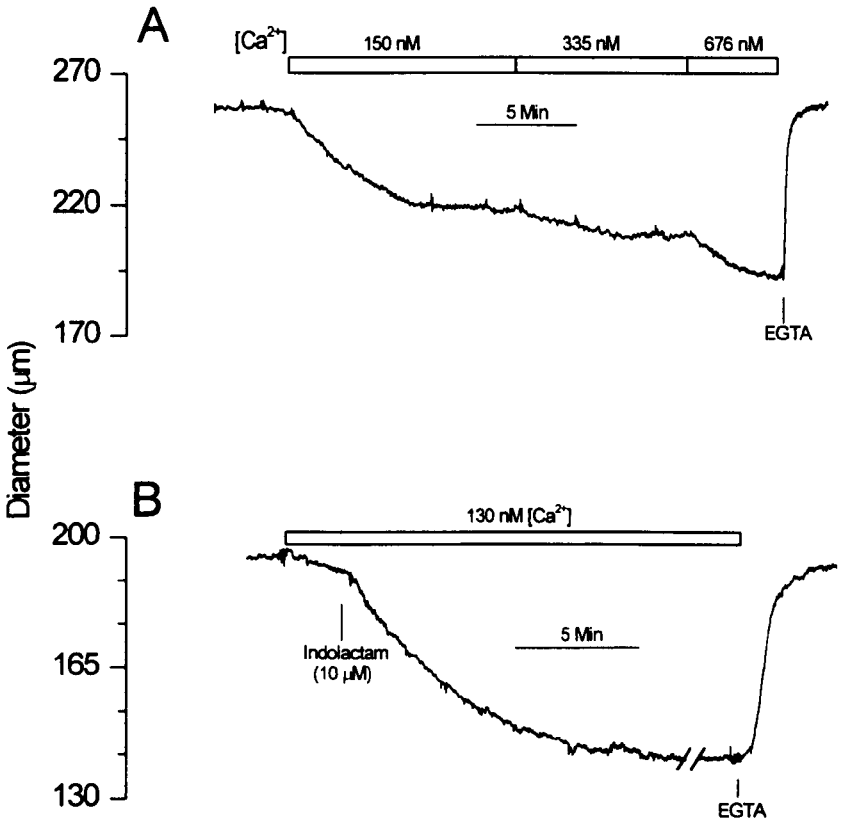


Figure 5.1 (A) Typical recording showing a concentration dependent constriction of rat permeabilised cerebral resistance artery to an increasing Ca^{2+} concentration (150 to 676 nM). (B) The protein kinase C stimulator indolactam (10 µM), under $[Ca^{2+}]_i$-clamped conditions, produced a constriction of rat permeabilised cerebral resistance arteries.

site of the kinase (House & Kemp, 1987). A concentration dependent reversal of the Ca^{2+}-induced constriction was evoked by $PKC_{(19-36)}$ but only at high concentrations (100 μM; figure 5.2A). Summarised concentration response relations displaying the ability of $PKC_{(19-36)}$ to only partially reverse the Ca^{2+}-induced constriction are shown in figure 5.2C, with a maximum reversal of 45 ± 12% ($P<0.05$, n=6) achieved at 100μM. However $PKC_{(19-36)}$ at a much lower concentration almost fully reversed the indolactam-induced constriction (85 ± 4 % at 1μM; figure 5.1B). Summarised data is presented in figure 5.2D, with a maximum reversal of 85 ± 4% ($P<0.01$, n=6) achieved at 1μM. This value is consistent with the suggested affinity of $PKC_{(19-36)}$ for PKC (IC_{50} 0.3μM, Smith *et al.*, 1990). These results suggest that the indolactam-induced constriction of the cerebral resistance artery occurred via an activation of PKC. Some of the possible mechanisms by which PKC may evoke a constriction at a fixed Ca^{2+} may include: 1) Direct activation of myosin light chain kinase (MLCK) or an inhibition of light chain phosphatase (Stull *et al.*, 1993; Somlyo & Somlyo, 1994). 2) a relief of the contractile inhibition by the thin filament associated proteins caldesmon (Adam et al., 1989) and calponin (Winder & Walsh, 1990) or 3) a direct phosphorylation of myosin (Singer *et al.*, 1989).

The cerebral artery, after beta-escin permeabilisation, was contracted by 24% with 506nM Ca^{2+}. SM-1 produced a concentration-dependent reversal of the Ca^{2+}-induced constriction of the artery (figure 5.3A). Summarised

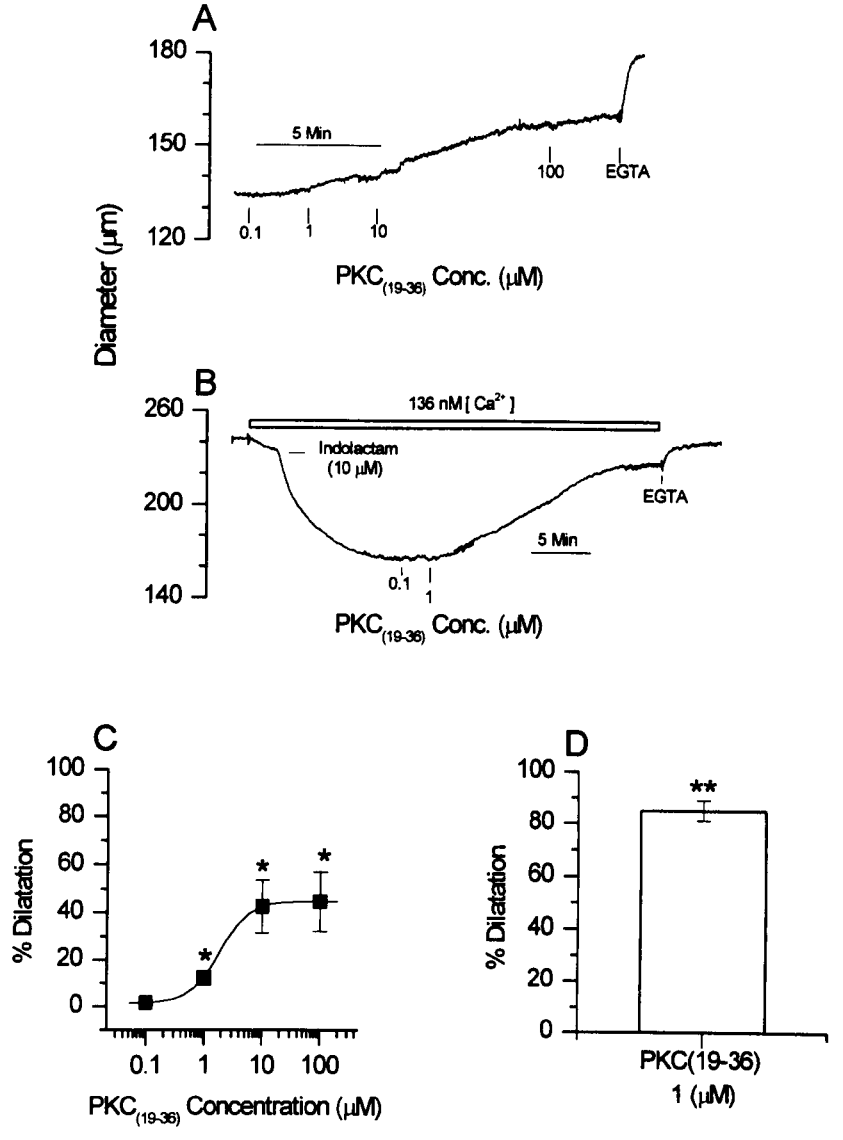


Figure 5.2 (A) Concentration dependent reversal of a Ca^{2+}-induced constriction of the rat permeabilised cerebral resistance artery to increasing concentrations of the protein kinase C inhibitor $PKC_{(19-36)}$ (0.1µM to 100µM). (B) Ability of the protein kinase C inhibitor (0.1 and 1 µM) to reverse an indolactam (10 µM) induced constriction of the rat permeabilised cerebral resistance artery, under $[Ca^{2+}]_i$-clamped conditions. (C) Summarised concentration response relation, showing the ability of the protein kinase C inhibitor to partially reverse the Ca^{2+}-induced constriction of the rat permeabilised cerebral resistance artery. The maximum reversal achieved was 45 ± 12% at 100µM (*P<0.05, n=6). (D) Summarised data showing the ability of the protein kinase C inhibitor (1 µM) to almost completely reverse the indolactam (10 µM) induced constriction under $[Ca^{2+}]_i$-clamped conditions (85 ± 4%, **P<0.01, n=6).

concentration response relations are shown in figure 5.3C, illustrating the ability of SM-1 to almost completely reverse the Ca^{2+}-induced constriction of the artery, with an IC_{50} = 1 ± 0.4μM and a maximum reversal of 88 ± 5%, ($P<0.01$, n=5), achieved at 100μM. SM-1 (100μM) also reversed the PKC induced constriction of the rat permeabilised cerebral resistance artery (figure 5.3B). After permeabilisation the [Ca^{2+}] was increased to 166nM and after which indolactam was introduced. Indolactam (1μM) evoked a 21 ± 5% constriction of the artery (n=5). SM-1 (100μM) reversed this constriction by 71 ± 2% ($P<0.01$, n=5). Summarised data is presented in figure 5.3D.

The role of the calmodulin in both Ca^{2+} and PKC induced constriction was next examined using RS-20. The cerebral artery after being permeabilised was contracted by 22% with 506nM Ca^{2+}. RS-20 produced a concentration dependent reversal of the Ca^{2+}-induced constriction of artery (figure 5.4A). Summarised concentration response relations are shown in figure 5.4C, illustrating the ability of RS-20 to almost completely reverse the Ca^{2+}-induced constriction of the artery, with an IC_{50} = 5 ± 3μM, and a maximum reversal of 68 ± 9%, ($P<0.01$, n=5), achieved at 100 μM. However, in contrast, RS-20 (300μM) had little effect on the PKC induced constriction of the rat permeabilised cerebral resistance artery (figure 5.4B), with a maximum reversal of only 21 ± 13% (n=5) achieved at 300 μM. 100μM RS-20 was also investigated but this too had little effect on the PKC induced constriction

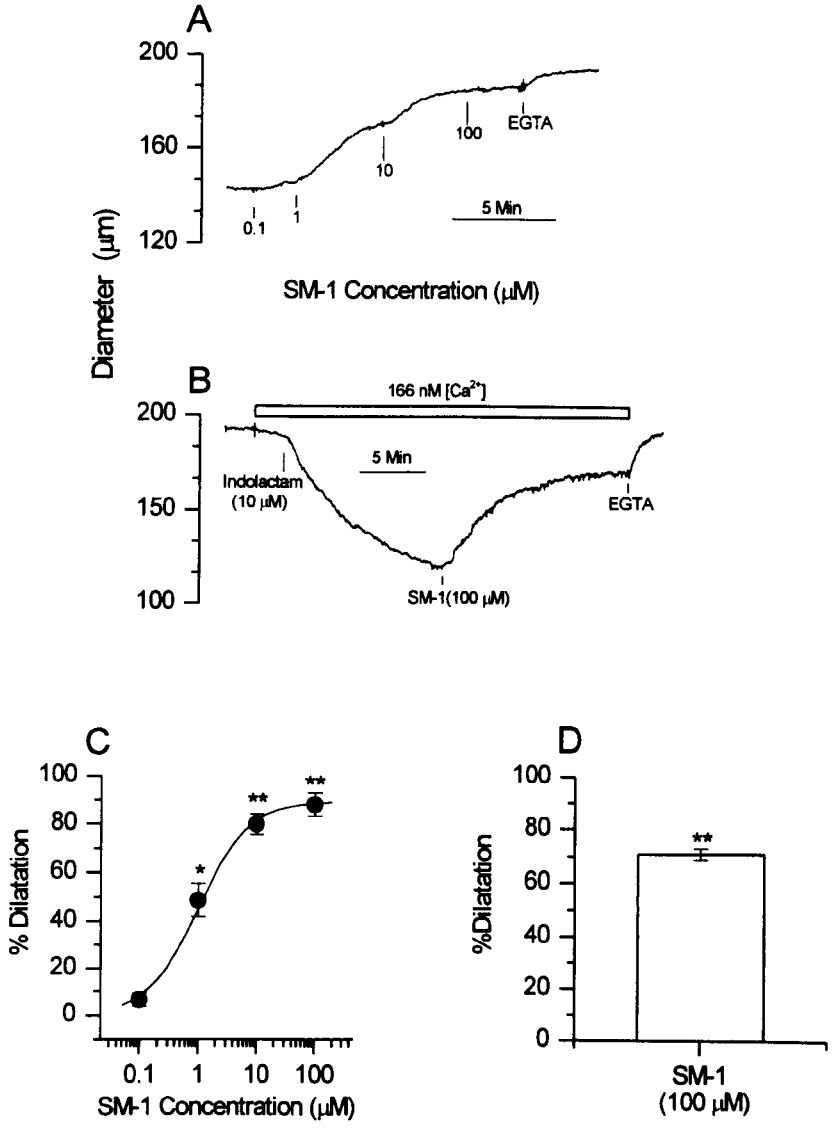


Figure 5.3 (A) Concentration dependent reversal of a Ca^{2+}-induced constriction of the rat permeabilised cerebral resistance artery to increasing concentrations of the myosin light chain kinase antagonist SM-1 (0.1μM to 100μM). (B) Ability of SM-1 (100μM) to reverse an indolactam (10μM) induced constriction of the rat permeabilised cerebral resistance artery, under $[Ca^{2+}]_i$-clamped conditions. (C) Summarised concentration response relation, showing the ability of SM-1 to almost completely reverse the Ca^{2+}-induced constriction of the rat permeabilised cerebral resistance artery. The maximum reversal achieved was 88 ± 5% at 100μM, IC_{50} = 1 ± 0.4μM (**P<0.01, n=5). (D) Summarised data showing the ability of SM-1 (100μM) to almost completely reverse the indolactam (10 μM) induced constriction under $[Ca^{2+}]_i$-clamped conditions (71 ± 2%, **P<0.01, n=5).

producing a maximal reversal of only 23 ± 13% (n=6), data not shown.

Summarised data is presented in figure 5.4D.

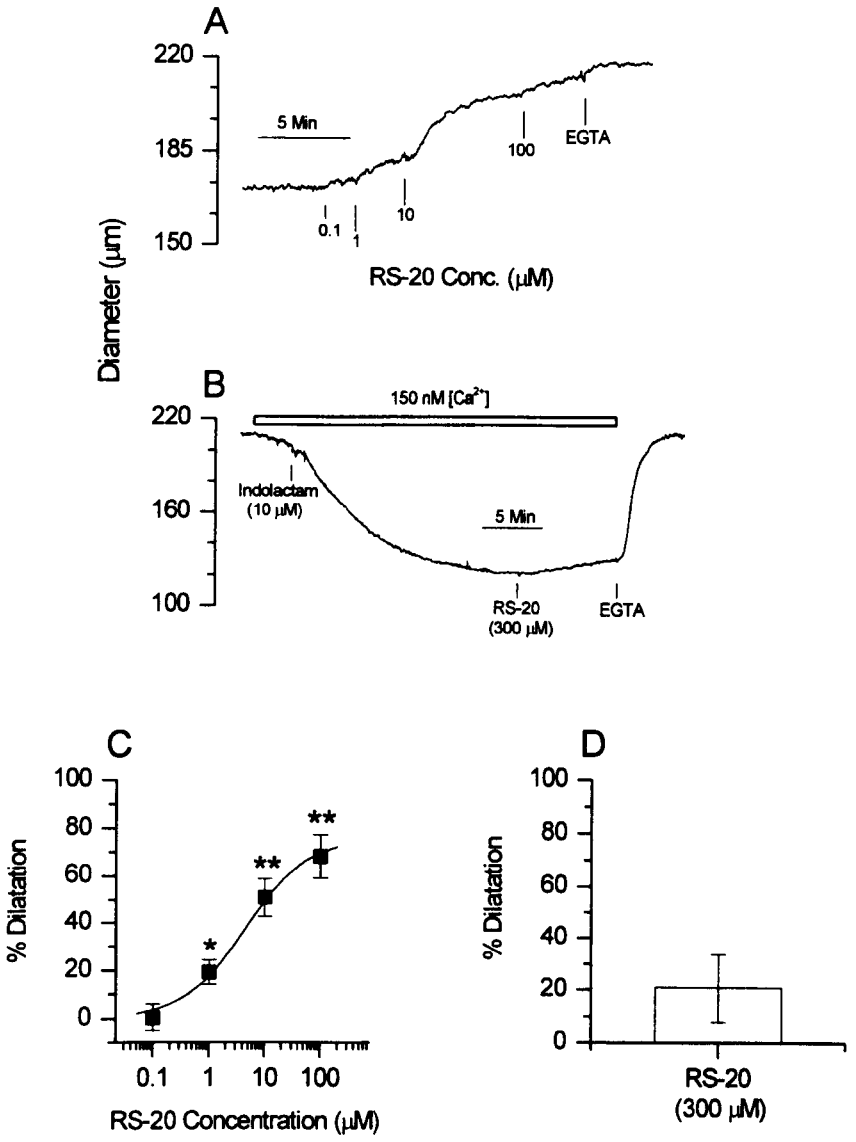


Figure 5.4 (A) Concentration dependent reversal of a Ca^{2+}-induced constriction of the rat permeabilised cerebral resistance artery to increasing concentrations of the calmodulin binding antagonist RS-20 (0.1µM to 100µM). (B) In contrast to SM-1, RS-20 (300µM) produced only a very small reversal of the indolactam (10µM) induced constriction of the rat permeabilised cerebral resistance artery, under $[Ca^{2+}]_i$-clamped conditions. (C) Summarised concentration response relation, showing the ability of RS-20 to reverse the Ca^{2+}-induced constriction of the rat permeabilised cerebral resistance artery. The maximum reversal achieved was 68 ± 9% at 100µM, IC_{50} = 5 ± 3µM (**P<0.01, n=5). (D) Summarised data showing that RS-20 (300µM) produced only a small reversal of the indolactam (10 µM) induced constriction under $[Ca^{2+}]_i$-clamped conditions (23 ± 13%, n=5).

5.5 Discussion

In this study the effect of peptide antagonists of myosin light chain kinase and calmodulin were examined upon both Ca^{2+}- and protein kinase C- evoked contraction of rat permeabilised cerebral resistance arteries. Initial findings demonstrated that increased Ca^{2+} concentration and PKC activation each produced a contraction of the permeabilised cerebral artery. The MLCK antagonist SM-1 and the calmodulin antagonist RS-20 each inhibited the Ca^{2+}-evoked contraction but only SM-1 inhibited the PKC-evoked contraction. These results suggest that MLCK but not calmodulin plays an obligatory role in PKC-evoked smooth muscle contraction.

Ca^{2+}-dependent phosphorylation of myosin light chain is the major activation pathway of smooth muscle contraction (Hartshorne, 1987). However, a secondary pathway independent of myosin light chain phosphorylation due to increased $[Ca^{2+}]_i$ may exist (Lee & Severson, 1994). For example, a number of studies have demonstrated that agonist-induced contractions do not always correlate with myosin light chain phosphorylation (Jiang & Morgan, 1987; Wagner & Ruegg, 1986) and with little or no change in the intracellular Ca^{2+} concentration (Kamm & Stull, 1989; Bradley & Morgan, 1987). Other studies have demonstrated that agonist-induced contractions at low intracellular Ca^{2+} concentrations can be prevented by the inhibition of PKC (Collins *et al.*, 1992) and that constitutively active forms of PKC produce Ca^{2+}-independent contraction of smooth muscle (Horowitz *et al.*, 1996a).

There may be a number of possible mechanisms for PKC-evoked contraction. Firstly it has been suggested that a direct phosphorylation of myosin light chain (MLC) may be responsible. PKC can produce a direct phosphorylation of $Ser^{1,2}$ and Thr^{9} of myosin light chains *in vitro* (Singer *et al.*, 1989; Sutton & Haeberle, 1990). However, phosphorylation of MLC at these residues was associated with a decrease, rather than an increase, in actomyosin ATPase activity (Ikebe *et al.*, 1987a). Furthermore, agonist activation of smooth muscle resulted in the phosphorylation of MLC at the consensus sequences for MLCK, and not the consensus sequences for PKC (Singer *et al.*, 1989; Kamm *et al.*, 1989). Secondly, it has been proposed that PKC-evoked contraction may occur via a direct phosphorylation and activation of MLCK (Stull et al., 1993; Somlyo & Somlyo, 1994). However, this proposal has also been questioned. Phosphorylation of MLCK by PKC decreases the Ca^{2+} sensitivity of MLCK (Stull *et al.*, 1990; Van Riper *et al.*, 1995).

Another possibility is that PKC may phosphorylate MLCP decreasing its activity. Indeed, prostaglandin $F_{2\alpha}$ evoked a Ca^{2+}-independent contraction in ferret permeabilised aorta cells as a result of PKC activation and subsequent phosphatase inhibition (Katsuyama & Morgan, 1993). Similarly, phorbol ester-evoked Ca^{2+}-independent phosphorylation of MLC, and contraction in aortic strips, may also have been the result of an inhibition of MLCP activity (Itoh *et al.*, 1993).

Caldesmon (CaD) and calponin (CaP) are thin filament-associated proteins that inhibit the actomyosin ATPase activity. Phosphorylation of CaD or CaP relieves their contractile inhibition. Both caldesmon (Adam et al., 1989) and calponin (Winder & Walsh, 1990) are phosphorylated *in vitro* by PKC, which removes the inhibition of actomyosin ATPase resulting in muscle contraction (Sobue & Sellers, 1991; Winder & Walsh, 1993).

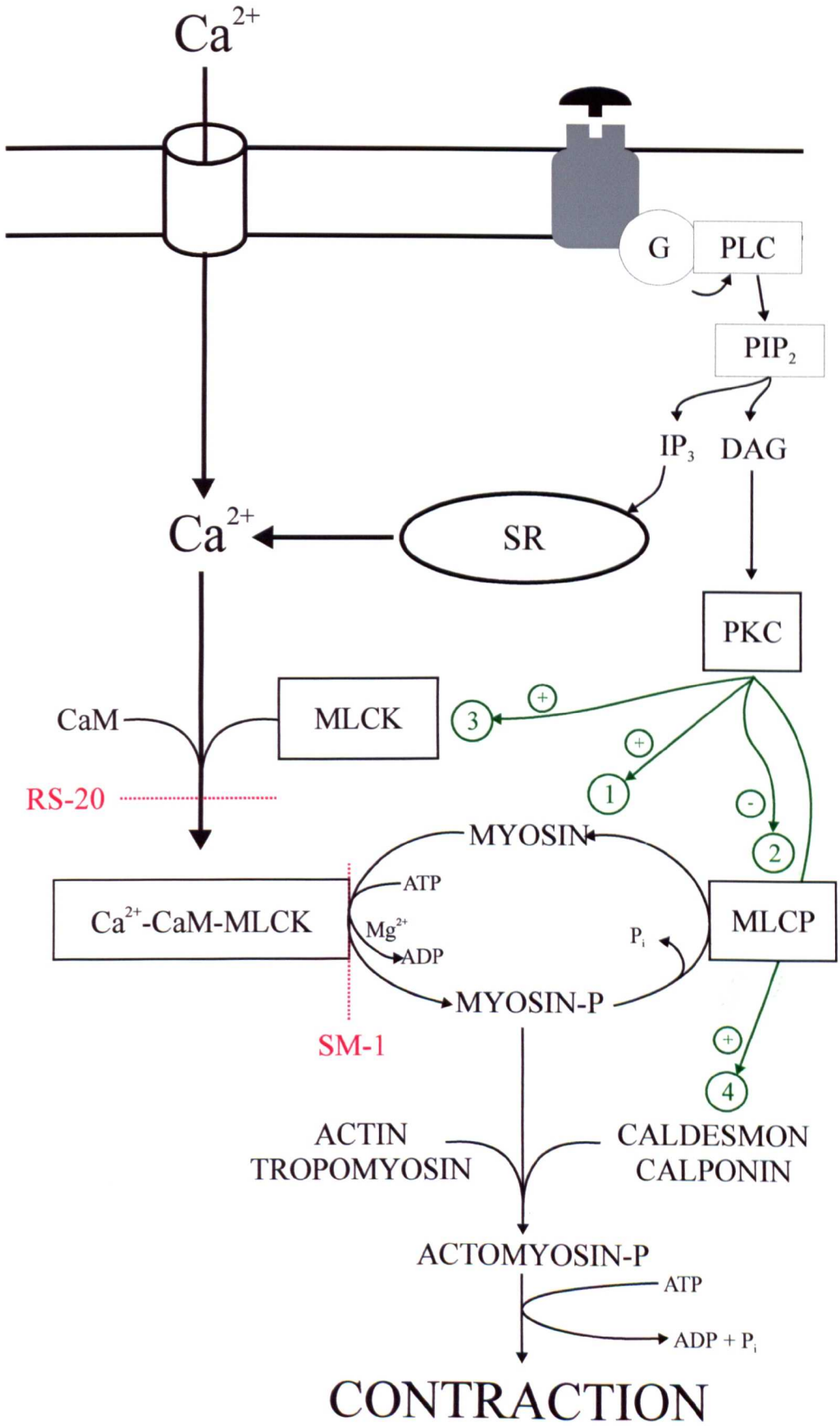
To examine the possible mechanisms of PKC-evoked contraction in the present study, isolated rat cerebral arteries were chemically permeabilised using β-escin, and the effect of the calmodulin and MLCK inhibitors, RS-20 and SM-1 respectively, were examined. Indolactam was used as the PKC activator - its effects were fully reversed by the PKC inhibitor $PKC_{(19-36)}$. High concentrations of $PKC_{(19-36)}$ produced only a moderate inhibition of the Ca^{2+}-evoked contraction. One possible explanation for this inhibition is that $PKC_{(19-36)}$ is not solely selective for PKC and could be acting via an inhibition of MLCK at a high concentrations (100 μM; House & Kemp, 1987; Smith *et al.,* 1990). The inhibition of Ca^{2+}-evoked contraction required concentrations more than 100 times higher than that required to inhibit PKC.

SM-1 and RS-20 each demonstrated a concentration-dependent reversal of the Ca^{2+}-evoked contraction, confirming the ability of both these peptides to enter the permeabilised cells of the artery. SM-1 (100μM) produced an 88% reversal of Ca^{2+}-activated contraction, a value consistent with studies by Itoh *et al.,*

(1989). In the latter study an 84% reversal of Ca^{2+}-induced shortening of permeabilised toad stomach smooth muscle cells was reported.

The MLCK inhibitor SM-1 but not the calmodulin inhibitor RS-20 reversed the PKC-evoked constriction. Therefore, MLCK but not calmodulin may contribute to PKC-evoked smooth muscle contraction. These results would point to either a phosphorylation and activation of MLCK as the major mechanism of PKC-evoked contraction (but see Stull *et al.*, 1990; Van Riper *et al.*, 1995) or possibly a phosphorylation and inhibition of MLCP by PKC. However, SM-1, while fully reversing the Ca^{2+}-induced constriction, did not fully reverse the PKC-evoked constriction of the cerebral artery. This result suggests that an SM-1 resistant mechanism, working in parallel with MLCK, may also contribute to the PKC-evoked contraction. This mechanism may include a direct phosphorylation and activation of MLC or a direct phosphorylation of the inhibitory, thin filament associated proteins CaD and CaP. As illustrated in figure 5.5 each of these pathways would be unaffected by either RS-20 of SM-1.

In summary, the results presented suggest that MLCK but not CaM plays a major role in the PKC-evoked contraction of cerebral resistance arteries. Furthermore the SM-1 resistant component of the PKC-evoked contraction may involve a direct phosphorylation of either MLC, or the inhibitory thin filament-associated proteins CaD or CaP.


Ca2+
G
PLC
PIP2
IP3
DAG
Ca2+
SR
PKC
CaM
MLCK
3
+
+
1
-
2
RS-20
MYOSIN
ATP
Ca2+-CaM-MLCK
Mg2+
ADP
Pi
MLCP
MYOSIN-P
SM-1
+
4
ACTIN
TROPOMYOSIN
CALDESMON
CALPONIN
ACTOMYOSIN-P
ATP
ADP + Pi
CONTRACTION

Chapter 6

General Discussion

Arterioles and small arteries with diameters of less than 500 μm play an important role in maintaining the vascular peripheral resistance (Mulvany & Aalkjaer, 1990) and hence the regulation of blood flow. These blood vessels respond to increases and decreases in intravascular pressure with a constriction and dilatation respectively; a process known as the myogenic response (Johansson, 1989). The myogenic response is accepted as being important in the maintenance of constant blood flow regardless of changes in blood pressure. However, many aspects of the control of the response remain unclear. The main objectives of this thesis were directed towards an understanding of the effect of the metabolic agent's adenosine and K^+ as well as second messenger systems in the control of myogenic tone in rat cerebral and mesenteric resistance arteries.

In chapter 3, it was reported that rat isolated cerebral and mesenteric arteries developed myogenic tone when pressurised. Adenosine dilated mesenteric but not cerebral arteries. Adenosine may act as a metabolic vasodilator in the mesenteric circulation, in agreement with other studies (reviewed by Jacobson & Pawlik, 1994). In contrast, adenosine did not dilate cerebral arteries a finding which challenges the view that it acts as a metabolic vasodilator in the cerebral circulation (Morii *et al.*, 1987; Simpson & Phillis, 1991; Coney & Marshall, 1998).

Adenosine-evoked dilatation of the mesenteric artery was independent of the endothelium, K^+ channel activation or a change in membrane potential.

Decreased myofilament Ca^{2+} sensitivity may contribute to adenosine-evoked dilatation of mesenteric arteries. Future studies may be directed towards the possible second messenger systems activated by adenosine, which may contribute to the decreased myofilament Ca^{2+} sensitivity. In particular, cAMP-dependent kinase, PKC and Ca^{2+}-calmodulin dependent kinase II, have each been demonstrated to produce a phosphorylation of MLCK (Conti & Adelstein, 1981; Nishikawa *et al.*, 1984; Hashimoto & Soderling, 1990; Ikebe & Reardon, 1990), decreasing its Ca^{2+}/CaM sensitivity (Tansey, 1994). Furthermore, cAMP can activate cGMP-dependent kinase (PKG; Francis *et al.*, 1988), resulting in a decrease in myosin phosphorylation and contractile force (Pfitzer & Boels, 1991). In addition to a decrease in the Ca^{2+} sensitivity, cAMP and cGMP have many possible sites of action by which they could regulate intracellular Ca^{2+} homeostasis, in non-permeabilised vascular smooth muscle (reviewed Lincoln *et al.*, 1996). Investigation into other regulatory sites could provide interesting areas at which future research may be directed.

The role of the putative metabolic vasodilator K^+ in the cerebral circulation was investigated in chapter 4. Isolated cortically penetrating cerebral arterioles were used, to our knowledge, for the first time. Several studies have demonstrated that increased extracellular K^+ concentration ($[K^+]_o$) evokes a dilatation of rat cerebral arteries, and that this dilatation is mediated via inward rectifier K^+ channel (K_{IR}) activation (Edwards *et al.*, 1988; McCarron & Halpern, 1990; Knot, Zimmerman & Nelson, 1994). Recently, it has been suggested that K_{IR} may be absent from cortically penetrating cerebral arteries (Quinn & Beech,

1998). In contrast, the results presented in chapter 4, established that increased $[K^+]_o$ evoked a dilatation of the rat thalamo-perforating artery. The K^+-evoked dilatation was most likely mediated via an activation of K_{IR} channels. During normal physiological neuronal activity, ischaemia, hypoxia or hypoglycemia $[K^+]_o$ increases (Somjen, 1979; Sieber et al., 1993). It is plausible, therefore, that K^+ and not adenosine link metabolic activity and blood flow within the rat cerebral circulation. An extension of these studies may be directed towards an understanding of the whole cell and single channel currents activated by an elevation in K^+. In particular the regulation of the gating of the channel by external K^+ as well as internal Mg^{2+} and polyamines could be examined.

In chapter 5, the regulation of myogenic tone by second messenger systems was examined. Agonist-induced contractions do not always correlate with myosin light chain phosphorylation (Wagner & Ruegg, 1986; Jiang & Morgan, 1987) or with increased intracellular Ca^{2+} (Bradley & Morgan, 1987; Kamm & Stull, 1989). It has been suggested that the second messenger enzyme protein kinase C (PKC) may be involved in these contractions (Collins *et al.*, 1992; Horowitz *et al.*, 1996a). In chapter 5 a PKC-evoked a contraction of the rat permeabilised cerebral artery under $[Ca^{2+}]_i$ clamped conditions was demonstrated.

Two specific kinase inhibitors were used to elucidate the mechanism of action of PKC-evoked contraction of the rat permeabilised cerebral artery. The first, SM-1, is based on the inhibitory region of myosin light chain kinase (MLCK)

and inhibits the action of MLCK (Ikebe *et al.*, 1987b; Kemp *et al.*, 1987; Pearson *et al.*, 1988). The second, RS-20, is based on the calmodulin binding site of MLCK and prevents calmodulin binding to MLCK (Lukas, *et al.*, 1986; Ikebe *et al.*, 1988). SM-1, but not RS-20, reversed the PKC-evoked contraction. The PKC-evoked contraction, may therefore involve an activation of MLCK, but not calmodulin. However, SM-1 did not fully reverse the PKC-evoked contraction, although it did completely block Ca^{2+}-evoked contraction. It is possible, therefore, that PKC may act via several mechanisms, which could include direct phosphorylation of myosin light chain or the inhibitory thin filament-associated proteins, caldesmon (CaD) or calponin (CaP). As illustrated in figure 5.5, these pathways would be unaffected by either SM-1 or RS-20. Future studies could be directed towards the examination of these possible sites of PKC regulation. For example, PKC can evoke a Ca^{2+}-independent contraction of vascular smooth muscle cells, and CaP is phosphorylated by PKC *in vitro* (Horowitz *et al.*, 1996a). Furthermore, exogenously applied CaP significantly reduced, or completely inhibited, a Ca^{2+}-independent, PKC-evoked contraction of isolated vascular smooth muscle cells (Horowitz *et al.*, 1996b). It was suggested that endogenous CaP, phosphorylated by PKC, translocates to the cell membrane. Exogenous CaP occupied the vacant binding sites, thus maintaining an inhibition of smooth muscle contraction (Horowitz *et al.*, 1996b). One area of future study, therefore, would be to examine the effect of exogenously applied CaP or CaD upon the SM-1 resistant contraction, with an aim to determine the role, if any,

of thin filament associated proteins CaD and CaP in PKC-evoked smooth muscle contraction.

Chapter 7

References

ADAM, L. P., HAEBERLE, J. R. & HATHAWAY, D. R. (1989). Phosphorylation of caldesmon in arterial smooth muscle. *J. Biol. Chem.* **264**, 7698-7703.

AKATSUKA, Y., EGASHIRA, K., KATSUDA, Y., NARISHIGE, T., UENO, H., SHIMOKAWA, H. & TAKESHITA, A. (1994). ATP sensitive potassium channels are involved in adenosine A_2 receptor mediated coronary vasodilatation in the dog. *Cardiovascular Research* **28**, 906-911.

ANREP, G. (1912). On local vascular reactions of the arterial wall to changes of internal pressure. *Journal of Physiology* **45**, 318-327.

ASHCROFT, F. M. (1988). Adenosine 5'-triphosphate-sensitive potassium channels. *Annu.Rev.Neurosci.* **11**, 97-118.

BAE, Y. M., PARK, M. K., LEE, S. H., HO, W. K. & EARM, Y. E. (1999). Contribution of Ca^{2+}-activated K^+ channels and non-selective cation channels to membrane potential of pulmonary arterial smooth muscle cells of the rabbit. *Journal of Physiology* **514**, 747-758.

BAYLISS, W. M. (1902). On the local reactions of the arterial wall to changes of internal pressure. *Journal of Physiology* **28**, 220-223.

BEAN, B. P., STUREK, M., PUGA, A. & HERMSMEYER, K. (1986). Calcium channels in muscle cells isolated from rat mesenteric arteries: Modulation by dihydropyridine drugs. *Circulation Research* **59**, 229-235.

BELLONI, F. L. & HINTZE, T. H. (1991). Glibenclamide attenuates adenosine-induced bradycardia and coronary vasodilatation. *Am.J.Physiol.* **261**, H720-H727.

BENHAM, C. D., Bolton, T. B., Lang, R. J. & Takewaki, T. (1986). Calcium-activated potassium channels in single smooth muscle cells of rabbit jejunum and guinea-pig mesenteric artery. *Journal of Physiology* **371**, 45-67.

BENHAM, C. D., HESS, P. & TSIEN, R. W. (1987). Two types of calcium channels in single smooth muscle cells from rabbit ear artery studied with whole-cell and single channel recordings. *Circulation Research* **61**, I10-I16.

BENHAM, C. D. & TSIEN, R. W. (1987). A novel receptor-operated Ca^{2+}-permeable channel activated by ATP in smooth muscle. *Nature* **328**, 275-278.

BERCZI, V., GREENE, A. S., DORNYEI, G., CSENGODY, J., HODI, G., KADAR, A. & MONOS, E. (1992a). Venous myogenic tone: studies in human and canine vessels. *Am.J.Physiol.* **263**, H315-H320.

BERCZI, V., STEKIEL, J., CONTENY, S. J. & RUSCH, N. J. (1992b). Pressure-induced activation of membrane K^+ current in rat saphenous artery. *Hypertension* **19**, 725-729.

BERNE, R. M. (1963). Cardiac nucleotides in hypoxia: a possible role in regulation of coronary blood flow. *Am.J.Physiol.* **204**, 317-322.

BERNE, R. M. (1964). Regulation of coronary blood flow. *Physiol Rev* **44**, 1-29.

BERNE, R. M. (1980). The role of adenosine in the regulation of coronary blood flow. *Circulation Research* **47**, 808-813.

BERRIDGE, M. J. (1987). Inositol triphosphate and diacylglycerol: two interacting second messengers. *Annu.Rev.Biochem.* **56**, 159-193.

BERS, D. M. (1982). A simple method for the accurate determination of free [Ca^{2+}] in Ca-EGTA solutions. *Am.J.Physiol.* **242**, C404-C408.

BEVAN, J. A. (1982). Selective action of Diltiazem on cerebral vascular smooth muscle in the rabbit: Antagonism of extrinsic but not intrinsic maintained tone. *The American Journal of Cardiology.* **49,** 519-524.

BEVAN, J. A. & WELLMAN, G. C. (1993). Intraluminal flow-initiated hyperpolarization and depolarization shift the membrane potential of arterial smooth muscle toward an intermediate level. *Circulation Research.* **73**, 1188-1192.

BRADLEY, A. B. & MORGAN, K. G. (1987). Alterations in cytoplasmic calcium sensitivity during porcine coronary artery contraction as detected by aequorin. *Journal of Physiology* **385**, 437-448.

BRAYDEN, J. E. & NELSON, M. T. (1992). Regulation of Arterial Tone by Activation of Calcium-Dependent Potassium Channels. *Science* **256**, 532-535.

BRAYDEN, J. E. & WELLMAN, G. C. (1989). Endothelium-Dependent Dilation of Feline Cerebral Arteries: Role of Membrane Potential and Cyclic Nucleotides. *Journal of Cerebral Blood Flow and Metabolism* **9**, 256-263.

BULOW, A. (1996). Differentiated effects of vasodilators on myogenic reactivity during partial inhibition of myogenic tone in pressurised skeletal muscle small arteries of the rat. *Acta.Physiol.Scand.* **157**, 419-426.

BULOW, A. & NILSSON, H. (1991). Myogenic activity in isolated resistance arteries from skeletal muscle of the rat. *Blood Vessels* **28**, 278-279.

CAPDEVILA, J. H., FALCK, J. R. & ESTABROOK, R. W. (1992). Cytochrome P450 and the arachadonic acid cascade. *FASEB J.* **6**, 731-736.

CHEN, G. & SUZUKI, H. (1991). Endothelium-dependent hyperpolarization elicited by adenine compounds in rabbit carotid artery. *Am.J.Physiol.* **260**, H1037-H1042.

CHEN, W., BRACE, R. A., SCOTT, J. B., ANDERSON, D. K. & HADDY, F. J. (1972). The mechanism of the vasodilator action of potassium. *Proceedings of the Society of Experimental Biology and Medicine* **140**, 820-824.

CHENG, H. C., KEMP, B. E., PEARSON, R. B., SMITH, A. J., MISCONI, L., VANPATTEN, S. M. & WALSH, D. A. (1986). A potent synthetic peptide inhibitor of the cAMP-dependent protein kinase. *J. Biol. Chem.* **261**, 989-992.

CHENG, H. C., VANPATTEN, S. M., SMITH, A. J. & WALSH, D. A. (1985). An active twenty-amino-acid-residue peptide derived from the inhibitor protein of the cyclic AMP-dependent protein kinase. *Biochem. J.* **231**, 655-661.

CHLOPICKI, S., NILSSON, H. & MULVANY, M. J. (1996). The late part of the myogenic response of rat mesenteric small arteries is inhibited by Cytochrome P450 inhibitors. *J.Vasc.Res.* **33**, 48(Abstract)

CLAYTON, F. C., HESS, T. A., SMITH, M. A. & GROVER, G. J. (1992). Coronary reactive hyperemia and adenosine -induced vasodilation are mediated partially by a glyburide-sensitive mechanism. *Pharmacology* **44**, 92-100.

COLLINS, E. M., WALSH, M. P. & MORGAN, K. G. (1992). Contraction of single vascular smooth muscle cells by phenylephrine at constant $[Ca^{2+}]_i$. *Am.J.Physiol.* **262**, H754-H762.

CONEY, A. M. & MARSHALL, J. M. (1998). Role of adenosine and its receptors in the vasodilatation induced in the cerebral cortex of the rat by systemic hypoxia. *Journal of Physiology* **509.2**, 507-518.

CONTI, M. A. & ADELSTEIN, R. S. (1981). The relationship between calmodulin binding and phosphorylation of smooth muscle myosin kinase by the catalytic subunit of 3', 5'- cAMP-dependent protein kinase. *J. Biol. Chem.* **256,** 3178-3181.

D'ANGELO, G. & MEININGER, G. A. (1994). Transduction mechanisms involved in the regulation of myogenic activity. *Hypertension* **23**, 1096-1105.

DART, C. & STANDEN, N. B. (1993). Adenosine-activated potassium current in smooth muscle cells isolated from the pig coronary artery. *Journal of Physiology* **471**, 767-786.

DAUT, J., MAIER-RUDOLF, W., BECKERATH, N., MEHRKE, G., GUNTHER, K. & GOEDEL-MEINEN, L. (1990). Hypoxic dilation of coronary arteries is mediated by ATP-sensitive potassium channels. *Science* **247**, 1341-1344.

DAVIS, M. J., DONOVITZ, J. A. & HOOD, J. D. (1992a). Stretch-Activated Single-Channel and Whole Cell Currents in Vascular Smooth Muscle Cells. *Am.J.Physiol.* **262**, C1083-C1088.

DAVIS, M. J., FEERRER, P. N. & GORE, R. W. (1986). Vascular anatomy and hydrostatic pressure profile in the hamster cheek pouch. *Am.J.Physiol.* **250**, H291-H303.

DAVIS, M. J., MEININGER, G. A. & ZAWIEJA, D. C. (1992b). Stretch-Induced Increases in Intracellular Calcium of Isolated Vascular Smooth Muscle Cells. *Am.J.Physiol.* **263**, H1292-H1299.

DAVIS, M. J., SHI, X. & SIKES, P. J. (1992c). Modulation of bat wing venule contraction by transmural pressure changes. *Am.J.Physiol.* **262**, H625-H634.

DAVIS, M. J. & SIKES, P. J. (1990). Myogenic responses of isolated arterioles; test for a rate-sensitive mechanism. *Am.J.Physiol.* **259**, H1890-H1900.

DEAL, C. P. Jr. & GREEN, H. D. (1954). Effects of pH on blood flow and peripheral resistance in muscular and cutaneous vascular beds in the hind limb of the pentobarbitalized dog. *Circulation Research* **2,** 148-154.

DEFILY, D. V. & CHILIAN, W. M. (1995). Coronary microcirculation: autoregulation and metabolic control. *Basic Res. Cardiol.* **90**, 112-118.

DeLANEROLLE, P., NISHIKAWA, M., YOST, D. A. & ADELSTEIN, R. A. (1984). Increased phosphorylation of myosin light chain kinase after an increase in cyclic AMP in intact smooth muscle. *Science* **223**, 1415-1417.

DULING, B. R., GORE, R. W., DACEY, R. G. & DAMON, D. N. (1981). Methods for isolation, cannulation, and in vitro study of single microvessels. *Am.J.Physiol.* **241**, H108-H116.

DUNNE, M. J., WEST-JORDAN, J. A., ABRAHAM, R. J., EDWARDS, R. H. & PETERSEN, O. H. (1988). The gating of nucleotide-sensitive K^+ channels in insulin-secreting cells can. *J.Membrane Biol.* **104**, 165-177.

EDWARDS, F. R., HIRST, G. D. S. & SILVERBERG, G. D. (1988). Inward rectification in rat cerebral arterioles; involvement of potassium ions in autoregulation. *Journal of Physiology* **404**, 455-466.

FALCONE, J. C., DAVIS, M. J. & MEININGER, G. A. (1991). Endothelial independence of myogenic response in isolated skeletal muscle arterioles. *Am.J.Physiol.* **260**, H130-H135.

FALLOON, B. J., STEPHENS, N., TULIP, J. R. & HEAGERTY, A. M. (1995). Comparison of small artery sensitivity and morphology in pressurized and wire-mounted preparations. *Am.J.Physiol.* **268**, H670-H678.

FARACI. F. M., BREESE, K.R & HEISTAD, D.D. (1994). Cerebral vasodilatation during hypercapnia. Role of glibenclamide-sensitive potassium channels and nitric oxide. *Stroke* **25,** 1679-1683.

FOLKOW, B. (1949). Intravascular pressure as a factor regulating the tone of the small vessels. *Acta.Physiol.Scand.* **17**, 289-310.

FORSTER, H. G., TERWEE, P. M., TAKENAKA, T., HOHMAN, T. C. & EPSTEIN, M. (1994). Impairment of afferent arteriolar myogenic responsiveness in the galactose-fed rat. *Proceedings of the Society of Experimental Biology and Medicine* **206**, 365-374.

FOX, P. T. & RAICHLE, M. E. (1984). Stimulus rate dependence of regional cerebral blood flow in human striate cortex, demonstrated by positron emission tomography. *Journal of Neurophysiology* **51**, 1109-1120.

FRANCIS, S. H., NOBLETT, B. D. TODD, B. W. WELLS, J. N. & CORBIN, J. D. (1988). Relaxation of vascular and tracheal smooth muscle by cyclic nucleotide analogs that preferentially activate cGMP-dependent protein kinase. *Mol. Pharmacol.* **34,** 506-517.

FREDRICKS, K. T., LIU, Y.P. & LOMBARD, J.H. (1994). Response of extraparenchymal resistance arteries of rat skeletal muscle to reduced PO_2. *Am.J.Physiol.* **267**, H706-H715.

FURCHGOTT, R. F. (1983). Role of endothelium in responses of vascular smooth muscle. *Circulation Research* **53**, 557-573.

GANITKEVICH, V. Y. & ISENBERG, G. (1990). Contribution of two types of calcium channels to membrane conductance of single myocytes from guinea pig coronary artery. *Journal of Physiology* **426**, 19-42.

GEBREMEDHIN, D., MA, Y., FALCK, J. R., ROMAN, R. J., VANROLLINS, M. & HARDER, D. R. (1992). Mechanism of action of cerebral epoxyeicosatrienoic acids on cerebral arterial smooth muscle. *Am.J.Physiol.* **263**, H519-H525.

GEBREMEDHIN, D., LANGE, A.R., LOWRY, T.F., TAHERI, M.R., BIRKS, E.K., HUDETZ, A.G., NARAYANAN, J., FALCK, J.R., OKAMOTO, H., ROMAN, R.J., NITHIPATIKOM, K., CAMPBELL, W.B. & HARDER, D. R. (2000). Production of 20-HETE and its role in autoregulation of cerebral blood flow. *Circulation Research.* **87.** 60-65.

GOKINA, N. I., WELLMAN, T. D., BEVAN, R. D., WALTERS, C. L., PENAR, P. L. & BEVAN, J. A. (1996). Role of Ca^{2+}-activated K^+ channels in the regulation of membrane potential and tone of smooth muscle in human pial arteries. *Circulation Research* **79**, 881-886.

GREENWOOD, I. A. & LARGE, W. A. (1998). Properties of a Cl^- current activated by cell swelling in rabbit portal vein vascular smooth muscle cells. *Am.J.Physiol.* **275**, H1524-H1532.

GUHARAY, F. & SACHS, F. (1984). Stretch-activated single ion channel currents in tissue-cultured embryonic chick skeletal muscle. *Journal of Physiology* **352**, 685-701.

GUSTAFSSON, D., GRANDE, P., BORGSTROM, P. & LINDBERG, L. (1988). Effects of calcium antagonists on myogenic and neurogenic control of resistance and capacitance vessels in cat skeletal muscle. *Journal of Cardiovascular Pharmacology* **12**, 413-422.

GUYTON, A. C., ROSS, J. M., CARRIER, O, Jr. & WALKER, J.R. (1964). Evidence for tissue oxygen demand as the major factor causing autoregulation. *Circulation Research* **14-15** (suppl 1), I60-I68.

HADDY, F.J. & SCOTT, J. B. (1968). Metabolically linked vasoactive chemicals in local regulation of blood flow. *Physiological Reviews.* **48.** 688-707.

HALPERN, W. & KELLEY, M. (1991). In vitro methodology for resistance arteries. *Blood Vessels* **28**, 245-251.

HALPERN, W., OSOL, G. & COY, G. S. (1984). Mechanical behavior of pressurised in vitro prearteriolar vessels determined with a video system. *Annals of Biomedical Engineering* **12**, 463-479.

HARDER, D. R. (1984). Pressure-Dependent Membrane Depolarization in Cat Middle Cerebral Artery. *Circulation Research* **55**, 197-202.

HARDER, D. R. & MADDEN, J. A. (1985). Cellular mechanism of force development in cat middle cerebral artery by reduced pCO_2. *Pflügers Arch.* **403**, 402-404.

HARDER, D. R. (1987). Pressure-induced myogenic activation of cat cerebral arteries is dependent on intact endothelium. *Circulation Research* **60**, 102-107.

HARDER, D. R., GILBERT, R. & LOMBARD, J. H. (1987). Vascular muscle cell depolarization and activation in renal arteries on elevation of transmural pressure. *Am.J.Physiol.* **253**, F778-F781.

HARDER, D. R., KAUSER, K., ROMAN, R. J. & LOMBARD, J. H. (1989a). Mechanisms of pressure-induced myogenic activation of cerebral and renal arteries: role of the endothelium. *Journal of Hypertension* **7**, S11-S15.

HARDER, D. R., SANCHEZ-FERRER, C., KAUSER, K., STEKIEL, W. J. & RUBANYI, G. M. (1989b). Pressure releases a transferable endothelial contractile factor in cat cerebral arteries. *Circulation Research* **65**, 193-198.

HARDER, D. R., ROMAN, R. J. & LOMBARD, J. H. (1991). Cellular and biochemical mechanisms of pressure-induced myogenic tone in arteries. *In: Resistance arteries, structure and function.* Elsevier Science Publishers B.V. pp 178-182.

HARDER, D. R., GEBREMEDHIN, D., NARAYANAN, J., JEFCOAT, C., FALCK, J. R., CAMPBELL, W. B. & ROMAN, R. J. (1994). Formation and action of a P-450 4A metabolite of arachadonic acid in cat cerebral microvessels. *Am.J.Physiol.* **266**, H2098-H2107.

HARDER, D. R., CAMPBELL, W. B. & ROMAN, R. J. (1995). Role of cytochrome P450 enzyme metabolites of arachadonic acid in the control of vascular tone. *J.Vasc.Res.* **32**, 79-92.

HARDIE, G. (1988). Pseudosubstrates turn off protein kinases. *Nature* **355**, 592-593.

HARTSHORNE, D. J. (1987). Biochemistry of the contractile process in smooth muscle. In *Physiology of the gastrointestinal tract*, ed. JOHNSON, L. R. pp. 423-482. New York: Raven.

HASHIMOTO, Y. & SODERLING, T. R. (1990). Phosphorylation of smooth muscle myosin light chain kinase by Ca^{2+}/calmodulin-dependent protein kinase II: comparative study of the phosphorylation sites. *Arch. Biochem. Biophys.* **278**, 41-45.

HAYASHI, K., EPSTEIN, M. & LOUTZENHISER, R. (1992a). Enhanced myogenic responsiveness of renal interlobular arteries in spontaneously hypertensive rats. *Hypertension* **19**, 153-160.

HAYASHI, K., EPSTEIN, M., LOUTZENHISER, R. & FORSTER, H. G. (1992b). Impaired myogenic responsiveness of the afferent arteriole in streptozotocin-induced diabetic rats: role of eicosanoid derangement's. *J.Am.Soc.Nephrol.* **2**, 1578-1586.

HENDRICKX, H. & CASTEELS, R. (1974). Electrogenic sodium pump in arterial smooth muscle cells. *Pflügers Arch.* **346**, 299-306.

HENRION, D., LAHER, I., KLAASEN, A. & BEVAN, J. A. (1994). Myogenic tone of rabbit facial vein and posterior cerebral artery is influenced by changes in extracellular sodium. *Am.J.Physiol.* **266**, H377-H383.

HERLIHY, J. T., BOCKMAN, E. L., BERNE, R. M. & RUBIO, R. (1976). Adenosine relaxation of isolated vascular smooth muscle. *Am.J.Physiol.* **230**, 1239-1243.

HILL, C. E., HIRST, G. D. S., SILVERBERG, G. D. & VAN HELDEN, D. F. (1986). Sympathetic innervation and excitability of arterioles originating from the rat middle cerebral artery. *Journal of Physiology* **371**, 305-316.

HILL, M. A., DAVIS, M. J. & MEININGER, G. A. (1990a). Cyclooxygenase inhibition potentiates myogenic activity in skeletal muscle arterioles. *Am.J.Physiol.* **258**, H127-H133.

HILL, M. A., FALCONE, J. C. & MEININGER, G. A. (1990b). Evidence for protein kinase C involvement in arteriolar myogenic reactivity. *Am.J.Physiol.* **259**, H1586-H1594.

HILL, M. A. & MEININGER, G. A. (1993). Impaired arteriolar myogenic reactivity in early experimental diabetes. *Diabetes* **42**, 1226-1232.

HIRST, G. D. S., SILVERBERG, G. D. & VANHELDEN, D. F. (1986). The action potential and underlying ionic currents in proximal rat middle cerebral arterioles. *Journal of Physiology* **371**, 289-304.

HIRST, G. D. S. & VAN HELDEN, D. F. (1982). Ionic basis of the resting potential of submucosal arterioles in the ileum of the guinea pig. *Journal of Physiology* **333**, 53-67.

HISADA, T., ORDWAY, R. W., KIRBER, M. T., SINGER, J. J. & WALSH, J. V. (1991). Hyperpolarization-activated cationic channels in smooth muscle cells are stretch sensitive. *Pflügers Arch.* **417**, 493-499.

HORI, M. & KITAKAZE, M. (1991). Adenosine, the heart, and coronary circulation. *Hypertension* **18**, 565-574.

HOROWITZ, A., CLEMENT-CHOMIENNE, O., WALSH, M. P. & MORGAN, K. G. (1996a). ε-Isoenzyme of protein kinase C induces a Ca^{2+}-independent contraction in vascular smooth muscle. *Am.J.Physiol.* **271**, C589-C594.

HOROWITZ, A., CLEMENT-CHOMIENNE, O., WALSH, M. P., TAO, T., KATSUYAMA, H. & MORGAN, K. G. (1996b). Effects of calponin on force generation by single smooth muscle cells. *Am.J.Physiol.* **270**, H1858-H1863.

HOSTETTER, T. H., RENNKE, H. G. & BRENNER, B. M. (1982). The case for intrarenal hypertension in the initiation and progression of diabetic and other glomerulopathies. *Am.J.Med.* **72**, 375-380.

HOUSE, C. & KEMP, B. E. (1987). Protein kinase C contains a pseudosubstrate prototope in its regulatory domain. *Science* **238**, 1726-1728.

HU, S. & KIM, H. S. (1993). Activation of K^+ channel in vascular smooth muscles by cytochrome P450 metabolites of arachadonic acid. *European Journal of Pharmacology* **230**, 215-221.

HWA, J. J. & BEVAN, J. A. (1986a). A nimodipine-resistant Ca^{2+} pathway is involved in myogenic tone in a resistance array. *Am.J.Physiol.* **251**, H182-H189.

HWA, J. J. & BEVAN, J. A. (1986b). Dihydropyridine calcium agonists selectively enhance resistance artery myogenic tone. *European Journal of Pharmacology* **126**, 231-238.

HWA, J. J. & BEVAN, J. A. (1986c). Stretch-dependent (myogenic) tone in rabbit ear resistance arteries. *Am.J.Physiol.* **250**, H87-H95.

IKEBE, M. & HARTSHORNE, D. J. (1985). Phosphorylation of smooth muscle myosin at two distinct sites by myosin light chain kinase. *J. Biol. Chem.* **260**, 10027-10031.

IKEBE, M., HARTSHORNE, D. J. & ELZINGA, M. (1986). Identification, phosphorylation, and dephosphorylation of a second site for myosin light chain kinase on the 20,000 Dalton light chain of smooth muscle myosin. *J. Biol. Chem.* **261**, 36-39.

IKEBE, M., HARTSHORNE, D. J. & ELZINGA, M. (1987a). Phosphorylation of the 20,000-dalton light chain of smooth muscle myosin by the calcium-activated, phospholipid-dependent protein kinase. *J. Biol. Chem.* **262**, 9569-9573.

IKEBE, M. & HORNICK, T. (1991). Determination of the phosphorylation sites of smooth muscle caldesmon by protein kinase C. *Archives of Biochemistry and Biophysics* **288**, 538-542.

IKEBE, M., KEMP, B. E. & HARTSHORNE, D. J. (1988). Study of the inhibitory region of smooth-muscle myosin light chain kinase. *Biophysical journal* **53**, 182a

IKEBE, M. & REARDON, S. (1990). Phosphorylation of smooth muscle myosin light chain kinase by smooth muscle Ca^{2+}/calmodulin-dependent multifunctional protein kinase. *J. Biol. Chem.* **265,** 8975-8978.

IKEBE, M., STEPINSKA, M., KEMP, B. E., MEANS, A. R. & HARTSHORNE, D. J. (1987b). Proteolysis of smooth muscle myosin light chain kinase. Formation of inactive and calmodulin-independent fragments. *J. Biol. Chem.* **262**, 13828-13834.

IMIG, J. D., ZOU, A., ORTIZ DE MONTELLANO, P. R., SUI, Z. & ROMAN, R. J. (1994). Cytochrome P-450 inhibitors alter afferent arteriolar responses to elevations in pressure. *Am.J.Physiol.* **266**, H1879-H1885.

ISHIZAKA, H. & KUO, L. (1996). Acidosis-induced coronary arteriolar dilatation is mediated by ATP-sensitive potassium channels in vascular smooth muscle. *Circulation Research* **78,** 50-57.

ITOH, T., IKEBE, M., KARGACIN, G. J., HARTSHORNE, D. J., KEMP, B. E. & FAY, F. S. (1989). Effects of modulators of myosin light-chain kinase activity in single smooth muscle cells. *Nature* **338**, 164-167.

ITOH, H., SHIMOMURA, A., OKUBO, S., ICHIKAWA, K., ITO, M., KONISHI, T. & NAKANO, T. (1993). Inhibition of myosin light chain

phosphatase during Ca^{2+}-independent vasoconstriction. *Am.J.Physiol.* **265**, C1319-C1324.

JACKSON, P. A. & DULING, B. R. (1989). Myogenic response and wall mechanics of arterioles. *Am.J.Physiol.* **257**, H1147-H1155.

JACKSON, W. F. (1993). Arteriolar tone is determined by activity of ATP-sensitive potassium channels. *Am.J.Physiol.* **34**, H1797-H1803.

JACKSON, W. F. & DULING, B. R. (1983). The oxygen sensitivity of hamster cheekpouch arterioles: *in vitro* and *in vivo* studies. *Circulation Research* **53**, 515-525.

JACOBSON, E. D. & PAWLIK, W. W. (1994). Adenosine regulation of mesenteric vasodilation. *Gastroenterology* **107**, 1168-1180.

JIANG, M. J. & MORGAN, K. G. (1987). Intracellular calcium levels in phorbol ester-induced contractions of vascular muscle. *Am.J.Physiol.* **253**, H1365-H1371.

JOHANSSON, B. (1989). Myogenic tone and reactivity: definitions based on mussel physiology. *Journal of Hypertension* **7**, S5-S8.

JOHNSON, P. C. (1964). Autoregulation of blood flow. *Circulation Research* **14-15** (suppl 1), I1-291.

JOHNSON, P. C. (1981). The Myogenic Response. In *Handbook of Physiology, The Cardiovascular System. Vascular Smooth Muscle.* chap 15, pp 409-442. Bethesda, MD: Am. Physiol. Soc.

JOHNSON, P. C. (1986). Autoregulation of blood flow. *Circulation Research* **59**, 483-495.

KAMM, K. E. & STULL, J. T. (1985). The function of myosin and myosin light chain kinase phosphorylation in smooth muscle. *Annu. Rev. Pharmacol. Toxicol.* **25**, 593-620.

KAMM, K. E. & STULL, J. T. (1989). Regulation of smooth muscle contractile elements by second messengers. *Annu.Rev.Physiol.* **51**, 299-313.

KARARA, A., DISHMAN, E., JACOBSON, H., FALCK, J. R. & CAPDEVILA, J. H. (1990). Arachadonic acid epoxygenase. Stereochemical analysis of the endogenous epoxyeicosatrienoic acids of human kidney cortex. *FEBS Lett.* **268**, 227-230.

KATSUYAMA, H. & MORGAN, K. G. (1993). Mechanisms of Ca^{2+}-independent contraction in single permeabilized ferret aorta cells. *Circulation Research* **72**, 651-657.

KATUSIC, Z. S., SHEPARD, J. T. & VANHOUTTE, P. M. (1987). Endothelium-dependent contraction to stretch in canine basilar arteries. *Am.J.Physiol.* **H21**, H671-H673.

KAUSER, K., CLARK, J. E., MASTERS, B. S., ORTIZ DE MONTELLANO, P. R., MA, Y., HARDER, D. R. & ROMAN, R. J. (1991). Inhibitors of cytochrome P-450 attenuate the myogenic response of dog renal arcuate arteries. *Circulation Research* **68**, 1154-1163.

KEMP, B. E., PARKER, M. W., HU, S., TIGANIS, T. & HOUSE, C. (1994). Substrate and pseudosubstrate interactions with kinases: determinants of specificity. *Trends Biochem. Sci.* **19**, 440-444.

KEMP, B. E., PEARSON, R. B., GUERIECO, J. V., BAGUCHI, I. C. & MEANS, A. R. (1987). The calmodulin binding domain of chicken smooth

muscle myosin light chain kinase contains a pseudosubstrate sequence. *J. Biol. Chem.* **262**, 2542-2548.

KINOSHITA, H. & KATUSIC, Z. S. (1997). Role of potassium channels in relaxations of isolated canine basilar arteries to acidosis. *Stroke* **28,** 433-438

KIRBER, M. T., WALSH, J. V. & SINGER, J. J. (1988). Stretch-activated ion channels in smooth muscle: a mechanism for the initiation of stretch-induced contraction. *Pflügers Arch.* **412**, 339-345.

KLEPPISCH, T. & NELSON, M. T. (1995). Adenosine activates ATP-sensitive potassium channels in arterial myocytes via A_2 receptors and cAMP-dependent protein kinase. *Proc.Natl.Acad.Sci.* **92**, 12441-12445.

KNOT, H. J. & NELSON, M. T. (1995). Regulation of membrane potential and diameter by voltage-dependent K^+ channels in rabbit myogenic cerebral arteries. *Am.J.Physiol.* **269**, H348-H355.

KNOT, H. J. & NELSON, M. T. (1998). Regulation of arterial diameter and wall [Ca^{2+}] in cerebral arteries of rat by membrane potential and intravascular pressure. *Journal of Physiology* **508.1**, 199-209.

KNOT, H. J., ZIMMERMAN, P. A. & NELSON, M. T. (1994). Activation of inward rectifier K^+ channels (K_{IR}) dilates small cerebral and coronary arteries in response to elevated potassium. *Biophysical journal* **66**, A144

KNOT, H. J., ZIMMERMAN, P. A. & NELSON, M. T. (1996). Extracellular K^+-induced hyperpolarizations and dilatations of rat coronary and cerebral arteries involve inward rectifier K^+ channels. *Journal of Physiology* **492**, 419-430.

KONTOS, H., RAPER, A. J. & PATTERSON, J. L. (1977). Analysis of vasoactivity of local pH, PCO_2 and bicarbonate on pial vessels. **8,** 258-360.

KONTOS, H., WEI. E. P., RAPER, A. J., ROSENBLUM, W. I., NAVARI, R. M. & PATTERSON, J. L. (1978). Role of tissue hypoxia in local regulation of cerebral microcirculation. *Am.J.Physiol.* **234**, H582-H591.

KONTOS, H. (1981). Regulation of the cerebral circulation. *Annu.Rev.Physiol.* **43**, 397-407.

KUKOVETZ, W. R., POCH, G., HOLZMANN, S., WURM, A. & RINNER, I. (1978). Role of cyclic nucleotides in adenosine-mediated regulation of coronary flow. *Adv. Cyclic Nucleotide Res.* **9**, 297-409.

KULIK, T. J., EVANS, J. N. & GAMBLE, W. J. (1988). stretch-induced contraction in pulmonary arteries. *Am.J.Physiol.* **255**, H1391-H1398.

KUO, L., CHILIAN, W. M. & DAVIS, M. J. (1990a). Coronary arteriolar myogenic response is independent of endothelium. *Circulation Research* **66**, 860-866.

KUO, L., DAVIS, M. J. & CHILIAN, W. M. (1990b). Endothelium-dependent, flow-induced dilatation of isolated coronary arteries. *Am.J.Physiol.* **259**, H1063-H1070.

KUSCHINSKY, W., WHAL, M., BOSSE, O. & THURAU, K. (1972). Perivascular potassium and pH as determinants of local pial arterial diameter in cats. *Circulation Research* **16**, 240-247.

LAHER, I. & BEVAN, J. A. (1987). Protein kinase C activation selectively augments a stretch-induced, calcium-dependent tone in vascular smooth

muscle. *The Journal of Pharmacology and Experimental Therapeutics* **242**, 566-572.

LAHER, I. & BEVAN, J. A. (1989a). Stretch of vascular smooth muscle activates tone and $45Ca^{2+}$ influx. *Hypertension* **7**, 17-20.

LAHER, I. & BEVAN, J. A. (1989b). Staurosporine, a protein kinase C inhibitor, attenuates Ca^{2+}-dependent stretch-induced vascular tone. *Biochemical and Biophysical Research Communications* **158**, 58-62.

LAHER, I., HWA, J. & BEVAN, J. A. (1988a). Calcium and vascular myogenic tone. *Annals New York Academy of Sciences* 216-225.

LAHER, I., VANBREEMAN, C. & BEVAN, J. A. (1988b). stretch-dependent calcium uptake associated with myogenic tone in rabbit facial vein. *Circulation Research* **63**, 669-672.

LANGTON, P. D. (1993). Calcium channel currents recorded from isolated myocytes of rat basilar artery are stretch sensitive. *Journal of Physiology* **471**, 1-11.

LARGE, W. A. & WANG, Q. (1996). Characteristics and physiological role of the Ca^{2+}-activated Cl^- conductance in smooth muscle. *Am.J.Physiol.* **271**, C435-C454.

LEDINGHAM, I. M., MCBRIDE, T. I., PARRATT, J. R. & VANCE, J. P. (1970). The effect of hypercapnia on myocardial blood flow and metabolism. *Journal of Physiology* **210**, 87-105.

LEE, M. W. & SEVERSON, D. L. (1994). Signal transduction in vascular smooth muscle: diacylglycerol second messengers and PKC action. *Am.J.Physiol.* **267**, C659-C678.

LEE, R. M., GARFIELD, R. E., FORREST, J. B. & DANIEL, E. E. (1983). Morphmetric study of structural changes in mesenteric blood vessels of spontaneously hypertensive rats. *Blood Vessels* **20**, 57-71.

LINCOLN,T.M., CORNWELL,T.W., KOMALAVILAS,P., MACMILLAN-CROW, A & BOERTHE,N. (1996). The Nitric Oxide-Cyclic GMP Signaling System. In *Biochemistry of Smooth Muscle*, ed. BARANY, M. pp. 257-268. Academic Press Inc.

LIU, J., HILL, M. A. & MEININGER, G. A. (1994). mechanisms of myogenic enhancement by norepherine. *Am.J.Physiol.* **266**, H440-H446.

LIU, Y., HARDER, D. R. & LOMBARD, J. H. (1994). Myogenic activation of canine small renal arteries after non-chemical removal of the endothelium. *Am.J.Physiol.* **267**, H302-H307.

LOMBARD, J. H., SMEDA, J., MADDEN, J. A. & HARDER, D.R. (1986). Effect of reduced oxygen availability upon myogenic depolarization and contractility of cat middle cerebral artery. *Circulation Research* **58**, 565-569.

LOUTZENHISER, R. D. & PARKER, M. J. (1994). Hypoxia inhibits myogenic reactivity of renal afferent arterioles by activating ATP-sensitive K^+ channels. *Circulation Research* **74**, 861-869.

LUKAS, T. J., BURGESS, W. H., PRENDERGAST, F. G., LAU, W. & WATTERSOM, D. M. (1986). Calmodulin binding domains: characterization of a phosphorylation and calmodulin binding site from myosin light chain kinase. *Biochemistry* **25**, 1458-1464.

LUKAS, T. J., HAIECH, J., LAU, W., CRAIG, T. A., ZIMMER, W. E., SHATTUCK, R. L., SHOWMAKER, M. O. & WATTERSON, D. M. (1988).

Calmodulin and calmodulin-regulated protein kinases as transducers of intracellular calcium signals. *Cold Springs Harbour Symposium.* **53,** 185-193.

MA, Y., GEBREMEDHIN, D., SCHWARTZMAN, M. L., FALCK, J. R., CLARK, J. E., MASTERS, B. S., HARDER, D. R. & ROMAN, R. J. (1993). 20-Hydroxyeicosatetraenoic acid is an endogenous vasoconstrictor of canine renal arcuate arteries. *Circulation Research* **72,** 126-136.

MACPHERSON, R. D., MCLEOD, L. J. & RASIAH, R. L. (1991). Myogenic response of isolated pressurized rabbit ear artery is independent of endothelium. *Am.J.Physiol.* **H29,** H779-H784.

MARSHALL, J. M., THOMAS, T. & TURNER, L. (1993). A link between adenosine, potassium and ATP-sensitive potassium channels in muscle vasodilatation in the rat in systemic hypoxia. *Journal of Physiology* **472,** 1-9.

MCCARRON, J. G. & HALPERN, W. (1990). Impaired potassium-induced dilatation in hypertensive rat cerebral arteries does not reflect altered Na^+,K^+-ATPase dilation. *Circulation Research* **67,** 1035-1039.

MCCARRON, J. G., OSOL, G. & HALPERN, W. (1989). Myogenic responses are independent of the endothelium in rat pressurized posterior cerebral arteries. *Blood Vessels* **26,** 315-319.

MCCARRON, J. G., CRICHTON, C. A., LANGTON, P. D., MACKENZIE, A. & SMITH, G. L. (1997). Myogenic contraction by modulation of voltage-dependent calcium currents in isolated rat cerebral arteries. *Journal of Physiology* **498,** 371-379.

MCCARRON, J. G. & HALPERN, W. (1990). Potassium dilates rat cerebral arteries by two independent mechanisms. *Am.J.Physiol.* **259,** H902-H908.

MCCARRON, J. G., MCGEOWN, J. G., WALSH, J. V. & FAY, F. S. (1997). Modulation of high- and low-voltage-activated calcium currents in smooth muscle by calcium. *Am.J.Physiol.* **273**, C883-C892.

MCCARRON, J. G., QUAYLE, J. M., HALPERN, W. & NELSON, M. T. (1991). Cromakalim and pinacidil dilate small mesenteric arteries but not small cerebral arteries. *Am.J.Physiol.* **261**, H287-H291.

MCGIFF, J. C. (1991). Cytochrome P450 metabolites of arachadonic acid. *Annu. Rev. Pharmacol. Toxicol.* **31**, 339-369.

MCPHERSON, G. A. & STORK, A. P. (1992). The resistance of some rat cerebral arteries to the vasorelaxant effect of cromakalim and other K^+ channel openers. *Br. J. Pharmacol.* **104**, 51-58.

MEININGER, G. A. & DAVIS, M. J. (1992). Cellular Mechanisms Involved in the Vascular Myogenic Response. *Am.J.Physiol.* **263**, H647-H659.

MELLANDER, S. (1989). Functional aspects of myogenic vascular control. *Journal of Hypertension* **7**, S21-S30.

MILLER, D. J. & SMITH, G. L. (1985). The contractile behavior of EGTA- and detergent-treated heart muscle. *J.Muscle Res. and Cell Motility* **6**, 541-67.

MILLER, F. J., DELLSPERGER, K. C. & GUTTERMAN, D. D. (1997). Myogenic constriction of human coronary arterioles. *Am.J.Physiol.* **273**, H257-H264.

MORFF, R. J. & GRANGER, H. J. (1982). Autoregulation of blood flow within individual arterioles in the rat cremaster muscle. *Circulation Research* **51**, 43-55.

MORII, S., NGAI, A. C., KO, K. R. & WINN, H. R. (1987). Role of adenosine in regulation of cerebral blood flow: effects of theophylline during normoxia and hypoxia. *Am.J.Physiol.* **253**, H165-H175.

MULLER, J.M., DAVIS, M.J. & CHILIAN, W.M. (1996). Integrated regulation of pressure and flow in the microcirculation. *Cardiovascular Research* **32,** 668-678

MULVANY, M. J. & AALKJAER, C. (1990). Structure and function of small arteries. *The American Physiological Society* **70**, 921-961.

NAGAO, T., SADOSHIMA, S., KAMOUCHI, M. & FUJISHIMA, M. (1991). Cromakalim dilates rat cerebral arteries in vitro. *Stroke* **22**, 221-224.

NAGAO, T., IBAYASHI, S., SADOSHIMA, S., FUJII, K., FUJII, K., OHYA, Y. & FUJISHIMA, M. (1996). Distribution and physiological roles of ATP-sensitive K^+ channels in the vertebrobasilar system of the rabbit. *Circulation Research* **78**, 238-243.

NAKAYAMA, K. (1982). Calcium-dependent contractile activation of cerebral artery produced by quick stretch. *Am.J.Physiol.* **242**, H760-H768.

NAKHOSTINE, N. & LAMONTAGNE, D. (1993). Adenosine contributes to hypoxia-induced vasodilatation through ATP-sensitive K+ channel activation. *Am.J.Physiol.* **265**, H1289-H1293.

NARAYANAN, J., IMIG, M., ROMAN, R. J. & HARDER, D. R. (1994). Pressurization of isolated renal arteries increases inositol triphosphate and diacylglycerol. *Am.J.Physiol.* **266**, H1840-H1845.

NELSON, M. T., BLAUSTEIN, M. P. & SANTIAGO, E. M. (1980). Properties of sodium pumps in internally perfused barnacle muscle fibers. *Journal of General Physiology* **75**, 183-206.

NELSON, M. T. & BRAYDEN, J. E. (1993). Regulation of arterial tone by calcium-dependent K^+ channels and ATP-sensitive K^+ channels. *Cardiovascular Drugs and Therapy* **7**, 605-610.

NELSON, M. T., CONWAY, M. A., KNOT, H. J. & BRAYDEN, J. E. (1997). Chloride channel blockers inhibit myogenic tone in rat cerebral arteries. *Journal of Physiology* **502**, 259-264.

NELSON, M. T., MCCARRON, J. G. & QUAYLE, J. M. (1991). Ion channels in resistance arteries. In *The resistance vasculature*, eds. BEVAN, J. A., HALPERN, W. & MULVANY, M. J. pp. 265-279. Totawa, NJ. Humana.

NELSON, M. T., PATLAK, J. B., WORLEY, J. F. & STANDEN, N. B. (1990). Calcium Channels, Potassium Channels, and Voltage Dependence of Arterial Smooth Muscle Tone. *Am.J.Physiol.* **259**,

NELSON, M. T. & QUAYLE, J. M. (1995). Physiological roles and properties of potassium channels in arterial smooth muscle. *Am.J.Physiol.* **268**, C799-C822.

NELSON, M. T., STANDEN, N. B., BRAYDEN, J. E. & WORLEY, J. F. (1988). Noradrenaline contracts arteries by activating voltage-dependent calcium channels. *Nature* **336**, 382-385.

NEWMAN, E. A. (1986). High potassium conductance in astrocyte endfeet. *Science* **233**, 453-454.

NICHOLS, C. G. & LEDERER, W. J. (1991). Adenosine triphosphate-sensitive potassium channels in the cardiovascular system. *Am.J.Physiol.* **261**, H1675-H1686.

NISHIKAWA, M., de LANEROLLE. P., LINCOLN. T. M. & ADELSTEIN, R. S. (1984). Phosphorylation of mammalian myosin light chain kinase by the catalytic subunit of cyclic AMP-dependent protein kinase and by cyclic GMP-dependent protein kinase. *J. Biol. Chem.* **259,** 8429-8436.

NISHIMURA, J. & VANBREEMAN, C. (1989). Direct regulation of smooth muscle contractile elements by second messengers. *Biochemical and Biophysical Research Communications* **163**, 929-935.

NOMA, A. (1983). ATP-regulated K^+ channels in cardiac muscle. *Nature* **305**, 147-148.

NORDLANDER, M. & THALEN, P. (1987). Effects of felodipine on local and neurogenic control of vascular resistance. *Journal of Cardiovascular Pharmacology* **10**, 100-106.

OLSSON, R.A. (1981). Local factors regulating cardiac ad skeletal muscle blood flow. *Annual Review of Physiology* **43,** 385-395.

OLSSON, R. A. & PEARSON, J. D. (1990). Cardiovascular Purinoceptors. *Physiological Reviews* **70**, 761.

OSOL, G., CIPOLLA, M. & KNUTSON, S. (1989). A new method for mechanically denuding the endothelium of small (50-150 μm) arteries with a human hair. *Blood Vessels* **26**, 320-324.

OSOL, G. & HALPERN, W. (1985). Myogenic properties of cerebral blood vessels from normotensive and hypertensive rats. *Am.J.Physiol.* **249**, H914-H921.

OSOL, G., LAHER, I. & CIPOLLA, M. (1991). Protein Kinase C modulates basal myogenic tone in resistance arteries from the cerebral circulation. *Circulation Research* **68**, 359-367.

OSOL, G., LAHER, I. & KELLEY, M. (1993). myogenic tone is coupled to phospholipase C and G protein activation in small cerebral arteries. *Am.J.Physiol.* **265**, H415-H420.

PACAUD, P., LOIRAND, G., BARON, A., MIRONNEAU, C. & MIRONNEAU, J. (1991). Ca^{2+} channel activation and membrane depolarization mediated by Cl^- channels in response to noradrenaline in vascular myocytes. *Br.J.Pharmacol.* **104**, 1000-1006.

PARK, K. W., DAI, H. B., LOWENSTEIN, E. & SELLKE, F. W. (1996). Steady-state myogenic response of rat coronary microvessels is preserved by isoflurane but not by halothane. *Anesth. Analg.* **82**, 969-974.

PAULSON, O. B. & NEWMAN, E. A. (1987). Does the release of potassium from astrocyte endfeet regulate cerebral blood flow? *Science* **237**, 896-898.

PEARSON, J. D., WETTENHALL, R. E., MEANS, A. R., HARTSHORNE, D. J. & KEMP, B. E. (1988). Autoregulation of enzymes by pseudosubstrate prototopes: myosin light chain kinase. *Science* **241**, 970-973.

PENNANEN, M. F., BASS, B. L., DZIKI, A. J. & HARMON, J. W. (1994). Adenosine: differential effect on blood flow to subregions of the upper gastrointestinal tract. *J.Surg.Res* **56**, 461-465.

PFITZER, G. & BOELS, P. J. (1991). Differential skinning of smooth muscle: a new approach to excitation contraction coupling. *Blood Vessels* **28,** 262-267.

PLUM, F., POSNER, J. B. & TROY, B. (1968). Cerebral metabolic and circulatory responses to induced convulsions in animals. *Arch.Neurol.* **18,** 1-14.

PRIOR, H. M., YATES, M. S. & BEECH, D. J. (1999). Role of K^+ channels in A_{2A} adenosine receptor-mediated dilation of the pressurized renal arcuate artery. *Br. J. Pharmacol.* **126,** 494-500.

QUAST, U. & COOK, N. S. (1989). Moving together: K^+ channel openers and ATP-sensitive K^+ channels. *Trends in Pharmacological Science* **10**, 431-435.

QUAYLE, J. M., MCCARRON, J. G., ASBURY, J. R. & NELSON, M. T. (1993a). Single calcium channels in resistance-sized cerebral arteries from rats. *Am.J.Physiol.* **264**, H470-H478.

QUAYLE, J. M., MCCARRON, J. G., BRAYDEN, J. E. & NELSON, M. T. (1993b). Inward rectifier K^+ currents in smooth muscle cells from resistance-sized cerebral arteries. *Am.J.Physiol.* **265**, C1363-C1370.

QUAYLE, J. M., MCCARRON, J. G. & HALPERN, W. (1990). Calcium channels and tone in normotensive and hypertensive rat resistance sized arteries. *Biophysical journal* **57**, 301a(Abstract)

QUAYLE, J. M., NELSON, M. T. & STANDEN, N. B. (1997). ATP-sensitive and inwardly rectifying potassium channels in smooth muscle. *Physiological Reviews* **77**, 1165-1232.

QUAYLE, J. M. & STANDEN, N. B. (1994). K_{ATP} channels in vascular smooth muscle. *Cardiovascular Research* **28**, 797-804.

QUINN, K. & BEECH, D. J. (1998). K^+ channels determining resting potential in arterioles from rabbit cerebral cortex. *Journal of Physiology* **509.P**, 35P-36P.

ROUTLEDGE, H. S., MUIR, T. C., BRAIN, N. J. R., SMITH, G. L. & MCCARRON, J. G. (1998). Effect of adenosine on rat cerebral and mesenteric arteries. *Journal of Physiology* **509P**, P111

RUBANYI, G. M., ROMERO, J. C. & VANHOUTTE, P. C. (1986). Flow-induced release of endothelium-derived relaxing factor. *Am.J.Physiol.* **250**, H1145-H1149.

RUBIO, R., BERNE, R. M. & DOBSON, D. G. J. (1973). Sites of adenosine production in cardiac and skeletal muscle. *Am.J.Physiol.* **225**, H938-H953.

RUEGG, U. T. & BURGESS, G. M. (1989). Staurosporine, K-252 and UCN-01: Potent but nonspecific inhibitors of protein kinases. *Trends in Physiology* **10**, 218-220.

SETOGUCHI, M., OHYA, Y., ABE, I. & FUJISHIMA, M. (1997). Stretch-activated whole-cell currents in smooth muscle cells from mesenteric resistance artery of guinea-pig. *Journal of Physiology* **501.2**, 343-353.

SIEBER, F. E., WILSON, D. A., HANLEY, D. F. & TRAYSTMAN, R. J. (1993). Extracellular potassium activity and cerebral blood flow during moderate hypoglycemia in anesthetized dogs. *Am.J.Physiol.* **264**, H1774-H1780.

SILVER, P. L., KAZMEREZ, W. & DISALVO, J. (1983). *The Journal of Pharmacology and Experimental Therapeutics* **228**, 342-347.

SIMPSON, R. E. & PHILLIS, J. W. (1991). Adenosine deaminase reduces hypoxic and hypercapnic dilatation of pial arterioles: evidence for mediation by adenosine. *Brain Research* **553**, 305-308.

SINGER, H. A., OREN, J. W. & BENSCOTER, H. A. (1989). Myosin light chain phosphorylation in ^{32}P-labelled rabbit aorta stimulated by phorbol 12,13-dibutyrate and phenylephrine. *J. Biol. Chem.* **264**, 21215-21222.

SMITH, G. L. & MILLER, D. J. (1985). Potentiometic measurements of stoichiometric and apparent affinity constants of EGTA for protons and divalent ions including calcium. *Biochem. Biophys. Acta* **839**, 287-299.

SMITH, M. K., COLBRAN, R. J. & SODERLING, T. R. (1990). Specificity's of autoinhibitory domain peptides for four protein kinases. *J. Biol. Chem.* **265**, 1837-1840.

SOMJEN, G. G. (1979). Extracellular potassium in the mammalian central nervous system. *Annu.Rev.Physiol.* **41**, 159-177.

SOMLYO, A. P. & SOMLYO, A. V. (1994). Signal transduction and regulation in smooth muscle. *Nature* **372**, 231-236.

SOUBE, K. & SELLARS, J. R. (1991). Caldesmon, a novel regulatory protein in smooth muscle and nonmuscle actomyosin systems. *J. Biol. Chem.* **266**, 12115-12118.

STANDEN, N. B., QUAYLE, J. M., DAVIES, N. W., BRAYDEN, J. E., HUANG, Y. & NELSON, M. T. (1989). Hyperpolarizing vasodilators activate ATP-sensitive K^+ channels in arterial smooth muscle. *Science* **245**, 177-180.

STANDEN, N. B. & STANFIELD, P. R. (1978). A potential- and time-dependent blockade of inward rectification in frog skeletal muscle fires by barium and strontium ions. *Journal of Physiology* **280**, 169-191.

STULL, J. T., HSU, L.-C., TANSEY, M. G. & KAMM, K. E. (1990). Myosin light chain kinase phosphorylation in tracheal smooth muscle. *J. Biol. Chem.* **265**, 16683-16690.

STULL, J. T., TANSEY, M. G., TANG, D.-C., WORD, R. A. & KAMM, K. E. (1993). Phosphorylation of myosin light-chain kinase - a cellular mechanism for Ca^{2+}-desensitization. *Mol.Cell.Biochem.* **128**, 229-237.

SUN, D., KALEY, G. & KOLLER, A. (1994). Characteristics and origin of myogenic response in isolated gracilis muscle arterioles. *Am.J.Physiol.* **266**, H1177-H1183.

SUN, D., MESSINA, E. J., KALEY, G. & KOLLER, A. (1992). Characteristics and origin of myogenic response in isolated mesenteric arterioles. *Am.J.Physiol.* **263**, H1486-H1491.

SUTTON, T. A. & HAEBERLE, J. R. (1990). Phosphorylation by protein kinase C of the 20'000-Dalton light chain of myosin in intact and chemically skinned vascular smooth muscle. *J. Biol. Chem.* **265**, 2749-2754.

SYKOVA, E. (1983). Extracellular K^+ accumulation in the central nervous system. *Prog.Biophys.Molec.Biol.* **42**, 135-189.

TABRIZCHI, R. & LUPICHUK, S. M. (1995). Vasodilation produced by adenosine in isolated rat perfused mesenteric artery: a role for endothelium. *Naunyn-Schmiedeberg's Arch.Pharmacology* **352**, 412-418.

TAGUCHI, H., HEISTAD, D. D., KITAZONO, T. & FARCACI, F. M. (1994). ATP-Sensitive K^+ channels mediate dilatation of cerebral arterioles during hypoxia. *Circulation Research* **74**, 1005-1008.

TAKENAKA, T., KANNO, Y., KITAMURA, Y., HAYASHI, K., SUZUKI, H. & SARUTA, T. (1996). Role of chloride channels in afferent arteriolar constriction. *Kidney International* **50**, 864-872.

TANSEY, M. G., LUBY-PHELPS, K., KAMM, K. E. & STULL, J. T. (1994). Ca^{2+}-dependent phosphorylation of myosin light chain kinase decreases the Ca^{2+} sensitivity of light chain phosphorylation within smooth muscle cells. *J. Biol. Chem.* **269**, 9912-9920.

THOMAS, R. C. (1972). Electrogenic sodium pump in nerve muscle cells. *Physiological Reviews* **52**, 563-594.

TIAN, R., VOGEL, P., LASSEN, N. A., MULVANY, M. J., ANDREASEN, F. & AALKJAER, C. (1995). Role of extracellular and intracellular acidosis for hypercapnia-induced inhibition of tension of isolated rat cerebral arteries. *Circulation Research* **76**, 269-275.

TODA, N. (1976). Potassium-induced relaxation in isolated cerebral arteries contracted with prostaglandin $F_{2\alpha}$. *Pflügers Arch.* **364**, 235-242.

TODA, N. (1974). Responsiveness to potassium and calcium ions of isolated cerebral arteries. *Am. J. Physiol.* **227,** 1206- 1211.

TODA, N., HATANO, Y. & MORI, K. (1989). Mechanisms underlying responses to hypercapnia and bicarbonate of isolated dog cerebral arteries. *Am. J. Physiol.* **257,** H141- H146.

VAN RIPER, D. A., WEAVER, B. A., STULL, J. T. & REMBOLD, C. M. (1995). Myosin light chain kinase phosphorylation in swine carotid artery contraction and relaxation. *Am.J.Physiol.* **268**, H2466-H2475.

VANRENTERGHEM, C. & LAZDUNSKI, M. (1993). Endothelin and vasopressin activate low conductance chloride channels in aortic smooth muscle cells. *Pflügers Arch.* **425**, 156-163.

VARON, S. S. & SOMJEN, G. G. (1979). Neuron-glia interactions. *Neurosci. Res. Program Bull.* **17**, 1-239.

VILLARROEL, A., ALVAREZ, O., OBERHAUSER, A. & LATORRE, R. (1988). Probing a Ca^{2+}-activated K^+ channel with quaternary ammonium ions. *Pflügers Arch.* **413**, 118-126.

VONBECKERATH, N., CYRYS, S., DISCHNER, A. & DAUT, J. (1991). Hypoxic vasodilatation in isolated, perfused guinea-pig heart: an analysis of the underlying mechanisms. *Journal of Physiology* **442**, 297-319.

WAGNER, J. & RUEGG, J. C. (1986). Skinned smooth muscle: calcium-calmodulin activation independent of myosin phosphorylation. *Pflügers Arch.* **407**, 569-571.

WALLIS, S. J., FIRTH, J. & DUNN, W. R. (1996). Pressure induced myogenic responses in human isolated cerebral resistance arteries. *Stroke* **27**, 2287-2291.

WALMSLEY, J. G., GORE, R. W., DACEY, R. G., DAMON, D. N. & DULING, B. R. (1982). Quantitative morphology of arterioles from the hamster cheek pouch related to mechanical analysis. *Microvascular Research* **24**, 249-271.

WATANABE, J., HORIGUCHI, S., KARIBE, A., KEITOKU, M., TAKEUCHI, M., SATOH, S., TAKISHIMA, T. & SHIRATO, K. (1994). Effects of ryanodine on development of myogenic response in rat small skeletal muscle arteries. *Cardiovascular Research* **28**, 480-484.

WEI, E. P., KONTOS, H. A. & BECKMAN, J. S. (1996). Mechanisms of cerebral vasodilation by superoxide, hydrogen peroxide and peroxynitrate. *Am.J.Physiol.* **271**, H1262-H1266.

WELSH, D. G., NELSON, M. T., ECKMAN, D. M. & BRAYDEN, J. E. (2000). Swelling-activated cation channels mediate depolarization of rat cerebrovascular smooth muscle by hyposmolarity and intravascular pressure. *Journal of Physiology* **527**, 139-148.

WINDER, S. J. & WALSH, M. P. (1990). Smooth muscle calponin. *J. Biol. Chem.* **265**, 10148-10155.

WINDER, S. J. & WALSH, M. P. (1993). Calponin; thin filament-linked regulation of smooth muscle contraction. *Cell Signal* **6**, 677-686.

WINN, H. R., RUBIO, R. & BERNE, R. M. (1981). Brain adenosine concentration during hypoxia in rats. *Am.J.Physiol.* **241**, H235-H242.

WRAY, S. (1988). Smooth muscle intracellular pH: measurement, regulation, and function. *Am.J.Physiol.* C213-C225.

YAMAZAKI, J., DUAN, D., JANIAK, R., KUENZLI, K., HOROWITZ, B. & HUME, J. R. (1998). Functional and molecular expression of volume-regulated chloride channels in canine vascular smooth muscle cells. *Journal of Physiology* **507**, 729-736.

ZARITSKY, J. J., ECKMAN, D. M., WELLMAN, G. C., NELSON, M. T. & SCHWARZ, T. L. (2000). Targeted disruption of Kir2.1 and Kir2.2 genes reveals the essential role of the inwardly rectifying K^+ current in K^+-mediated vasodilation. *Circulation Research* **87**, 160-166.

ZATZ, R., MEYER, T., RENNKE, H. & BRENNER, B. M. (1985). Predominance of hemodynamic rather than metabolic factors in the pathogenesis of diabetic glomerulopathy. *Proc.Natl.Acad.Sci.* **82**, 5963-5967.

ZHANG, G., MIYAHARA, H. & SUZUKI, H. (1989). Inhibitory actions of adenosine differ between ear and mesenteric artery of the rabbit. *Pflügers Arch.* **415**, 56-62.

ZHANG, L., BONEV, A. D., MAWE, G. & NELSON, M. T. (1994). Protein kinase A mediates activation of ATP-sensitive K^+ currents by CGRP in gallbladder smooth muscle. *Am.J.Physiol.* **267**, G494-G499.

ZOU, A., FLEMING, J. T., FALCK, J. R., JACOBS, E. R., GEBREMEDHIN, D., HARDER, D. R. & ROMAN, R. J. (1994). regional stereospecific effects of 11,12-epoxyeicosatrienoic acids on vascular tone and K^+ channel activity in renal arterioles. *Hypertension* **24**, 382(Abstract)