Mechanisms of agonist-induced calcium sensitisation in small arteries.

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ABSTRACT

Agonists acting through G-protein-coupled receptors contract vascular smooth muscle by elevating intracellular calcium and modulating the sensitivity of the contractile apparatus to calcium (calcium sensitisation). In this thesis the receptor agonists noradrenaline, endothelin-1 (ET-1) and vasopressin increased calcium sensitivity in α-toxin permeabilised rat mesenteric small arteries. The phorbol ester phorbol 12,13-dibutyrate (PdBu) and the fatty acid arachidonate (but not the receptor agonist angiotensin II, the phorbol ester 12-O-tetradecanoylphorbol-13-acetate or the lipids phosphatidic acid and lysophosphatidylcholine) increased calcium sensitivity also. The effect of down-regulation of protein kinase C (PKC) and the PKC inhibitor RO-31-8330 on the contractile response to receptor agonists and PdBu in rat mesenteric small arteries was investigated.

Down-regulation of PKCs α and δ by overnight incubation with PdBu completely abolished the contraction to PdBu in intact arteries but had no effect on the contraction to noradrenaline, ET-1 and vasopressin implying phorbol esters and receptor agonists were acting via different mechanisms and/or PKC isotypes to bring about smooth muscle contraction. $3x10^{-7}$M RO-31-8330 significantly reduced the tension developed to maximal doses of ET-1 (100nM) by approximately 30% in both intact and permeabilised rat mesenteric small arteries consistent with a partial role for PKC in the receptor agonist-induced calcium sensitisation in vascular smooth muscle. However, these data suggest another mechanism was additionally involved in this response, for example activation of tyrosine kinases (TKs). Therefore the effect of the TK inhibitors (TKIs) tyrphostin A23 and A47 plus the inactive analogue tyrphostin A1 on the contractile response to ET-1 in rat mesenteric small arteries was investigated.

100μM TKIs A23 and A47 (but not A1) significantly reduced the maximum tension developed to 100nM ET-1 by approximately 90% in intact and 50% in permeabilised arteries implying TKs played a substantial role in the receptor agonist-induced calcium sensitisation in vascular smooth muscle. The maximum ET-1-induced tension in permeabilised rat mesenteric small arteries was reduced by 65% in the presence of both the PKC inhibitor and the active TKI A23 suggesting activation of both PKC and TKs was predominantly responsible for the ET-1-induced increase in calcium sensitivity. However, these data suggest another mechanism was also involved in this effect.

Finally, the possibility that abnormalities in smooth muscle contraction occur in hypertension was investigated in mesenteric, renal and femoral small arteries from SHRs and WKYs at the onset and after hypertension had become established. It was found that the vascular responsiveness, myofilament calcium sensitivity and agonist-induced calcium sensitisation were not enhanced in the SHR compared to WKY at either age or in any vascular bed therefore these factors probably did not contribute to the hypertensive state of the SHR.

Declaration:

I declare that no portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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ABBREVIATIONS

AA	arachidonic acid
AII	angiotensin II
ACh	acetylcholine
ADP	adenosine 5′-diphosphate
AlF_4^-	aluminum fluoride
ATP	adenosine 5′-triphosphate
ATPase	adenosine 5′-triphosphatase
ATPγS	adenosine 5′-O-(3-thiotriphosphate)
AVP	vasopressin
$[Ca^{2+}]_i$	intracellular calcium concentration
CaM	calmodulin
DAG	1,2-diacylglycerol
DMSO	dimethyl sulfoxide
DPB	12-deoxyphorbol 13-isobutyrate
DPG	diphosphoglycerol
EC_{50}	concentration required for 50% contraction
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol-bis(ß-amino-ethyl ether) N,N,N′,N′-tetraacetic acid
ET-1	endothelin-1
G-protein	guanine nucleotide-binding regulatory protein
G_α	G-protein α subunit
$G_{\beta\gamma}$	G-protein βγ subunits
GAP	GTPase-activating protein
GDP	guanosine 5′-diphosphate
GDPßS	guanosine 5′-O-(ß-thiodiphosphate)

GTP	guanosine 5′-triphosphate
GTPase	guanosine 5′-triphosphatase
GTPγS	guanosine 5′-O-(3-thiotriphosphate)
IP_3	inositol 1,4,5-trisphosphate
KCl	potassium chloride
LysoPC	lysophosphatidylcholine
MAP kinase	mitogen-activated protein kinase
MLC	myosin light chain
MLCK	myosin light chain kinase
NA	noradrenaline
PA	phosphatidic acid
PC	phosphatidylcholine
PdBu	phorbol 12,13-dibutyrate
4αPdD	4-α-phorbol 12,13-didecanoate
PDGF	platelet-derived growth factor
$PGF_{2\alpha}$	prostaglandin F2 alpha
PI 3-kinase	phosphatidylinositol 3-kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PIP_3	phosphatidylinositol 3,4,5-trisphosphate
PKC	protein kinase C
PLC	phospholipase C
PLD	phospholipase D
PLA_2	phospholipase A_2
TK	tyrosine kinase
TKI	tyrosine kinase inhibitor
TPA	12-O-tetradecanoylphorbol-13-acetate
SHR	spontaneously hypertensive rat
WKY	Wistar-kyoto rat

CHAPTER ONE

INTRODUCTION

The contractile state of small arteries is a major regulator of peripheral vascular resistance and depends on a variety of stimuli including circulating hormones, neuronally released vasoconstrictor agonists, pressure and additional vasoactive factors released by the endothelium. Abnormalities in these regulatory mechanisms have been implicated in many cardiovascular diseases such as hypertension, arterial narrowing and coronary vasospasm.

Smooth muscle contraction is mediated by two major mechanisms: pharmacomechanical coupling (in response to stimulation by receptor agonists) and electromechanical coupling (Somlyo and Somlyo 1968). Over the past decade the intracellular signal transduction pathways involved in pharmacomechanical coupling have been the subject of intense research. The observation that receptor agonists activate the phosphoinositol cascade (Griendling et al 1986) suggests protein kinase C (PKC) may be involved in the regulation of vascular smooth muscle tone. However, this idea is controversial as later studies showed that the contractile response to receptor agonists in intact vascular smooth muscle tissue was not dependent on the activation of PKC (Shimamoto et al 1992, Hori et al 1993).

More recently receptor agonists have been shown to activate tyrosine kinases (TKs)(Tsuda et al 1991, Koide et al 1992) leading to a role for this signalling pathway in vascular smooth muscle contraction. This hypothesis is supported by

the observation that growth factors, whose effects are mediated by tyrosine phosphorylation, can increase vascular tone (Hollenberg 1994, Hughes 1995). However, the precise role of TKs in the receptor agonist-induced contraction in vascular smooth muscle has yet to be determined.

A major component of pharmacomechanical coupling in smooth muscle is a G-protein-mediated increase in the sensitivity of the contractile apparatus to calcium. Calcium sensitisation has been clearly demonstrated in permeabilised vascular smooth muscle preparations in which receptor agonists can potentiate a fixed, sub-maximal calcium-induced contraction in the presence of GTP (Nishimura et al 1988, Kitazawa et al 1989). A potential role for PKC, TKs and arachidonic acid in the receptor agonist-induced calcium sensitisation has been proposed, but such investigations have only concentrated on one mechanism at a time.

The vasoconstrictor agonist-induced contraction in small arteries has been extensively studied using the myograph but few investigators have used this technique to examine the signal transduction pathways involved in pharmacomechanical coupling. Therefore, the purpose of this thesis was to study the involvement of both PKC and TKs in the contractile response to vasoconstrictor agonists in intact and permeabilised rat mesenteric small arteries mounted in a wire myograph (Mulvany and Halpern 1977). Finally, the possibility that abnormalities in vascular responsiveness, myofilament calcium sensitivity and agonist-induced calcium sensitisation occur in hypertension was also investigated.

1. Excitation-contraction coupling.

The primary event initiating smooth muscle contraction is an increase in the intracellular calcium concentration ($[Ca^{2+}]_i$). Calcium binds to calmodulin (CaM) to form Ca_4^{2+}.CaM inducing a conformational change in CaM which exposes the site(s) of interaction with the target protein - in this case an enzyme myosin light chain kinase (MLCK)(Walsh 1994). The ternary complex Ca_4^{2+}.CaM.MLCK is generated which catalyses the phosphorylation of Ser^{19} on the two 20kDa myosin light chains (MLCs) of myosin. This activates actomyosin ATPase resulting in cross-bridge cycling and smooth muscle contraction (Walsh 1994). Relaxation occurs when $[Ca^{2+}]_i$ falls leading to a dissociation of the Ca_4^{2+}.CaM.MLCK complex and regeneration of the inactive MLCK. Myosin then becomes dephosphorylated by smooth muscle phosphatase (smooth muscle PP1M)(Alessi et al 1992), the myosin cross-bridges detach from the actin filaments and the muscle relaxes (Walsh 1994).

1.1. Electromechanical coupling.

Regulation of $[Ca^{2+}]_i$ by changes in membrane potential has been termed 'electromechanical coupling' (Somlyo and Somlyo 1968). The resting membrane potential of smooth muscle is negative with respect to the extracellular space and more positive potentials depolarise the membrane opening voltage-gated calcium channels. This causes calcium influx to increase $[Ca^{2+}]_i$ and trigger smooth muscle contraction (Somlyo and Himpens 1989).

1.2. Pharmacomechanical coupling.

Regulation of $[Ca^{2+}]_i$ independent of changes in membrane

potential has been termed 'pharmacomechanical coupling' (Somlyo and Somlyo 1968). The major mechanism of pharmacomechanical coupling involves the binding of a contractile agonist eg vasopressin (AVP), endothelin (ET-1), angiotensin II (AII) and α_1 adrenoceptor agonists such as noradrenaline (NA) and phenylephrine (PE) to its membrane receptor leading to the activation of guanine nucleotide-binding regulatory proteins (G-proteins). This increases the production of inositol 1,4,5-trisphosphate (IP_3) via the phosphoinositol cascade which releases calcium from the intracellular stores. Contractile agonists can also open receptor-operated calcium channels but calcium influx through these channels probably does not make a significant contribution to excitation-contraction coupling (Somlyo and Somlyo 1994). However, the depolarisation due to the opening of receptor-operated calcium channels may cause calcium influx via the opening of voltage-gated calcium channels (Somlyo and Somlyo 1994). Finally, pharmacomechanical mechanisms can also contract or relax smooth muscle without a change in $[Ca^{2+}]_i$ via a contractile agonist-induced sensitisation or desensitisation of MLC phosphorylation to calcium (Somlyo and Somlyo 1994).

2. <u>G-proteins.</u>

There are two major families of G-protein involved in signal transduction: 1. heterotrimeric G-proteins which act as transducers for the seven-transmembrane-spanning receptors and 2. 'small' cytoplasmic G-proteins found as single polypeptides composed of about 200 amino acids, such as the ras p21 and rho p21 gene products (Simon et al 1991).

Heterotrimeric G-proteins are composed of α, ß and γ subunits and are classified by the identity of their distinct α subunits. To date seventeen different G_α subunits have been identified in mammalian tissue which can be divided into four sub-families on the basis of their derived amino acid sequences: G_s, G_i/G_o, G_q and G_{12} (Simon et al 1991). These sub-families can be separated into two further groups depending on their sensitivity or insensitivity to pertussis toxin. This toxin catalyses the ADP-ribosylation of the G_α subunit thereby uncoupling the G-protein from the receptor. $G_{\alpha q}$, which is insensitive to pertussis toxin, was first characterised by Strathmann and Simon in 1990 and is the α subunit of the heterotrimeric G-protein involved in the phosphoinositol cascade (Rhee and Choi 1992).

3. Phosphoinositol cascade.

The binding of a vasoconstrictor agonist to its specific receptor on the smooth muscle cell membrane causes a conformational change in the receptor which initiates the exchange of GDP for GTP on the $G_{\alpha q}$ subunit (Simon et al 1991). This decreases the affinity of $G_{\alpha q}$ for its ßγ subunits and they dissociate. GTP-bound $G_{\alpha q}$ is then able to interact with the effector protein - the enzyme phospholipase C (PLC) in this case. Hydrolysis of GTP to GDP by the intrinsic GTPase activity of $G_{\alpha q}$ increases the affinity of $G_{\alpha q}$ for its ßγ subunits and the receptor finishes the cycle (Simon et al 1991). There are three major family members of PLC: ß, γ, and δ and multiple subtypes of each have been identified (Rhee and Choi 1992). $G_{\alpha q}$ activates PLCß1 and PLCß3 whereas the $G_{\beta\gamma}$ subunits activate PLCß2 and PLCß3 (Smrcka and Sternweis

1993). PLCß catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate two intracellular messengers: IP_3 and 1,2-diacylglycerol (DAG)(Figure 1.1). IP_3 is water- soluble and diffuses into the cytoplasm to interact with the IP_3 receptor located on the sarcoplasmic reticulum triggering the release of calcium (Berridge 1993). DAG remains within the membrane due to its lipophilic nature and activates the enzyme protein kinase C (Nishizuka 1986).

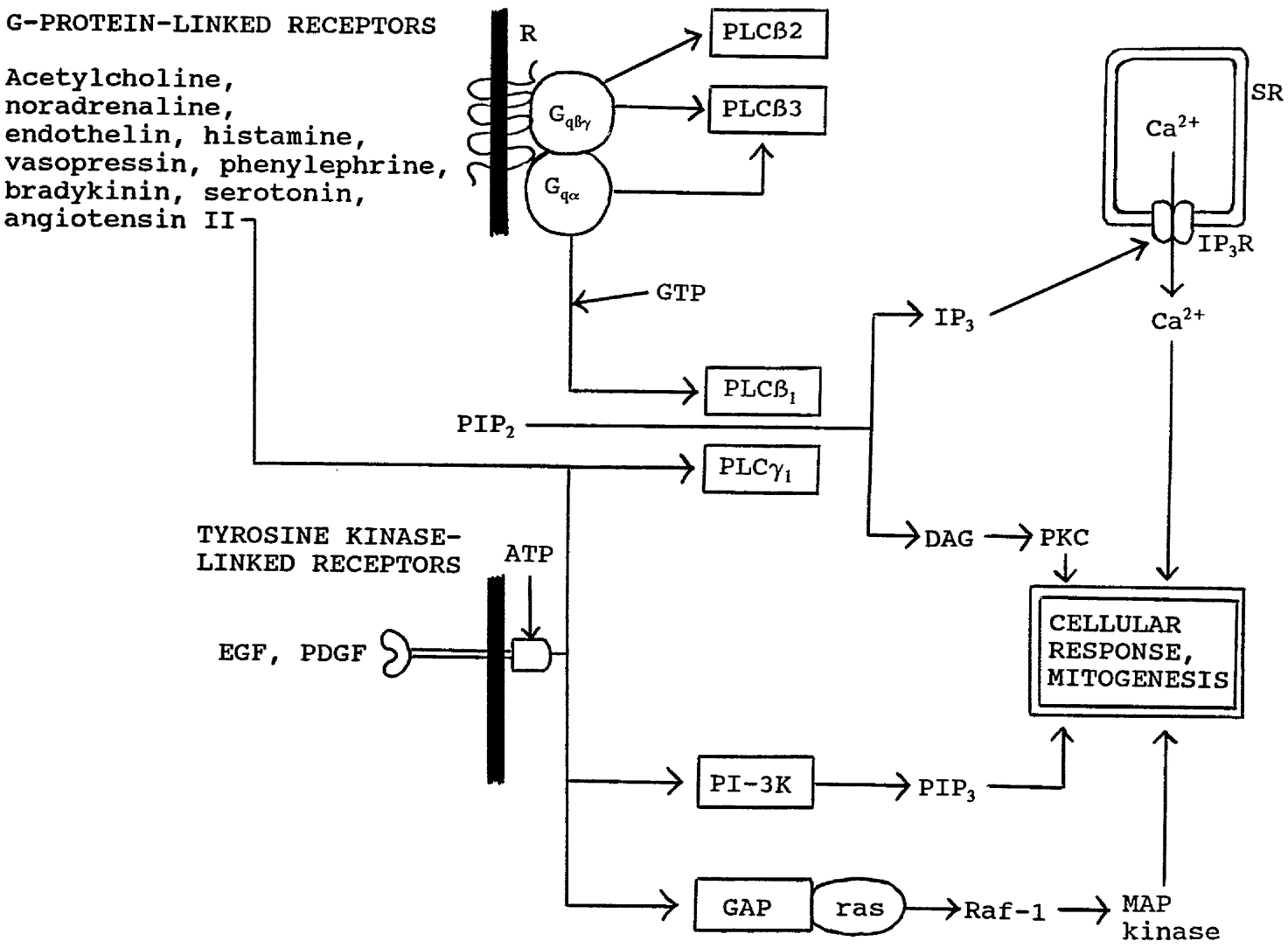
4. Phospholipase C.

It has been recently shown that AII can stimulate tyrosine phosphorylation of PLCγ in cultured rat aortic vascular smooth muscle cells (Marrero et al 1994, see Page 46). This lead to the production of IP_3 thereby questioning the involvement of PLCß in the AII-mediated hydrolysis of PIP_2. Indeed, Marrero et al (1994) failed to detect PLCß isoforms in their system confirming earlier studies using rat (Kato et al 1992) and rabbit (Homma et al 1993) aortic smooth muscle tissue. Despite the lack of expression of PLCß in vascular smooth muscle it has been detected in guinea pig intestinal (Murthy and Makhlouf 1995) and bovine iris sphincter (Zhou et al 1993) smooth muscle.

5. Calcium sensitisation.

In 1981 Dillon et al found the levels of MLC phosphorylation in swine carotid arterial strips rose initially upon stimulation with K^+ to reach maximum values at about 1 minute before declining slowly over a period of 20 minutes to values close to basal levels despite a sustained elevation of contractile force. This sustained contraction in the face of

FIGURE 1.1 Diagram summarising the major receptor activated pathways for stimulating the formation of inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG). Many agonists bind to 7-membrane-spanning domain receptors (R) which use a GTP-binding protein (G) to activate phospholipase C (PLC)ß1 whereas PLCγ1 is activated by the tyrosine kinase-linked receptors. Both PLCß1 and PLCγ1 cause the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate IP_3 which releases calcium from the intracellular stores and DAG which activates protein kinase C (PKC). The tyrosine kinase-linked receptors can also stimulate other effectors including phosphatidylinositol 3-kinase (PI-3K) which generates the lipid second messenger phosphatidylinositol 3,4,5-trisphosphate (PIP_3) and the GTPase-activating protein (GAP) which regulates ras. ras stimulates Raf-1 which in turn stimulates mitogen-activated protein kinase (MAP kinase). SR, sarcoplasmic reticulum; IP_3R, IP_3 receptor; PDGF, platelet-derived growth factor; EGF, epidermal growth factor. Modified from Berridge (1993).

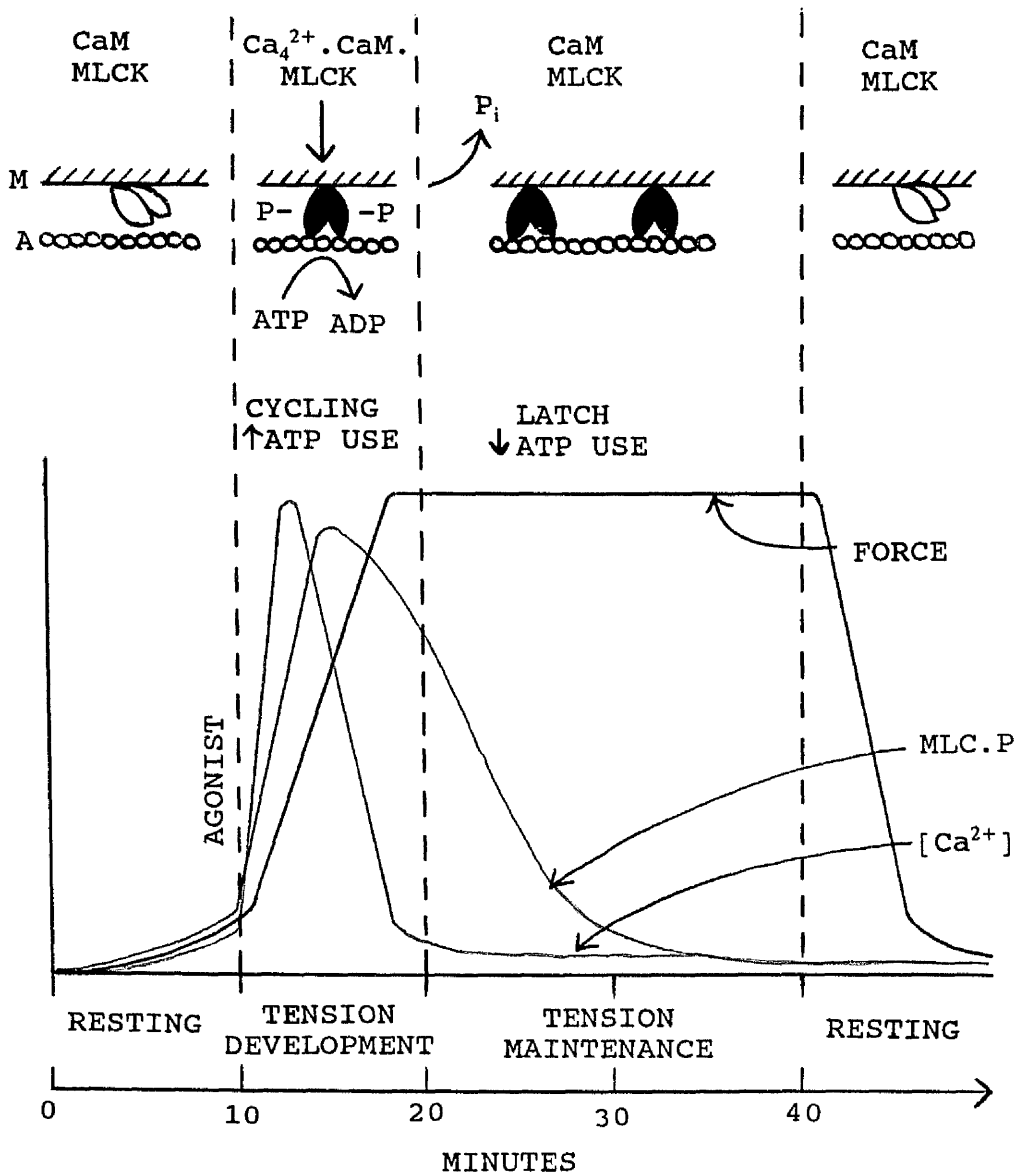

G-PROTEIN-LINKED RECEPTORS
Acetylcholine,
noradrenaline,
endothelin, histamine,
vasopressin, phenylephrine,
bradykinin, serotonin,
angiotensin II
R
PLCß2
PLCß3
$G_{q\beta\gamma}$
$G_{q\alpha}$
GTP
SR
Ca^{2+}
IP_3R
IP_3
Ca^{2+}
PLCß$_1$
PIP_2
PLCγ_1
TYROSINE KINASE-
LINKED RECEPTORS
ATP
DAG
PKC
CELLULAR
RESPONSE,
MITOGENESIS
EGF, PDGF
PI-3K
PIP_3
GAP
ras
Raf-1
MAP
kinase

transient levels of MLC phosphorylation led to the development of the latch bridge hypothesis (Figure 1.2). In this hypothesis following the formation of cycling actin-myosin cross-bridges responsible for initiating smooth muscle contraction the cross-bridges remained attached but became dephosphorylated to generate a non-cycling, long-lasting actin-myosin bridge - the latch bridge - which was capable of maintaining force (Dillon et al 1981).

In a later study the increase in $[Ca^{2+}]_i$, as measured by the photoprotein aequorin, following the addition of PE to cells of the ferret portal vein was also transient (Morgan and Morgan 1984). The $[Ca^{2+}]_i$ reached a peak within 1 minute following stimulation but then returned to values slightly above basal levels after about 5 minutes whereas the contractile force was maintained (Morgan and Morgan 1984). Subsequently it was postulated that the formation of latch bridges was highly sensitive to calcium therefore it still occurred when $[Ca^{2+}]_i$ fell to basal levels (Rasmussen et al 1987). However, as yet there has been no molecular evidence for such latch bridges therefore a second mechanism may have been involved in the sustained phase of the contractile response to vasoconstrictor agonists.

An alternative proposal is that certain agonists have the ability to sensitise the contractile apparatus to calcium thereby increasing the force/$[Ca^{2+}]_i$ ratio - known as 'calcium sensitisation'. Studies with fluorescent calcium indicators in intact vascular smooth muscle preparations have demonstrated the force/$[Ca^{2+}]_i$ relationship was indeed higher

FIGURE 1.2 Schematic representation of the latch bridge model of smooth muscle contraction. Depicted (*lower frame*) are the ————muscle force, ———— myosin light chain (MLC) phosphorylation (MLC.P), ————intracellular calcium concentration $[Ca^{2+}]_i$ when the muscle is resting (in the absence of agonist), *left*; during tension development, *centre*; during tension maintenance, *right*. The upper part of the diagram shows the states of interaction between myosin (M) and actin (A) filaments during each of these phases. At rest the $[Ca^{2+}]_i$ is low, calmodulin is not associated with myosin light chain kinase (MLCK), the MLCs are not phosphorylated (00) and hence there are no cross-bridges between M and A filaments. On the addition of an agonist at 10 minutes there is a transient increase in $[Ca^{2+}]_i$ and Ca_4.CaM becomes associated with MLCK. Ca_4.CaM.MLCK phosphorylates MLC (P-●●-P) and the M and A filaments form cross-bridges resulting in the development of tension. During tension development myosin ATPase activity is high, ATP utilisation is increased as well as oxygen consumption. During tension maintenance MLC.P becomes dephosphorylated with the formation of latch bridges (●●) between M and A filaments. As a result ATP utilisation and oxygen consumption fall but tension is maintained. Modified from Rasmussen et al (1987).


CaM
MLCK
Ca_4^{2+}.CaM.
MLCK
CaM
MLCK
CaM
MLCK
P_i
M
A
P-
-P
ATP
ADP
CYCLING
↑ATP USE
LATCH
↓ATP USE
FORCE
MLC.P
$[Ca^{2+}]_i$
AGONIST
RESTING
TENSION
DEVELOPMENT
TENSION
MAINTENANCE
RESTING
0
10
20
30
40
MINUTES

during agonist-induced than during high K^+-induced contractions (Bradley and Morgan 1987, Sato et al 1988). Furthermore, Jensen et al (1992) found the concentration/response curve to NA was shifted to the left in relation to the concentration/response curve to K^+ in rat mesenteric small arteries. In a recent study 0.1μM PE and 30mM K^+ increased the Fura-2 estimated $[Ca^{2+}]_i$ to similar levels in rat tail arteries but the K^+-induced contraction was only 47% as large as the PE-induced contraction (Chen and Rembold 1995). Consequently, Chen and Rembold (1995) postulated that approximately 50% of the contraction to PE was caused by an enhancement of the sensitivity of the contractile apparatus to calcium. Calcium sensitisation has been more clearly demonstrated using permeabilised vascular smooth muscle preparations in which calcium can be maintained at a constant level across the plasma membrane (Nishimura et al 1988, Kitazawa et al 1989).

6. <u>Methods of permeabilisation.</u>

The first permeabilisation experiment using smooth muscle was performed by Briggs (1963) by exposing guinea pig uterine fibres to 50% glycerol for four days up to four months. This technique was used to demonstrate the dependence of smooth muscle contractions on Mg_2ATP. Later Filo et al (1965) used glycerinated hog skeletal and smooth muscle fibres to demonstrate these contractions were dependent on calcium also. An alternative technique consisted of a brief exposure to EDTA which was employed to investigate the pH dependence of calcium activation and ATPase activity in skinned muscle fibre bundles of pig carotid arteries (Mrwa et al 1974).

However in both glycerol- and EDTA-treated smooth muscle the maximum tension developed to maximal calcium concentrations was only 5 to 10% of the maximum tension developed to contractile agonists in intact smooth muscle prior to the skinning procedure.

A more successful method for permeabilisation was subsequently discovered using detergents such as Triton X-100 and saponin. Gordon (1978) exposed strips of rabbit taenia coli to 1% Triton X-100 (a non-ionic detergent) overnight and found they developed up to 100% of the maximum tension in response to maximal calcium concentrations compared to that produced by intact strips in response to tetanus stimulation. Similarly, the maximum contraction induced by a high calcium concentration in fibres from guinea pig taenia coli skinned with the plant glycoside saponin was comparable to the potassium-induced contraction in intact fibres (Saida and Nonomura 1978). However, saponin uncouples receptors involved in the physiological activation of smooth muscle and therefore these preparations do not respond to vasoconstrictor agonists (Somlyo et al 1985).

Cassidy et al (1979) first permeabilised smooth muscle using the bacterial exotoxin Staphylococcal aureus α-toxin. This was achieved by exposing rabbit ileal strips to the toxin for 5 to 10 minutes. Incubation of the skinned strips in the ATP analogue ATPγS (this thiophosphorylated the light chains of myosin which became resistant to dephosphorylation by the phosphatase resulting in a locked state of myosin activation) caused a development of tension in the presence of calcium.

These results demonstrated that the calcium control of smooth muscle contraction involved a MLC kinase/phosphatase system.

Staphylococcal aureus α-toxin (α-toxin) is a single chain polypeptide of molecular weight (M_r) 34,000kDa which creates pores in the plasma membrane 3nM in diameter (Füssle et al 1981). This limited pore size permits the passage of low molecular weight solutes (such as ATP, inorganic ions and EGTA) into the cell, but high molecular weight solutes of M_r>4,000kDa (including soluble intracellular proteins such as calmodulin and PKC) are not lost from the cytosol (Füssle et al 1981, McEwen and Arion 1985, Ahnert-Hilger et al 1985, Bader et al 1986). It is unlikely the α-toxin molecules can penetrate into the cell and studies have demonstrated the intracellular membranes remained intact following treatment with α-toxin (McEwen and Arion 1985, Ahnert-Hilger et al 1985, Bader et al 1986).

Crude α-toxin was purified by Lind et al (1987) and subsequently used to investigate the temperature dependence of the intracellular calcium release mechanism 'calcium-induced calcium release' in skinned frog muscle fibres (Horiuti 1988). Fujii and Sakurai (1989) and Fink et al (1989) reported that purified α-toxin could stimulate the release of arachidonic acid (AA) in isolated rat aorta and cultured phaochromocytoma (PC12) cells respectively. This led to a role for AA metabolites in the contractile response to α-toxin (Fujii and Sakurai 1989) and in the pathogenic action of α-toxin (Fink et al 1989).

Nishimura et al (1988) and Kitazawa et al (1989) first demonstrated that α-toxin permeabilised vascular smooth muscle preparations retain receptor-effector coupling and responsiveness to vasoconstrictor agonists thereby allowing the process of calcium sensitisation to be investigated. They showed that both α adrenoceptor (Nishimura et al 1988, Kitazawa et al 1989) and muscarinic (Kitazawa et al 1989) agonists can markedly potentiate the contraction to a given, sub-maximal level of calcium. These potentiated contractions were mimicked by GTP and the non-hydrolysable GTP analogue GTPγS (Nishimura et al 1988, Kitazawa et al 1989) and inhibited by the non-hydrolysable GDP analogue GDPßS (Kitazawa et al 1989) which suggested the receptor agonist-induced calcium sensitisation was mediated by G-proteins.

However, it is still not known whether the G-protein involved in calcium sensitisation belongs to the membrane-associated heterotrimeric G-proteins or the 'small' cytoplasmic G-proteins. The fact that aluminum fluoride (AlF_4^-) caused contractions in intact (Boonen and De Mey 1990a, Jensen et al 1993) and permeabilised (Kawase and van Breeman 1992, Fujita et al 1995) vascular smooth muscle implied the G-protein was a heterotrimeric G-protein as AlF_4^- did not interact with the 'small' G-proteins (Kahn 1991). However, it has been shown that ras p21 can potentiate a sub-maximal calcium-induced contraction in ß-escin (see Material and Methods) skinned guinea pig mesenteric microarteries (Satoh et al 1993). Furthermore, the bacterial exoenzymes EDIN and C3 (which are known to ADP-ribosylate and inactivate the rho p21 family) inhibited the GTP- (Fujita et al 1995, Gong et al 1996),

GTPγS- (Hirata et al 1992, Fujita et al 1995, Noda et al 1995, Itagaki et al 1995), NA- (Fujita et al 1995), carbachol- (Itagaki et al 1995, Gong et al 1996) and ET-1- (Gong et al 1996) induced increase in calcium sensitivity of MLC phosphorylation. These studies suggested the 'small' G-proteins ras p21 and rho p21 were involved in receptor agonist-induced calcium sensitisation.

Fujiwara et al (1989) first reported using saponin-skinned strips from rabbit mesenteric arteries that the GTPγS-induced increase in calcium sensitivity of the contractile apparatus was associated with an increase in MLC phosphorylation. This finding was reproduced in subsequent investigations using both GTPγS and vasoconstrictor agonists in permeabilised vascular smooth muscle (Kitazawa and Somlyo 1990, Kitazawa et al 1991a, Kitazawa et al 1991b, Kubota et al 1992). Furthermore, Kubota et al (1992) demonstrated that the phosphorylation of MLC occurred at Ser^{19}, the site phosphorylated by MLCK, in saponin-skinned bovine tracheal smooth muscle stimulated with GTPγS. Since GTPγS did not significantly affect MLCK activity but inhibited MLC phosphatase activity it was proposed that the increased MLC phosphorylation during G-protein-mediated calcium sensitisation was due to inhibition of MLC phosphatase (Kubota et al 1992). This hypothesis was supported by an earlier report in which pretreatment with okadaic acid (a relatively specific inhibitor of MLC phosphatase) prevented the rapid decline in force developed to a sub-maximal calcium concentration in α-toxin permeabilised phasic smooth muscle preparations from the guinea pig ileum (Somlyo et al 1989).

Therefore, Somlyo et al (1989) concluded MLC phosphatase was the major factor controlling the regulation of the contractile response of smooth muscle to calcium.

The protein phosphatase responsible for dephosphorylation of smooth muscle MLC, smooth muscle PP1M, has recently been purified from chicken gizzard myofibrils and is a heterotrimer composed of three subunits with molecular masses of 130, 37 and 20 kDa (Alessi et al 1992) which is bound to myosin filaments by 'targeting subunits' (Shirazi et al 1994). As MLC phosphatase is associated with the intracellular myosin filaments and the receptors and G-proteins for calcium-sensitising agonists are located at the plasma membrane there may be a messenger or cascade which relays the inhibitory message from the membrane to the filament-bound MLC phosphatase. Gong et al (1992) proposed this messenger to be AA and found it increased the sub-maximal calcium-induced contraction and MLC phosphorylation in α-toxin permeabilised rabbit femoral arteries. Furthermore, AA dissociated the trimeric MLC phosphatase into its constituent subunits in cell-free assays thereby preventing its inhibitory action on MLC (Gong et al 1992). Gong et al (1995) demonstrated a number of calcium-sensitising agents including GTPγS, PE, carbachol and the phorbol ester phorbol 12,13-dibutyrate (PdBu) increased AA and DAG levels in permeabilised arteries which reinforced a role for AA as a potential second messenger in the agonist-induced calcium sensitisation.

However, Moreland et al (1992a) found the increase in MLC

phosphorylation during the NA-induced contraction in α-toxin permeabilised rabbit mesenteric arteries was only transient despite a constant $[Ca^{2+}]_i$ and monotonic increase in force. Therefore it was speculated that the enhancement of myofilament calcium sensitivity which may have been responsible for the sustained phase of the contractile response to vasoconstrictor agonists in vascular smooth muscle required an additional component other than phosphorylation of MLC, for example phosphorylation of proteins by protein kinase C (Moreland et al 1992a).

7. Protein kinase C.

Protein kinase C (PKC) was first identified by Nishizuka and colleagues in 1977 (Inoue et al 1977) and was later found to be a calcium-activated, phospholipid-dependent enzyme (Takai et al 1979). Protein kinase C phosphorylates proteins on serine and threonine residues and consists of a single polypeptide chain of molecular weight 77,000 composed of two functionally different domains (Figure 1.3). The catalytic domain contains the ATP-binding site and the regulatory domain contains the binding sites for calcium, phosphatidylserine and DAG or phorbol esters plus an autoinhibitory pseudosubstrate sequence (Nishizuka 1992).

7.1. PKC isotypes.

Eleven different PKC isotypes have been identified in mammalian tissue so far which differ in tissue expression, intracellular location and enzymological properties (Nishizuka 1995). Group A consists of four classical or conventional PKCs (cPKC): α, ßI, ßII, and γ that require

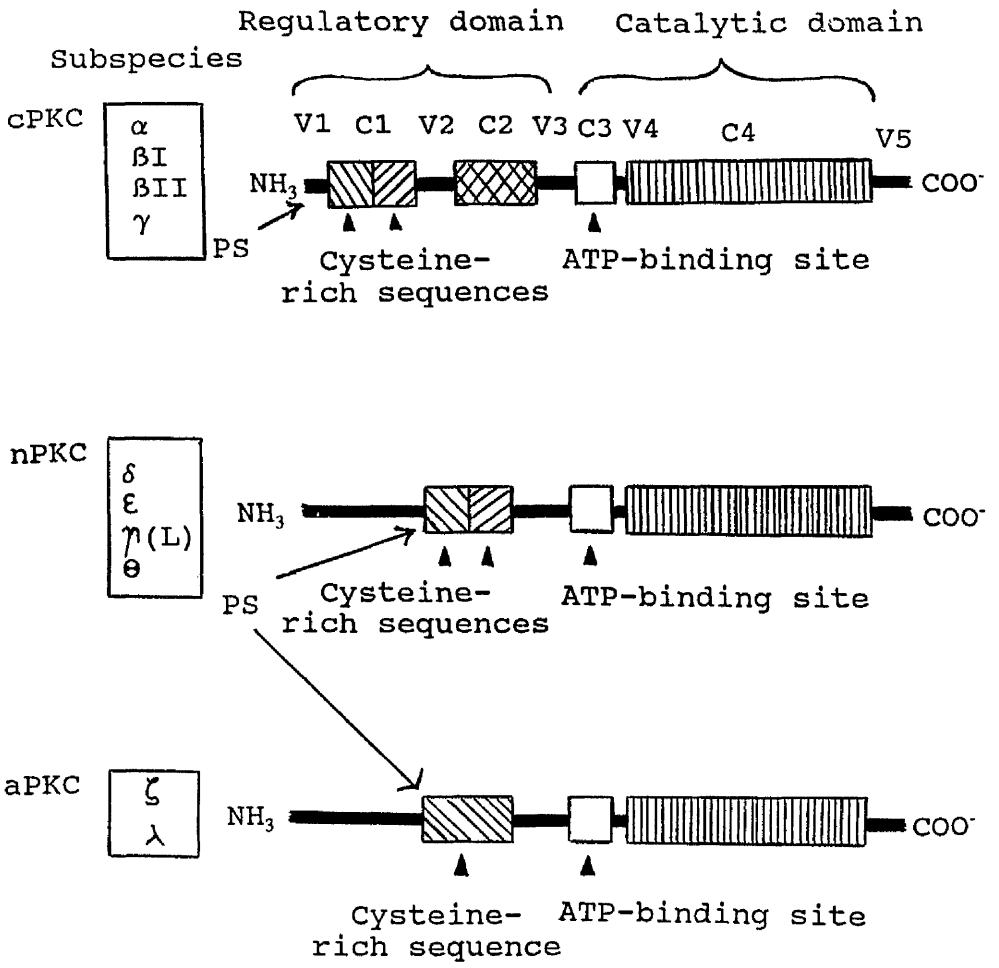


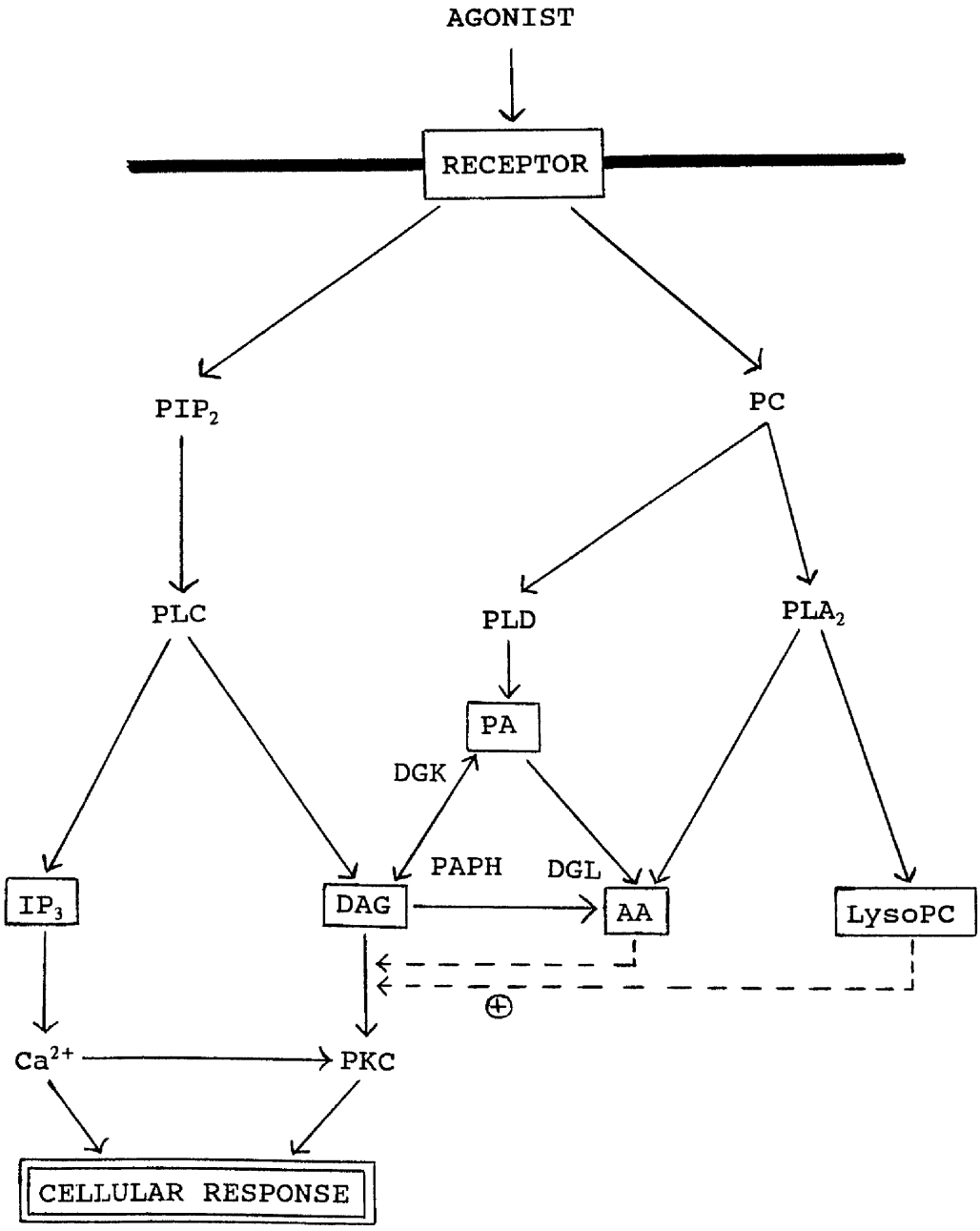
FIGURE 1.3 Structure of protein kinase C (PKC) isotypes. Each isotype has an amino (NH_3) terminal regulatory domain and a carboxy (COO^-) terminal catalytic domain. The cPKC subspecies has five variable (V1-V5) and four conserved (C1-C4) regions. Locations of the pseudosubstrate sequence (PS) and ATP-binding site are indicated. Cysteine-rich sequences are located in the C1 domain; two in each of α, β, γ, δ, ε, η and θ isotypes and one in the ζ and λ isotypes. The nPKC subspecies lack the C2 domain and exhibit calcium-independent activity. Modified from Nishizuka (1992).

calcium, phosphatidylserine and DAG or phorbol esters for their activation, for which activation is enhanced by cis-unsaturated fatty acids (such as AA) and lysophosphatidylcholine (Nishizuka 1995)(Figure 1.4). GroupB consists of five new PKCs (nPKC): δ, ε, η (L), θ and μ that are not dependent on calcium but require phosphatidylserine and DAG or phorbol esters for their activation (whether μ is a member of the PKC family is still a matter of debate however). The ε, but not δ, isotype is activated by cis-unsaturated fatty acids. δ, ε and η(L) isotypes are activated by phosphatidylinositol 3,4,5-trisphosphate (PIP_3) and to a lesser extent by PIP_2 (Nishizuka 1995). Finally, group C consists of two atypical PKCs (aPKC): ζ and λ. ζ is activated by phosphatidylserine, cis-unsaturated fatty acids, PIP_2 and PIP_3, but λ has not yet been characterised (Nishizuka 1995).

7.2. PKC and vascular smooth muscle contraction.

The potential involvement of PKC in the regulation of smooth muscle contraction was initially suggested following the observation that the tumour-promoting phorbol esters can activate PKC (Castagna et al 1982). Phorbol esters can induce slowly developing, sustained contractions in intact vascular smooth muscle (Danthuluri and Deth 1984, Rasmussen et al 1984) as a result of direct or indirect effects of PKC stimulation. As these contractions appeared to be dependent on extracellular calcium (Danthuluri and Deth 1984, Rasmussen et al 1984) this may have been a PKC-mediated phosphorylation of the voltage-gated calcium channels leading to an increase in $[Ca^{2+}]_i$ and phosphorylation of MLC.

FIGURE 1.4 Schematic representation of receptor agonist-induced membrane phospholipid degradation. Following the binding of an agonist to its membrane receptor phosphatidylinositol 4,5-bisphosphate (PIP_2) is hydrolysed by phospholipase C (PLC) to generate inositol 1,4,5-trisphosphate (IP_3) which releases calcium from the intracellular stores and 1,2-diacylglycerol (DAG) which activates protein kinase C (PKC). Agonist-receptor binding also results in the hydrolysis of phosphatidylcholine (PC) by phospholipase A_2 (PLA_2) to generate arachidonic acid (AA) and lysophosphatidylcholine (LysoPC) and both of these can stimulate the activation of PKC by DAG. Hydrolysis of PC by phospholipase D (PLD) results in the formation of phosphatidic acid (PA) which can be converted to DAG and AA by the action of phosphatidic acid phosphohydrolase (PAPH) and diacylglycerol lipase (DGL) respectively. DAG in turn can be converted to PA by the action of diacylglycerol kinase (DGK) and to AA by the action of DGL. + stimulation. Modified from Nishizuka (1995).


AGONIST
RECEPTOR
PIP2
PC
PLC
PLD
PLA2
PA
DGK
PAPH
DGL
IP3
DAG
AA
LysoPC
Ca2+
PKC
CELLULAR RESPONSE

However, in more recent studies the phorbol ester-induced contraction in intact and permeabilised vascular smooth muscle occurred without a change in $[Ca^{2+}]_i$ (Chatterjee and Tejada 1986, Jiang and Morgan 1987) or MLC phosphorylation (Chatterjee and Tejada 1986). Therefore, Chatterjee and Tejada (1986) and Jiang and Morgan (1987) proposed this contraction to be due to an increase in the sensitivity of the myofilaments to calcium mediated by PKC. This hypothesis was supported by later studies in which phorbol esters significantly shifted the pCa-tension curve to the left in permeabilised rat (Drenth et al 1989, Nishimura et al 1990, Jensen 1996) and rabbit (Nishimura et al 1991, Masuo et al 1994, Yoshida et al 1994) mesenteric arteries. Furthermore, synthetic DAGs, which also activate PKC directly, increased myofilament calcium sensitivity in permeabilised arteries (Masuo et al 1994, Jensen 1996).

7.3. Calcium-independent contractions.

Alternatively the phorbol ester-induced contraction may involve the activation of calcium-independent PKC isotypes. There is other evidence for this as the PE-induced contraction in isolated saponin-skinned ferret aortic smooth muscle cells occurred at constant $[Ca^{2+}]_i$ and was blocked by a pseudosubstrate peptide PKC inhibitor (Collins et al 1992). This was probably not due to an enhanced myofilament calcium sensitivity but to a calcium-independent contraction as the response to PE was unchanged over the pCa range 7.0-8.6 (Collins et al 1992). The PE-induced contraction was accompanied by a translocation of the calcium-independent PKC ε isotype to the plasma membrane therefore it was speculated

that PKCε was involved in the contractile response to PE in this system (Khalil et al 1992).

In the case of α_1 adrenoceptor agonists the activation of calcium-independent PKC isotypes may have been due to DAG generated by the phospholipase D (PLD)-catalysed hydrolysis of phosphatidylcholine (PC)(Figure 1.4). However, in a recent study stimulation of PLD-hydrolysis by NA in rat small arteries did not result in an increase in DAG levels (Ward et al 1995). The immediate product of PC hydrolysis by PLD is phosphatidic acid (PA) which induced contractions in isolated smooth muscle cells (Salmon and Honeyman 1980). The mechanism for this is still unclear but may have been via an increase in $[Ca^{2+}]_i$ (Salmon and Honeyman 1980) or enhancement of inositol lipid hydrolysis (Jackowski and Rock 1989). However, there is evidence PA can itself activate PKC (Nishizuka 1995). Ohanian et al (1990) showed that after the addition of the three vasoconstrictor agonists AII, AVP and NA to intact rat subcutaneous small arteries only AII led to an accumulation of DAG whereas DAG was converted to PA in the case of AVP and NA. Therefore it appears the second messengers generated upon cell stimulation is dependent on the agonist used which may lead to an agonist-specific activation of the PKC isotypes.

In phorbol ester-induced contractions where $[Ca^{2+}]_i$ was not increased and there were low levels of MLC phosphorylation other possible substrates for PKC were proposed - namely the thin filaments calponin and caldesmon or intermediate filaments.

7.4. Calponin.

Calponin is an actin- and calmodulin- binding protein found exclusively in smooth muscle which inhibited actomyosin ATPase via its interaction with actin (Winder and Walsh 1993). Calponin can be phosphorylated by PKC and calmodulin kinase II and phosphorylation by either reversed the inhibition of actomyosin ATPase resulting in cross-bridge cycling and smooth muscle contraction (Winder and Walsh 1993). Indeed, the phosphorylation of calponin in vivo by PKC may have modulated in part the contraction of strips of porcine coronary artery to ET-1 and PdBu (Mino et al 1995).

7.5. Caldesmon.

Caldesmon is another actin- and calmodulin-binding protein found in both smooth muscle and non-muscle cells which also inhibited actomyosin ATPase activity (Walsh 1994). Similar to calponin phosphorylation of caldesmon in vitro by PKC reversed its ability to inhibit actomyosin ATPase leading to smooth muscle contraction (Walsh 1994). However, the phosphopeptide maps of caldesmon phosphorylated in intact porcine carotid arteries showed little similarity to phosphopeptide maps of caldesmon phosphorylated in vitro by PKC or calmodulin kinase II (Adam et al 1989).

7.6. Intermediate filaments.

The skeletal framework of the cell (the cytoskeleton) is made up from two distinct fibrillar domains (Small et al 1986): the contractile filaments containing actin, myosin, tropomyosin and caldesmon and longitudinally arranged intermediate filaments (IFs) containing filamin, actin and

desmin (and other IF proteins for example synemin). It has been suggested that the contractile filament domain was responsible for initiation of contraction whereas the IF domain was responsible for the tonic phase of contraction via the phosphorylation of desmin and synemin by PKC (Rasmussen et al 1987). In the latter case a low rate of cross-bridge cycling resulted (latch state) which was not caused by specific latch bridges between actin and myosin filaments but by a reorganisation and stabilisation of the components of the IF domain (Rasmussen et al 1987).

The participation of PKC in the vasoconstrictor agonist-induced contraction in both intact and permeabilised vascular smooth muscle has been extensively investigated with the development of PKC inhibitors and the technique of PKC down-regulation.

7.7. PKC inhibitors.

The first PKC inhibitors included 1-(5-isoquinolinyl sulfonyl)-2-methyl-piperazine (H-7) (an isoquinolinesulfonamide) and staurosporine (a microbial compound) which interacted with the ATP-binding site on the catalytic domain of PKC (Hidaka et al 1984, Kobayashi et al 1989a). This region had a high degree of sequence homology with other protein kinases and both inhibitors were found to have non-specific effects on smooth muscle contraction (Rüegg and Burgess 1989). Indeed, the ability of staurosporine to prevent vasoconstriction has been attributed to the inhibition of MLC kinase and not PKC (Rüegg and Burgess 1989). In contrast, calphostin C, another microbial compound,

interacted with the regulatory domain of PKC which lacked sequence homology with other protein kinases and was a more specific PKC inhibitor (Kobayashi et al 1989a).

10^{-6}M calphostin C reduced the maximum ET-1-induced tension in intact rat aorta by 13.2% and 25.8% in calcium-containing medium and calcium-free Krebs containing 1mM EGTA respectively (Shimamoto et al 1992). Shimamoto et al (1993) found the same concentration of calphostin C completely inhibited the contraction to the phorbol esters 12-0-tetradecanoylphorbol-13-acetate (TPA) and PdBu in rat aorta but had no effect on the contraction to 40mM potassium chloride (KCl). However, the maximum tension developed to α adrenoceptors was significantly reduced by calphostin C in intact smooth muscle preparations (Shimamoto et al 1993, Burt et al 1996). Furthermore, it has been reported that the increase in calcium sensitivity of the myofilaments elicited by GTPγS, TPA, DAGs and lysophosphatidylinositol (Jensen 1996) and lysophosphatidylcholine, palmitoyl (Jensen et al 1996) in α-toxin permeabilised rat mesenteric small arteries was completely inhibited by calphostin C. It had no effect, however, on the acetylcholine-induced increase in myofilament calcium sensitivity in α-toxin permeabilised smooth muscle cells from the guinea pig stomach (Oishi et al 1992).

Synthetic peptides eg PKC_{19-36} and PKC_{19-31} were based on the autoinhibitory pseudosubstrate sequence of the regulatory domain of PKC and were therefore highly potent and specific inhibitors of PKC (House and Kemp 1987). PKC_{19-36} completely blocked the PdBu-induced calcium sensitisation in ß-escin

skinned rabbit mesenteric arteries but only partly blocked the ET-1-induced calcium sensitisation (Yoshida et al 1994). Similarly, Brozovich (1995) found PKC_{19-31} inhibited the increase in myofilament calcium sensitivity elicited by PE by approximately 30% in α-toxin permeabilised smooth muscle cells from the rabbit portal vein. Other studies using the synthetic peptide inhibitors PKC_{19-36} and PKC_{19-31} showed they had no effect on the potentiation of the sub-maximal calcium-induced contraction induced by GTPγS (Itoh et al 1994, Fujita et al 1995), NA (Fujita et al 1995) or AlF_4^- (Fujita et al 1995) in permeabilised vascular smooth muscle.

7.8. PKC down-regulation.

Protein kinase C becomes down-regulated in intact vascular smooth muscle cells when they are incubated with phorbol esters for long periods of time. Early studies showed that PKC down-regulation led to a reduction in the number of phorbol ester binding sites (Collins and Rozengurt 1982) and loss of PKC activity (Rodriguez-Pena and Rozengurt 1984). Stabel et al (1987) demonstrated this loss of PKC activity and immunoreactive polypeptide paralleled the phorbol ester-induced down-regulation of binding and responsiveness to PdBu in Swiss 3T3 cells. They also reported the depletion of PKC was not associated with changes in the amount of mRNA or rates of synthesis of PKC therefore was due to an increased rate of degradation of PKC (Young et al 1987). Since membrane-bound PKC is readily susceptible to proteolysis by the calcium-dependent neutral proteinases such as calpain Young et al (1987) proposed the mechanism of PKC down-regulation involved a phorbol ester-induced activation and

movement of PKC to the plasma membrane and subsequent degradation by calpain.

Hori et al (1993) found exposure of intact rat aortic smooth muscle strips to 3μM TPA for 24 hours completely abolished the contractile response to the phorbol ester 12-deoxyphorbol 13-isobutyrate (DPB) but had no effect on the contractile response to KCl, NA, ET-1 or prostaglandin F2 alpha ($PGF_{2\alpha}$). In the same study long term exposure to TPA completely blocked the DPB-induced calcium sensitisation but had no effect on the $PGF_{2\alpha}$-induced calcium sensitisation in α-toxin permeabilised rat aortic strips (Hori et al 1993). The maximum tension developed to NA and $PGF_{2\alpha}$ in intact rat aorta was also not altered following 17 hours incubation with 10μM PdBu (Rapoport et al 1995). However, other studies found prolonged exposure of intact smooth muscle preparations to PdBu had marked effects on the agonist-induced contraction (Merkel et al 1991, Marala et al 1993, Burt et al 1996).

The above investigations using the more specific PKC inhibitors and PKC down-regulation suggested PKC may have been involved in the vascular smooth muscle contraction in response to vasoconstrictor agonists in both intact and permeabilised preparations, but the evidence is conflicting. Therefore one aim of this thesis was to determine the exact role of PKC in the agonist-induced contraction in rat mesenteric small arteries.

7.9. PKC down-regulation and PKC isotypes.

Hori et al (1993) reported that chronic exposure of intact

rat aorta to TPA resulted in the down-regulation of all PKC isotypes. However, this conclusion was based solely on the assessment of PKC activity which may not have been sufficiently sensitive. Indeed, other studies using immunoblot analysis have shown that different PKC isotypes were down-regulated by prolonged exposure to phorbol esters depending on the species and tissue used (Brooks et al 1993, Duyster et al 1993, Pfeilschifter and Merriweather 1993, Kiss and Anderson 1994, Turner et al 1994). Furthermore, the PKC isotypes down-regulated depended on the length of time of incubation with the phorbol ester. Duyster et al (1993) showed following long-term treatment with TPA in cultured liver macrophages PKCß was depleted within 3 hours compared to PKCδ which took over 7 hours to become depleted. Furthermore, an 8 hour incubation with TPA was sufficient to down-regulate α and δ isotypes in cultured rat glomerular mesangial cells, whereas a 24 hour incubation with TPA was necessary to down-regulate PKC ε (Pfeilschifter and Merriweather 1993). In contrast, TPA treatment for 24 hours did not cause any depletion of PKC ζ in mesangial cells (Pfeilschifter and Merriweather 1993) probably because this isotype is not responsive to phorbol esters. In this thesis immunoblot analysis was used to identify not only the expression and intracellular distribution of the PKC isotypes present in rat mesenteric small arteries, but also which ones were down-regulated as a result of a fixed, long term incubation with the phorbol ester PdBu. Therefore it was possible to identify the specific PKC isotypes which were involved in the contractile response to PdBu and which ones were not involved in the contractile response to receptor

agonists in rat mesenteric small arteries.

The data in this thesis indicated that PKC was not the sole regulator of the agonist-mediated contraction in rat mesenteric small arteries therefore another mechanism may have been additionally responsible for this effect, for example the phosphorylation of proteins by tyrosine kinases.

8. Tyrosine kinases.

Tyrosine kinases (TKs) phosphorylate proteins on tyrosine residues and consist of three main subclasses: 1. membrane-associated receptor TKs including the epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and insulin receptors 2. cytoplasmic non-receptor TKs and 3. membrane-associated non-receptor TKs related closely to $pp60^{c\text{-}src}$. In smooth muscle cells activation of the membrane receptor TKs by the growth factors EGF and PDGF resulted in mitogenic responses (Dvir et al 1991, Bilder et al 1991) due to the activation of mitogen-activated protein kinase (MAP kinase) and PIP_3 (Figure 1.1).

8.1. TKs and vascular smooth muscle contraction.

Some investigators have reported that growth factors can also induce contractile responses in intact vascular smooth muscle (Berk and Alexander 1989, Yang et al 1992, Yang et al 1993, Sauro and Thomas 1993a, Merkel et al 1993, Hollenberg 1994, Laniyonu et al 1994a, Hughes 1995). Binding of EGF or PDGF to its membrane receptor resulted in the phosphorylation of tyrosine residues on PLCγ1 (Berridge 1993). This caused the hydrolysis of PIP_2 leading to an increase in the formation of

IP_3 and release of calcium from the intracellular stores (Figure 1.1).

It has been demonstrated in intact smooth muscle that contractile receptor agonists such as AII, AVP, ET-1, PE and NA can also stimulate tyrosine phosphorylation of proteins (Tsuda et al 1991, Koide et al 1992, Tsuda et al 1993, Molloy et al 1993, Di Salvo et al 1994). These receptor agonists are linked to the G_q class of heterotrimeric G-proteins which activate PLCß1 (Smrcka and Sternweis 1993). However, it has recently been shown that AII signal transduction through the AT_1 receptor also occurred via tyrosine phosphorylation of PLCγ1 (Marrero et al 1994). This was a similar pathway to that used by growth factors (Berridge 1993), but unlike the growth factor receptor the AT_1 receptor does not possess a TK activity. Hollenberg (1994) postulated the AII- and AVP-induced contraction in intact preparations of guinea pig gastric smooth muscle occurred via the recruitment of an intermediary, non-receptor TK. Indeed, receptor agonists increased $pp60^{c\text{-}src}$ activity in rat aortic vascular smooth muscle cells (Marrero et al 1995) and glomerular mesangial cells (Simonson and Herman 1993) suggesting the src family of TKs may have been involved in the tyrosine phosphorylation of PLCγ1.

Alternatively the heterotrimeric G-protein-coupled receptor agonists AII and AVP have been shown to activate MAP kinase in cultured rat vascular smooth muscle cells (Ishida et al 1992, Molloy et al 1993, Kribben et al 1993, Granot et al 1993, Jones et al 1994) which may have caused smooth muscle

contraction via the phosphorylation of caldesmon (Adam and Hathaway 1993, see Figure 1.5). This was dependent on the endogenous activation of PKC (Molloy et al 1993), mimicked by TPA (Ishida et al 1992, Granot et al 1993, Kribben et al 1993, Jones et al 1994) and blocked by staurosporine or down-regulation with TPA pretreatment (Kribben et al 1993) suggesting PKC may have been involved in the activation of MAP kinase. This was presumably due to the activation of ras p21 or Raf-1 (Lee and Severson 1994, see Figure 1.5). Therefore, it is becoming increasingly evident there is considerable "cross-talk" between signal transduction pathways so that regulation of cellular function is highly complex and sophisticated (Figure 1.5).

There is evidence that TKs can regulate the function of membrane channels including voltage-gated calcium channels (Wijetunge et al 1992, Wijetunge and Hughes 1995, Hatakeyama et al 1996), non-selective cation channels (Enoki et al 1995, Minami et al 1994) and K^+ channels (Smirnov and Aaronson 1995, Xiong et al 1995). Therefore stimulation of TKs may activate calcium permeable membrane channels leading to calcium influx and smooth muscle contraction.

The investigation of the involvement of tyrosine phosphorylation in the vasoconstrictor agonist-induced vascular smooth muscle contraction has been made possible with the development of TK inhibitors.

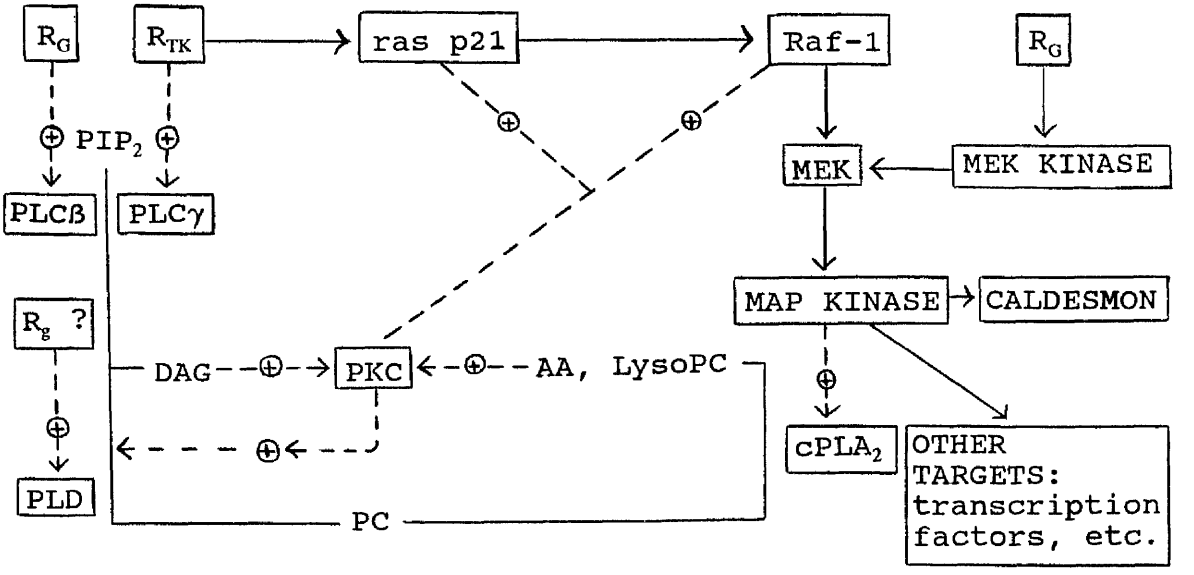


FIGURE 1.5 "Cross-talk" between signal transduction pathways involving phospholipid turnover. Receptors linked to heterotrimeric G-proteins (R_G) or receptors with tyrosine kinase (TK) activity (R_{TK}) cause the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C (PLC)ß and PLCγ respectively to generate 1,2-diacylglycerol (DAG) which activates protein kinase C (PKC). PKC in turn activates the phospholipase D (PLD)-catalysed hydrolysis of phosphatidylcholine (PC) which also yields DAG. PLD can also be stimulated by receptors linked to 'small' G-proteins (R_g). Autophosphorylation of R_{TK} activates ras p21 which then activates Raf-1. This initiates a kinase cascade with MAP kinase kinase (MEK) activating mitogen-activated protein kinase (MAP kinase). There are numerous cell targets for MAP kinase including phospholipase A_2 (PLA_2), caldesmon and nuclear transcription factors. Products of the PLA_2 reaction arachidonic acid (AA) and lysophosphatidylcholine (LysoPC) can further stimulate PKC and PKC can enhance MAP kinase activation by actions on ras p21 or Raf-1. MAP kinase can also be activated by stimulation of R_G via activation of MEK kinase. + stimulation. $cPLA_2$ cellular PLA_2. Modified from Lee and Severson (1994).

8.2. TK inhibitors.

The first generation TK inhibitors (TKIs) such as quercertin and the flavanoid genistein interacted with the ATP-binding site of TKs and were shown to inhibit other protein kinases (Gazit et al 1989). Recently tyrphostins, synthetic analogues of erbstatin, have been developed. They interacted with the substrate-binding site and were highly specific inhibitors of tyrosine phosphorylation (Levitzki and Gilon 1991).

Tyrosine kinase inhibitors blocked the contractile response to the growth factors EGF and PDGF in intact vascular smooth muscle (Yang et al 1992, Yang et al 1993, Sauro and Thomas 1993a, Merkel et al 1993, Hollenberg 1994, Laniyonu et al 1994a, Hughes 1995). Furthermore, vanadate and pervanadate (protein tyrosine phosphatase inhibitors) induced an increase in force generation in vascular smooth muscle preparations which was relaxed by the TKI genistein (Di Salvo et al 1993a, Di Salvo et al 1994, Laniyonu et al 1994b, Filipeanu et al 1995).

In the case of the G-protein-coupled receptor agonists low concentrations of genistein and tyrphostin had no effect on the contractile response to carbachol or bradykinin in circular and longitudinal preparations of guinea pig gastric smooth muscle (Yang et al 1992, Yang et al 1993, Hollenberg 1994). However, the AII-induced contraction was completely blocked in the longitudinal and partially blocked in the circular smooth muscle preparation in the presence of the TKIs (Yang et al 1992, Yang et al 1993, Hollenberg 1994). Laniyonu et al (1994a) found genistein and tyrphostin AG82

attenuated the contractile actions of AII, AVP, NA and $PGF_{2\alpha}$ in porcine coronary artery helical strips and the contractile action of AII in rat aorta helical strips. Moreover, the contraction to carbachol, NA (Di Salvo et al 1993b) and PE (Di Salvo et al 1993b, Filipeanu et al 1995) in vascular smooth muscle tissue was inhibited by higher concentrations of TKIs. In a recent study tyrphostin and genistein significantly relaxed the maximum NA-induced tension in intact rat mesenteric small arteries (Toma et al 1995). However, in other studies TKIs had no effect on the contractility of intact vascular smooth muscle to α adrenoceptor agonists (Sauro and Thomas 1993a, Merkel et al 1993, Jinsi and Deth 1995, Hughes 1995) and ET-1 (Jinsi and Deth 1995). The difference in results between all these investigations could be due to differences in tissue, species or TKIs used.

In permeabilised vascular smooth muscle preparations TKIs inhibited the increase in myofilament calcium sensitivity elicited by ras p21 (Satoh et al 1993), carbachol (Steusloff et al 1993) and NA (Toma et al 1995). Therefore the majority of investigations using TKIs suggested TKs may have also played a role in the contractile response to vasoconstrictor agonists in both intact and permeabilised vascular smooth muscle, although the evidence is again controversial. The precise involvement of TKs in the agonist-induced contraction in rat mesenteric small arteries was therefore another aim of this thesis.

9. Hypertension.

The elevated blood pressure in hypertension is associated with an increased total peripheral resistance and a normal cardiac output. The increase in peripheral resistance may be attributed to structural abnormalities: reduced lumen diameter and increased media to lumen ratio in small arteries (Heagerty et al 1993). However, other factors may also have been important including an enhanced reactivity of these arteries to vasoconstrictor agonists which has indeed been reported in adult SHRs compared to WKYs (Mulvany et al 1980, Tomobe et al 1988). This may have been due to an increased level of $[Ca^{2+}]_i$ available to the myofilaments as a result of abnormal calcium handling (Kwan and Daniel 1981) which has been demonstrated in freshly isolated smooth muscle cells from the SHR using ion selective electrodes (Losse et al 1984). However, Losse et al (1984) used aortic tissue whereas small arteries were used in this thesis given their importance in determining peripheral vascular resistance. In contrast, other studies have reported no enhanced reactivity in the SHR compared to WKY (Bund et al 1991, Mulvany and Aalkjaer 1990, Dohi and Lüscher 1991). Therefore the vascular responsiveness to vasoconstrictor agonists plus structural parameters were examined in this thesis in intact small arteries from SHRs and WKYs during the developmental and established phases of hypertension.

Yokokawa et al (1994) demonstrated that the PKC activity following stimulation with ET-3 in cultured aortic endothelial cells was higher in the SHR compared to WKY. Furthermore, aortic strips from the SHR showed a higher TK

activity, hyperresponsiveness to a PDGF-mediated contraction and hyporesponsiveness to vasorelaxation by tyrphostin than strips from the WKY (Sauro and Thomas 1993b). Recently, Kanagy and Webb (1994) reported that carotid arterial strips from stroke-prone spontaneously hypertensive rats (SHRSPs) developed greater force to mastoparan, a peptide which activates G-proteins directly, than strips from WKYs. These results suggested the increased vascular responsiveness observed in the SHR may have been due to enhanced intracellular signal transduction pathways involving G-proteins, PKC and TKs, for example the agonist-induced calcium sensitisation. Indeed, Satoh et al (1994a) found the reactivity to serotonin and GTPγS was increased in both intact and ß-escin skinned coronary arteries in 12 week SHRSPs compared to WKYs.

An augmented agonist-induced calcium sensitisation in hypertension may have also been due to an increased calcium sensitivity of the myofilaments themselves. Soloviev and Bershstein (1992) reported an increased myofilament calcium sensitivity in saponin-skinned smooth muscle cells from the SHR compared to WKY. However, other studies using Triton-X100 (Nghiem and Rapp 1983, McMahon and Paul 1985, Mrwa et al 1985), saponin (Satoh et al 1990) and ß-escin (Satoh et al 1994a) permeabilised preparations found no difference in calcium sensitivity between normotensive and hypertensive animals. Furthermore, in a recent study using intact rat mesenteric small arteries SHRs and WKYs were equally sensitive to calcium when challenged with increasing concentrations of NA in the presence of a constant level of

extracellular calcium (Bian and Bukoski 1995). Therefore, the overwhelming evidence suggested the calcium sensitivity of the contractile apparatus was not enhanced in hypertension. However, the majority of these studies using permeabilised preparations were carried out on aortas or large arteries in contrast to small arteries which were used in this study due to their important role in maintaining tone in the vasculature.

Therefore, the final aim of this thesis was to investigate the myofilament calcium sensitivity and the agonist-induced calcium sensitisation in young and adult SHRs and WKYs using α-toxin permeabilised small arteries. Ferrone et al (1979) found that different organs made unequal contributions to the increased total peripheral resistance observed in hypertension therefore all responses were examined in three different vascular beds, namely the mesenteric, renal and femoral vascular beds.

CHAPTER TWO

MATERIALS AND METHODS

1. The myograph.

The myograph was first developed in 1977 by Mulvany and Halpern for the measurement of structural and contractile properties of small arteries under standard isometric conditions. It consists of a 15ml capacity bath in which there are two separate pairs of mounting heads, allowing the study of two arteries simultaneously. Each mounting head has two screws positioned at either end which are used to clamp a 40μm diameter stainless steel wire onto which the artery is threaded. Therefore when mounted the artery is held by two wires which pass down the lumen (Figure 2.1). One mounting head is connected to a micrometer screw gauge while the other is connected to a force transducer via a connecting pin which passes through the side of the bath. The bath is heated to 37°C by heating blocks on either side which are warmed by circulating water. The myograph has a gas inlet for bubbling the bathing solution and two drainage ports attached to a suction pump for bath emptying. The force transducer is fixed into the wall of the bath and connected to a chart recorder (Graphic 2002, Lloyd Instruments, UK).

2. Morphology measurements.

The myograph is designed with a small perspex window situated directly beneath the mounting heads so that when it is mounted on the stage of a light microscope (Leitz UK Ltd., UK) the artery wall can be imaged using a water immersion objective (x40, Zeiss Ltd., UK). A calibrated micrometer eyepiece (x8, Zeiss Ltd., UK) is used to measure the

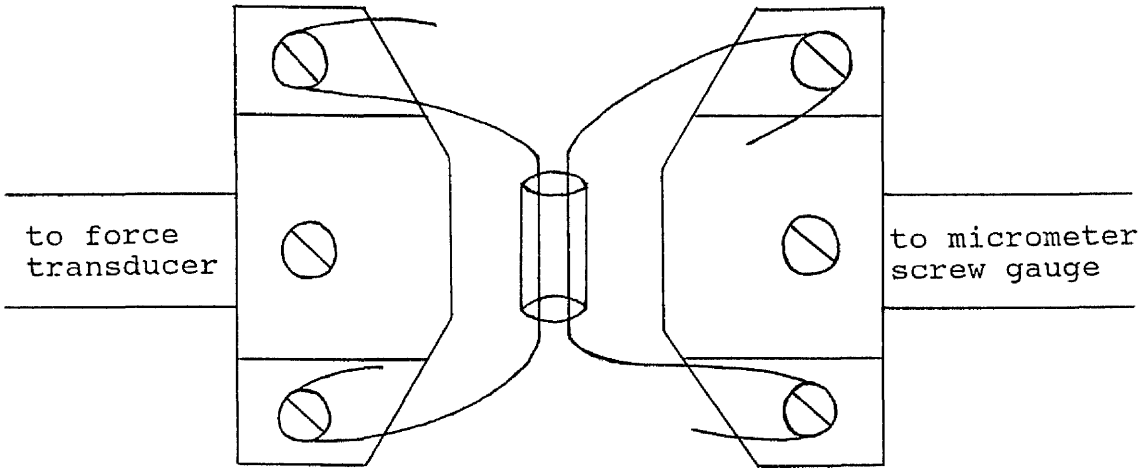


FIGURE 2.1 Diagram showing the mounting heads in a wire myograph. The vessel is held in position by two wires passing down the lumen which are clamped at each end by screws on the mounting head. The wires are shown to extend into the gap to illustrate their passage down the lumen of the vessel more clearly, but they are normally flat against the side of the mounting head.

thickness of the media (M_0), from which media area can be calculated (Mulvany et al 1978). All morphology measurements are made when the wires are separated so that the vessel is held just under tension. They can then be converted to normalised values using the following procedure.

3. Normalisation procedure.

This involves subjecting the vessel to a series of stretches and measuring the changes in tension and internal circumference on the chart recorder and micrometer screw gauge respectively. These readings are fed into a normalising programme run on an IBM compatible computer which calculates the effective pressure inside the vessel for each stretch. A tension-internal circumference curve is plotted and the internal circumference of the vessel when relaxed and under an effective transmural pressure of 100mmHg (L_{100}) is determined. The corresponding internal diameter (l_{100}) is calculated as L_{100}/π. The programme then calculates the micrometer reading at which the vessel would have an internal diameter (l_0) which is 90% of l_{100}. It has been demonstrated that active tension developed to contractile stimuli is maximum at this internal diameter (Mulvany and Halpern 1977). Assuming constant media volume the media thickness can then be calculated for l_0 (M_0).

4. Active tension.

This is calculated as force response divided by twice segment length as described previously (Mulvany et al 1978).

5. Solutions.

Vessels were dissected out and held in physiological saline solution (PSS) of the following composition (mM): NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 25, $MgSO_4$ 1.17, KH_2PO_4 1.18, K_2EDTA 0.026 and glucose 5.5 bubbled with 95% O_2 5% CO_2 to give a pH of 7.4 at 37°C. Potassium PSS (KPSS) has the same composition as PSS but with an equimolar substitution of KCl for NaCl.

6. Animals.

With the exception of Chapter Six female Sprague-Dawley rats aged 10-12 weeks were used. Third order mesenteric arteries were dissected out and segments 1-2mm long were mounted in the myograph and normalised to l_0 (as described on Page 56).

7. Permeabilisation.

All permeabilisation experiments were carried out at room temperature.

7.1. Alpha toxin.

7.1a. First method.

The first method for permeabilisation used Staphylococcal aureus α-toxin (α-toxin) obtained from GIBCO/BRL which has a concentration of 2,500 units/ml distilled water. The composition of solutions used in this method was modified from those used by Kitazawa et al (1989)(see overleaf).

	Relax		Preload	SolA	SolB	SolC
	mM	g/L	mM	mM	mM	mM
KCl	74.1	5.52	74.1	74.1	74.1	74.1
$MgSO_4$	4	0.0.986	4	4	4	4
$ATPNa_2$	4.5	2.479	4.5	4.5	4.5	4.5
EGTA	1		0.2	10	10	0.05
Hepes	25	5.96	25	25	25	25
Phosphocreatine	1	0.255	1	1	1	1
Glucose	5.5	1.0	5.5	5.5	5.5	5.5
$NaHCO_3$	10	0.84	10	10	10	10
KH_2PO_4	0.44	0.06	0.44	0.44	0.44	0.44
$CaCl_2$	0		0	0	10	0
PMSF			1			
Leupeptin			10^{-3}			
FCCP			10^{-3}			

pH 7.1 with KOH at room temperature.

All solutions were gassed with 95% O_2 5% CO_2.

A standard start was performed comprising 2 activations of 10μM noradrenaline (NA) in KPSS (NAK) followed by a single activation of 10μM NA in PSS, KPSS and finally NAK. Activations were for 2 minutes and the arteries were allowed to fully relax between each stimulation. Vessels were equilibrated in relaxing solution for 10 minutes then incubated in preload solution for 10 minutes containing the protease inhibitors phenylmethyl-sulfonyl fluoride (PMSF) and leupeptin plus a mitochondrial blocker carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP). Permeabilisation was achieved by adding 2,500 units α-toxin to 12.5ml pCa6.7 in the myograph bath (giving a final concentration of 200

units/ml α-toxin). A rapid contraction was observed caused by the calcium ions entering through the pores created by α-toxin in the artery wall (Figure 2.2).

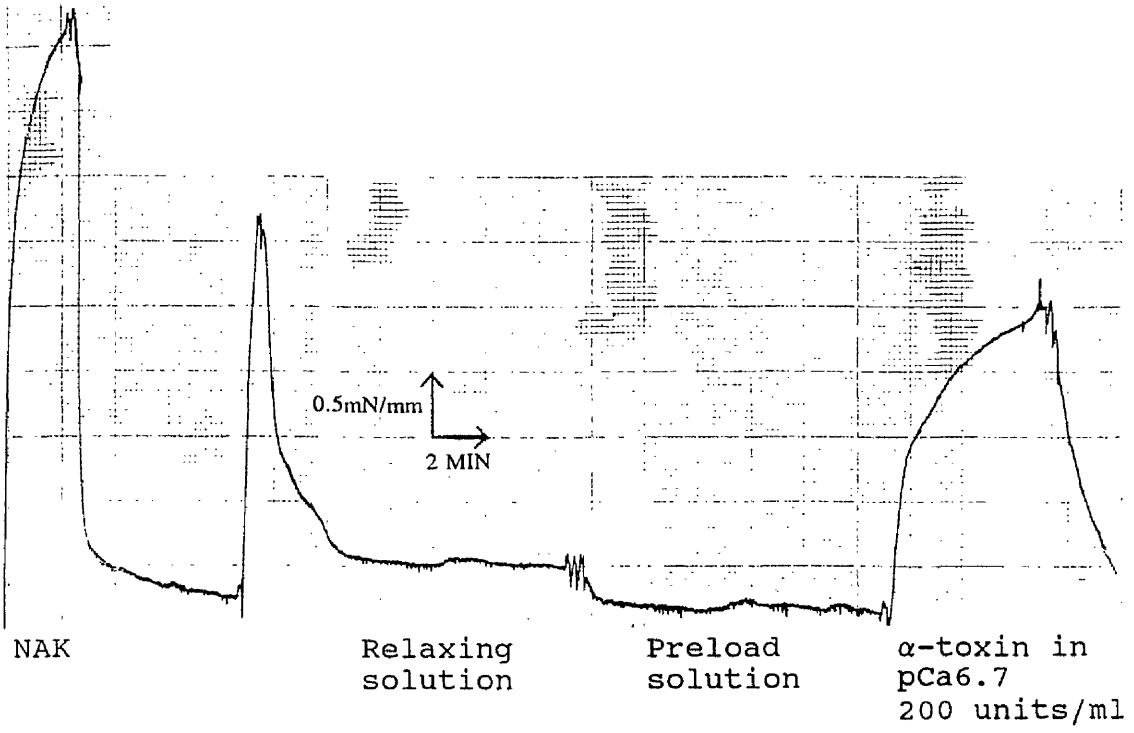


FIGURE 2.2 Representative tracing showing the protocol for the first method of permeabilisation of rat mesenteric small arteries using α-toxin.

After tension development had reached a plateau i.e., when full permeabilisation had taken place, (which took 5-6 minutes) the α-toxin was washed out and vessels were re-equilibrated in relaxing solution for 20 minutes. A calcium dose response curve was performed using concentrations of pCa7.5 to pCa5.0 made by mixing the following two solutions: solution A which has the same composition as relaxing solution except EGTA was 10mM and solution B which is identical to solution A with 10mM $CaCl_2$ added. The two solutions were mixed in the appropriate ratios (see overleaf).

	SolA	SolB
pCa7.5	0.931	0.069
pCa7.0	0.809	0.191
pCa6.3	0.430	0.570
pCa6.0	0.193	0.807
pCa5.5	0.116	0.884
pCa5.0	0.039	0.961

The calcium concentration was calculated using the apparent stability constant for Ca-EGTA at pH 7.0 and 20°C of $10^{6.4}$ M^{-1} (Horiuti 1988). The calcium dose response curves were repeated a number of times with the vessels re-equilibrated in relaxing solution between each one (Figure 2.3).

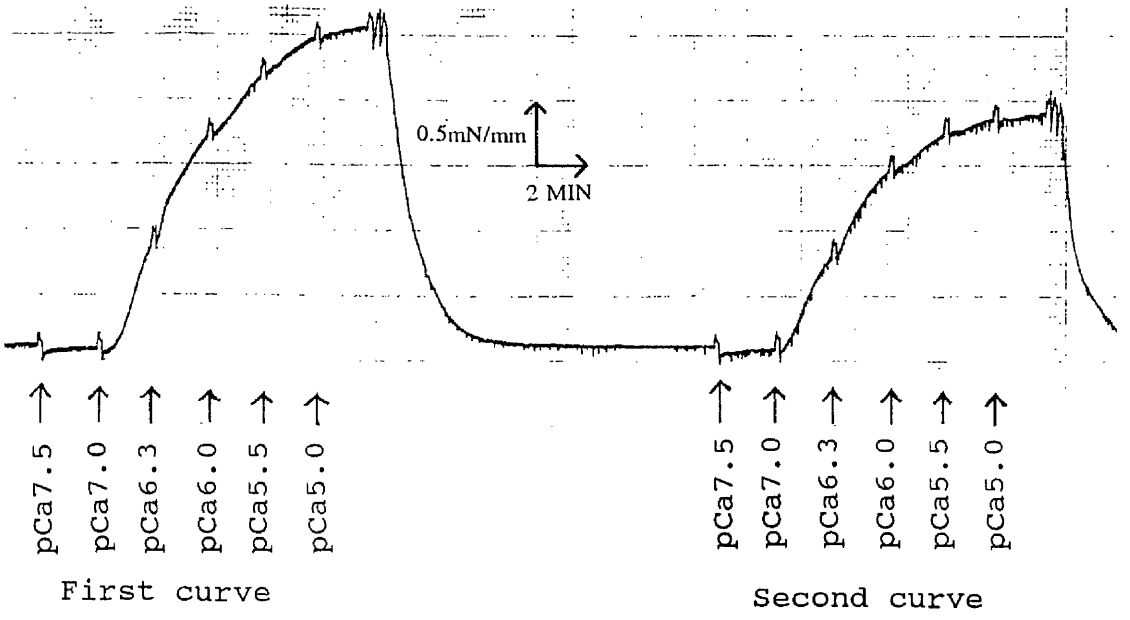


FIGURE 2.3 Representative tracing showing two consecutive calcium dose response curves in an α-toxin permeabilised rat mesenteric small artery.

The maximum contraction to each calcium concentration was calculated as active tension (as described on Page 56) and expressed as a % of the maximum tension developed to the final NAK stimulation in the standard start procedure:

		First curve (n=2)	Second curve (n=2)	Third curve (n=2)	Fourth curve (n=2)
Max NAK tension (mN/mm)		3.814	3.295	3.295	2.775
pCa7.5	(mN/mm)	0	0	0	0
	(%)	0	0	0	0
pCa7.0	(mN/mm)	0.593	0.354	0.169	0.057
	(%)	15.55	9.28	5.13	2.05
pCa6.3	(mN/mm)	1.229	0.792	0.619	0.273
	(%)	32.33	24.02	18.77	9.84
pCa6.0	(mN/mm)	1.527	1.019	0.839	0.390
	(%)	40.04	26.71	25.45	14.05
pCa5.5	(mN/mm)	1.739	1.561	0.941	0.469
	(%)	45.60	47.37	28.54	16.90
pCa5.0	(mN/mm)	1.823	1.676	0.970	0.567
	(%)	47.80	50.86	29.44	20.43

These results indicate that a certain degree of permeabilisation has occurred, but the responses to the maximum calcium concentration are reduced compared to the final NAK response. In addition, the amplitude of the pCa5.0-induced contraction decreased with time from about 50% of the NAK-induced contraction in the first curve to 20% of the NAK-induced contraction in the fourth curve (a period of about 2 hours) which can be seen more clearly from Figure 2.4.

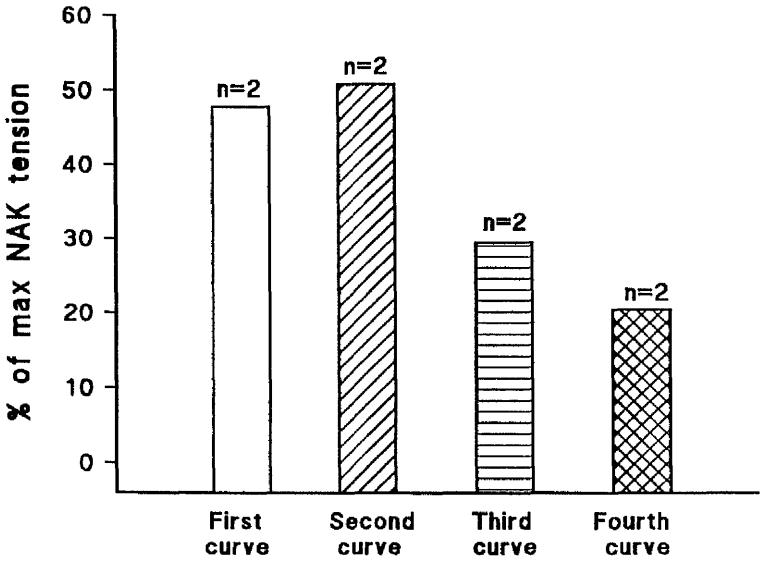


FIGURE 2.4 Bar graph demonstrating the decrease in the maximum tension developed to pCa5.0 with time in α-toxin permeabilised rat mesenteric small arteries (expressed as a % of the maximum NAK tension). Numbers of experiments are shown.

This loss of the vessels in their viability to contractile responses means that any experiments following permeabilisation would not be reproducible. In a separate experiment using the same method for permeabilisation sensitisation responses were tried using the α_1 adrenoceptor agonist phenylephrine (PE)(10μM) and caffeine (25mM) which releases calcium from the intracellular stores. This was achieved by incubating the vessels in pCa6.7 for 8 minutes then washing out with preload solution. Vessels were then placed in solution C plus 100μM GTP for 2 minutes before each agonist was added to the myograph bath. However, the responses to both PE and caffeine were very poor suggesting this permeabilisation procedure was not optimal for rat mesenteric vascular smooth muscle tissue. Therefore another

method was tried in order to achieve better responses to both calcium and receptor agonists following permeabilisation.

7.1b. Second method.

The α-toxin used in this method was obtained from List Laboratories (Camberley, California, USA) which has a similar concentration to the GIBCO/BRL α-toxin (1,500-4,000 units/ml distilled water). Rabbit mesenteric arteries were used in a 'bubble' plate system which was developed by Horiuti in 1988. Small strips about 100-200μm wide and 1-3mm long were dissected out and tied to two tungsten styluses by a single silk thread (Figure 2.5). They were then stretched to 1.2 times resting length and isometric force was measured with a force transducer (AE801, Norway). At x100 sensitivity 102mm on the chart recorder corresponded to 0.25mg of force.

The composition of solutions used in this method are the same as those described by Kitazawa et al (1989)(see Appendix). Strips were equilibrated in normal external solution (NES) for 10 minutes then activated with potassium external solution (KES) followed by 10μM PE in NES. Activations were for 2 minutes and the strips were allowed to fully relax between each stimulation. They were then incubated in relaxing solution (G_1) which caused an initial transient contraction followed by relaxation to baseline after about 15 minutes. Permeabilisation was achieved by adding 10μl of the α-toxin stock solution to 0.35ml of pCa6.7 (therefore giving a concentration of about 50-120 units/ml α-toxin) for 45 minutes. This concentration of α-toxin produced a gradual increase in tension which reached a plateau after 15-20

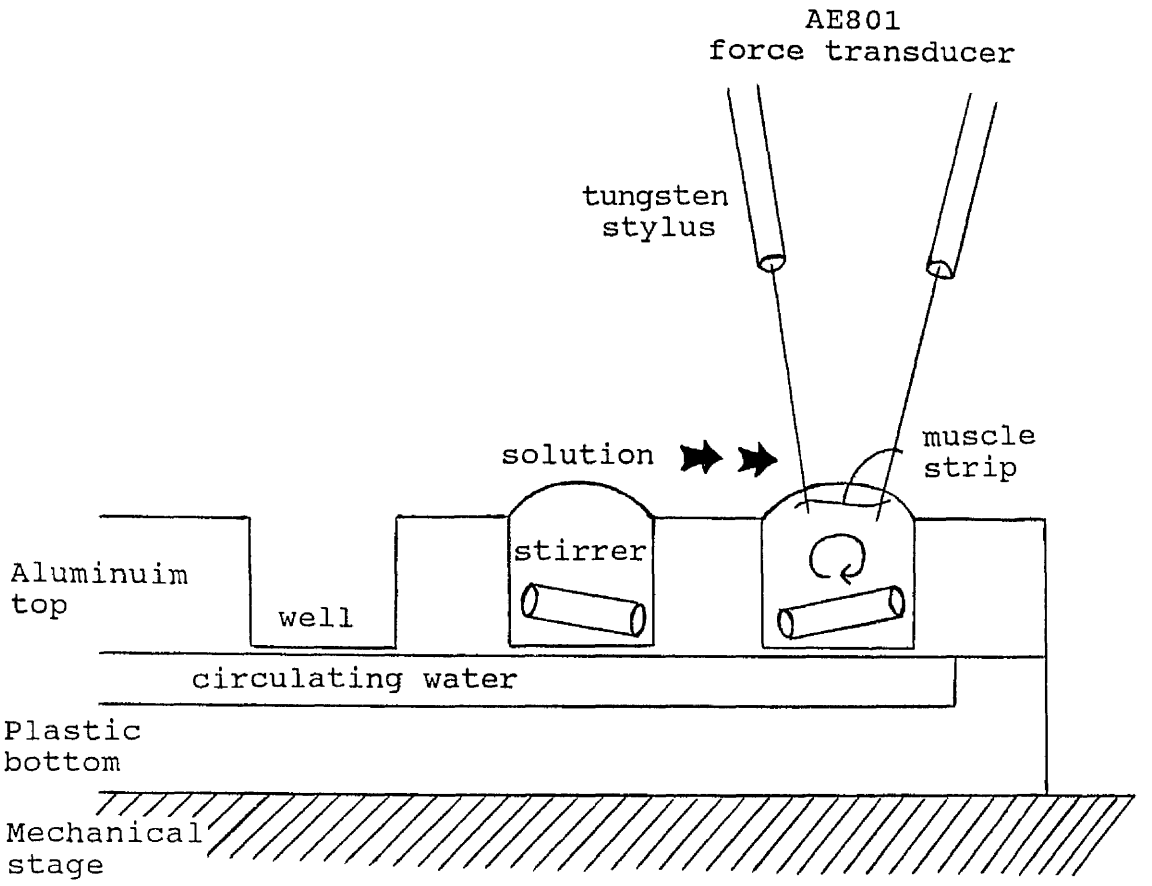


FIGURE 2.5 'Bubble' plate system (developed by Horiuti 1988). It consists of a number of small wells (measuring 7 x 9mm) bored into an aluminum plate (10mm thick) which contain the solutions for bathing. The volume of solution in each well (0.35ml) is such that the top level of the solution surface is higher than the plate surface by some 2-3mm. The strip is positioned in the convex meniscus of the solution so the latter can be changed by sliding the plate on the mechanical stage. A small magnetic stirrer bar (measuring 2 x 5mm) is placed in each well and driven by a rotating magnet (not shown) under the mechanical stage.

minutes then steadily declined over the following period of time (Figure 2.6).

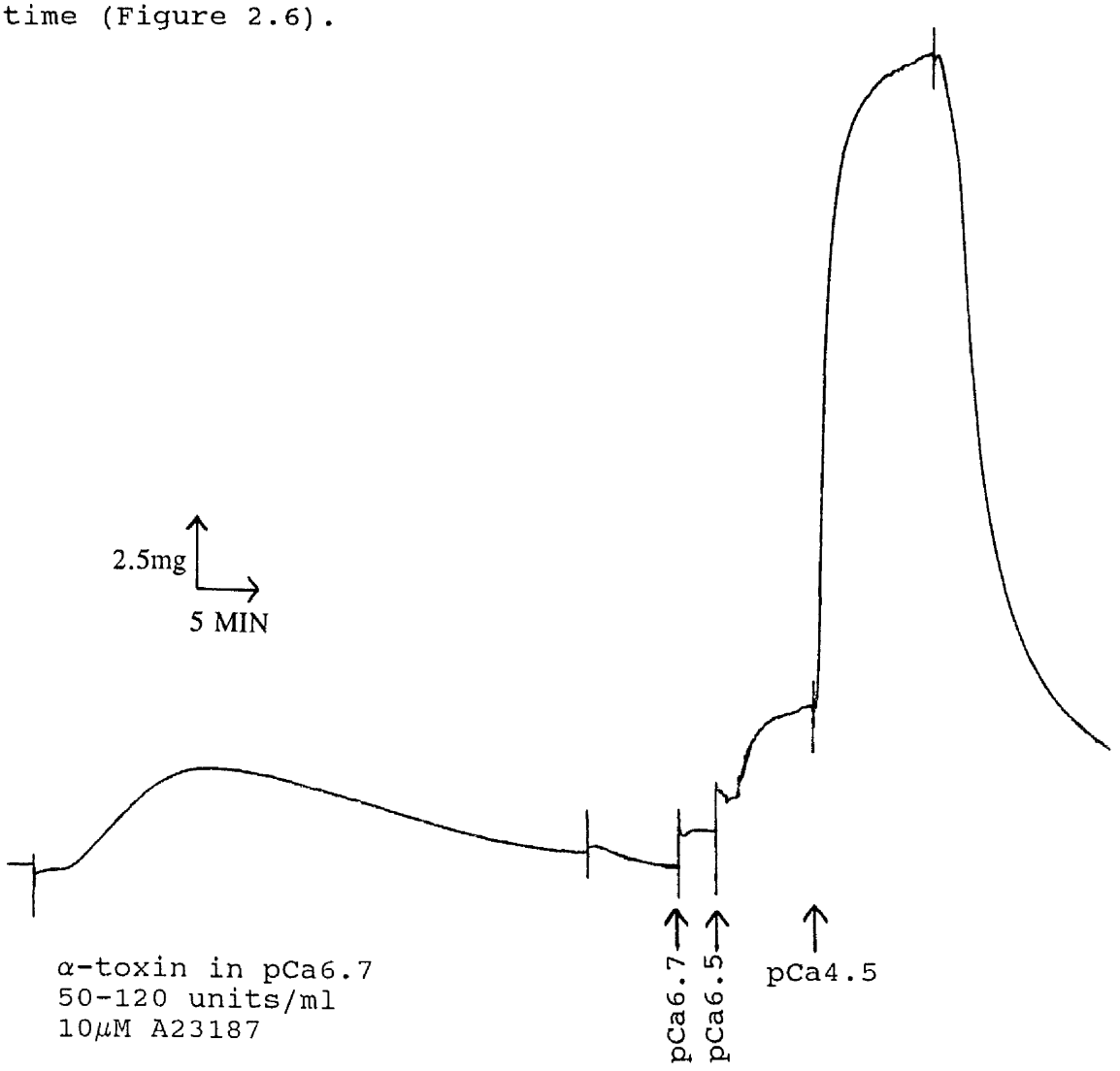


FIGURE 2.6 Representative tracing showing the profile of the response of a rabbit mesenteric arterial strip to α-toxin followed by increasing concentrations of calcium.

The calcium ionophore A23187 (10μM)(3.5μl of a 1mM stock solution) was included with the α-toxin to deplete the sarcoplasmic reticulum of calcium thereby ensuring organellar calcium did not contribute to the sensitisation response. After equilibrating the strips in G_1 for 5 minutes a dose response curve to calcium was performed using pCa6.7, pCa6.5

and pCa4.5 made up from a mixture of the calcium activating solutions G_{10} and CaG (as calculated using the method described on Pages 59 and 60). Increasingly larger developments in tension were observed (Figure 2.6), the final one to pCa4.5 being nearly 3 times greater than the tension developed to KES in the intact strips indicating the strips had been successfully permeabilised:

Max KES force = 15.00mg

pCa6.7 = 2.00mg = 13.33%

pCa6.5 = 8.25mg = 55.00%

pCa4.5 = 40.25mg = 268.33%

A calcium sensitisation experiment was then carried out with GTP (10μM), PE (10μM) and GTPγS (300μM)(Figure 2.7): vessels were pre-constricted with pCa6.7 and the agonists were added respectively when the previous contraction had reached a plateau. Each agonist induced a large contraction over and above the previous one therefore it appeared the strips had been permeabilised without disrupting the receptor-effector coupling:

pCa6.7 = 7.25mg = 48.33%

GTP (10μM) = 15.50mg = 103.33%

PE (10μM) = 25.50mg = 170.00%

GTPγS (300μM) = 32.00mg = 213.33%

The responses to calcium persisted over a time period of 8 hours therefore it proved a highly efficient method for permeabilisation of rabbit mesenteric arterial strips.

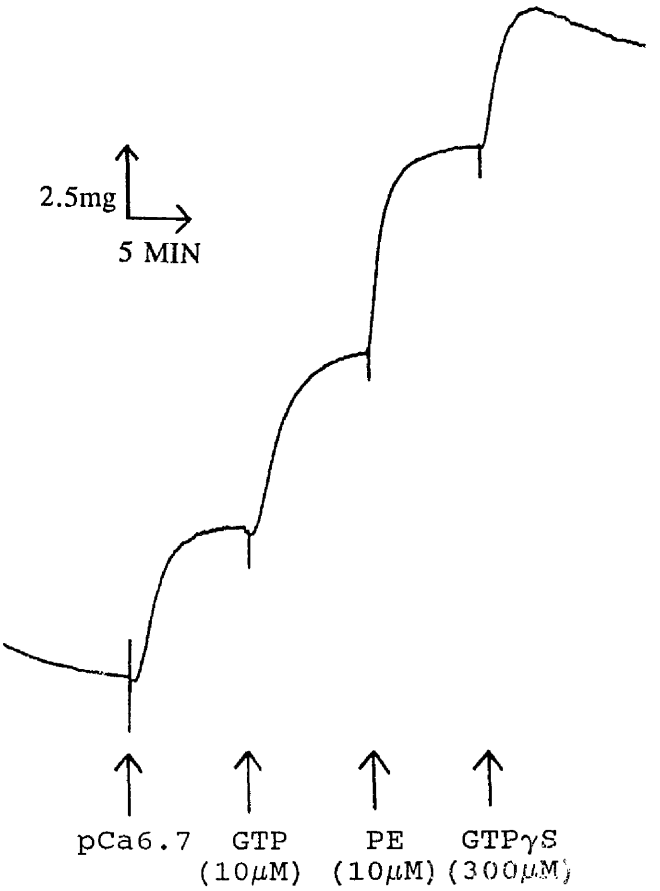


FIGURE 2.7 Representative tracing showing the response of an α-toxin permeabilised rabbit mesenteric arterial strip to pCa6.7, GTP (10μM), PE (10μM) and GTPγS (300μM).

When the same protocol was repeated using rat mesenteric arterial strips the contractile response to 10μM PE in the intact strips was very small. 10μM NA gave much better contractile responses maybe because it binds to both α_1 and α_2 adrenoceptors whereas PE binds specifically to α_1 adrenoceptors. In view of this plus the fact it is more physiological NA was used as the α adrenoceptor agonist in all future calcium sensitisation experiments. After the addition of α-toxin a similar development of tension was observed to that seen in the rabbit mesenteric arterial

strips which also took 15-20 minutes to reach a plateau before starting to decline (Figure 2.8).

FIGURE 2.8 Representative tracing showing the profile of the response of a rat mesenteric arterial strip to α-toxin.

When strips were activated with pCa6.7, pCa6.5 and pCa4.5 a dose response curve was obtained (Figure 2.9) with the contraction to pCa4.5 again being larger than the contraction to KES in intact strips:

Max KES force = 11.00mg

pCa6.7 = 0.75mg = 6.82%

pCa6.5 = 9.75mg = 88.64%

pCa4.5 = 16.30mg = 147.73%

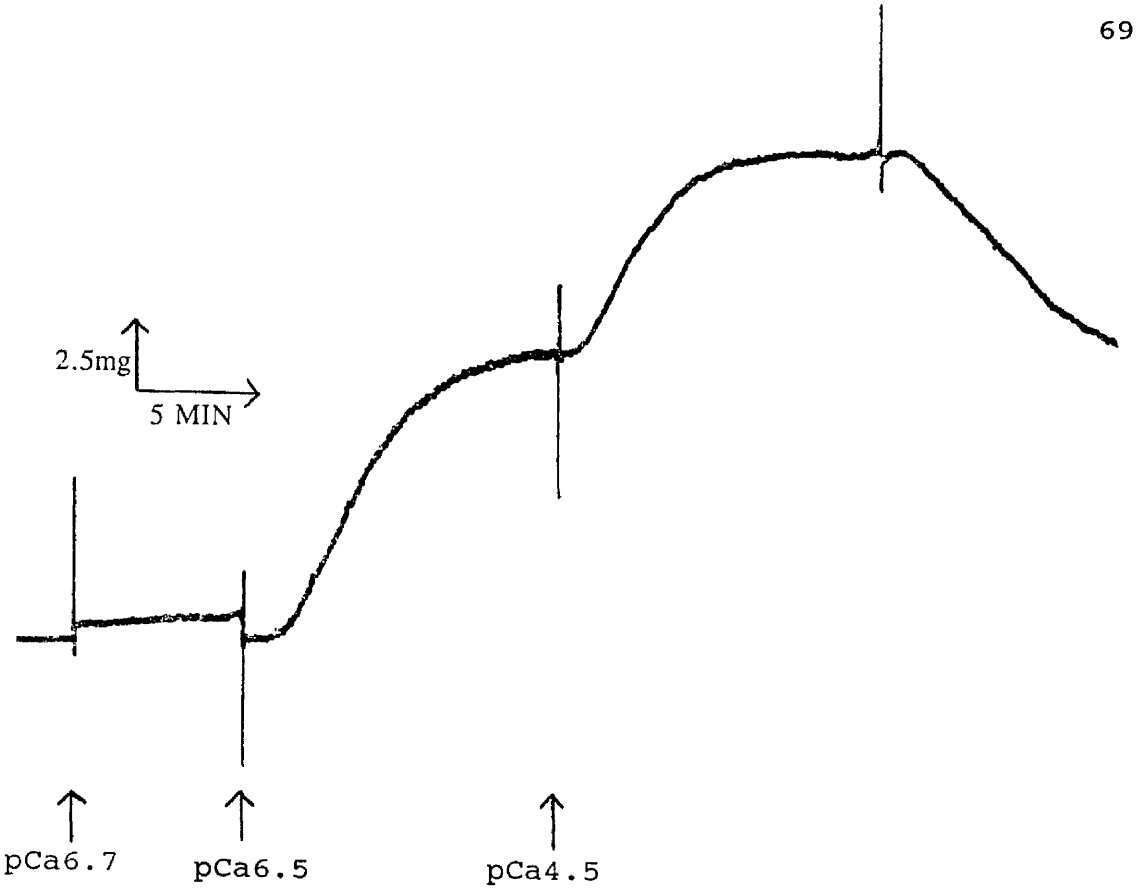


FIGURE 2.9 Representative tracing showing the response of an α-toxin permeabilised rat mesenteric arterial strip to increasing concentrations of calcium.

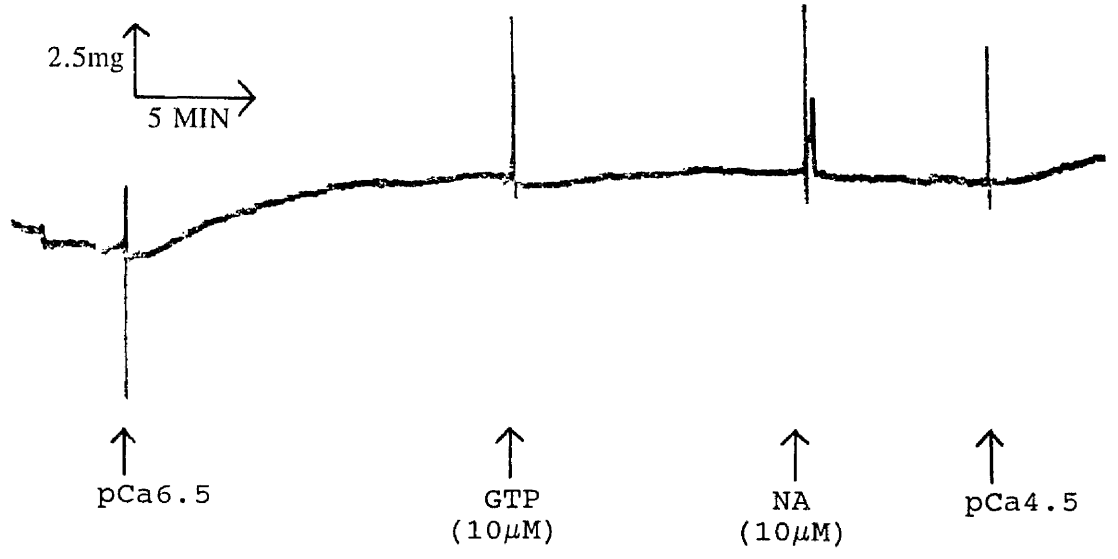


FIGURE 2.10 Representative tracing showing the response of an α-toxin permeabilised rat mesenteric arterial strip to pCa6.5, GTP (10μM), NA (10μM) and pCa4.5.

However, it can be seen there was no GTP- or NA- induced enhancement of calcium sensitivity followed by a loss of the contractile response to pCa4.5 (Figure 2.10) suggesting the rat arterial strip had been irreversibly damaged by the permeabilisation procedure. This may be because rat mesenteric smooth muscle cells are more susceptible to α-toxin-mediated damage following toxin binding than rabbit mesenteric smooth muscle cells. Therefore, instead of leaving the rat strips in α-toxin for 45 minutes they were re-equilibrated in G_I when tension development had reached a plateau. Using this method a similar dose response curve to calcium was obtained:

Max KES force = 10.00mg

pCa6.7 = 4.50mg = 45.00%

pCa6.5 = 6.25mg = 62.50%

pCa4.5 = 14.25mg = 142.50%

However, the calcium sensitisation responses to GTP (10μM), NA (10μM) and GTPγS (300μM) were much improved (Figure 2.11).

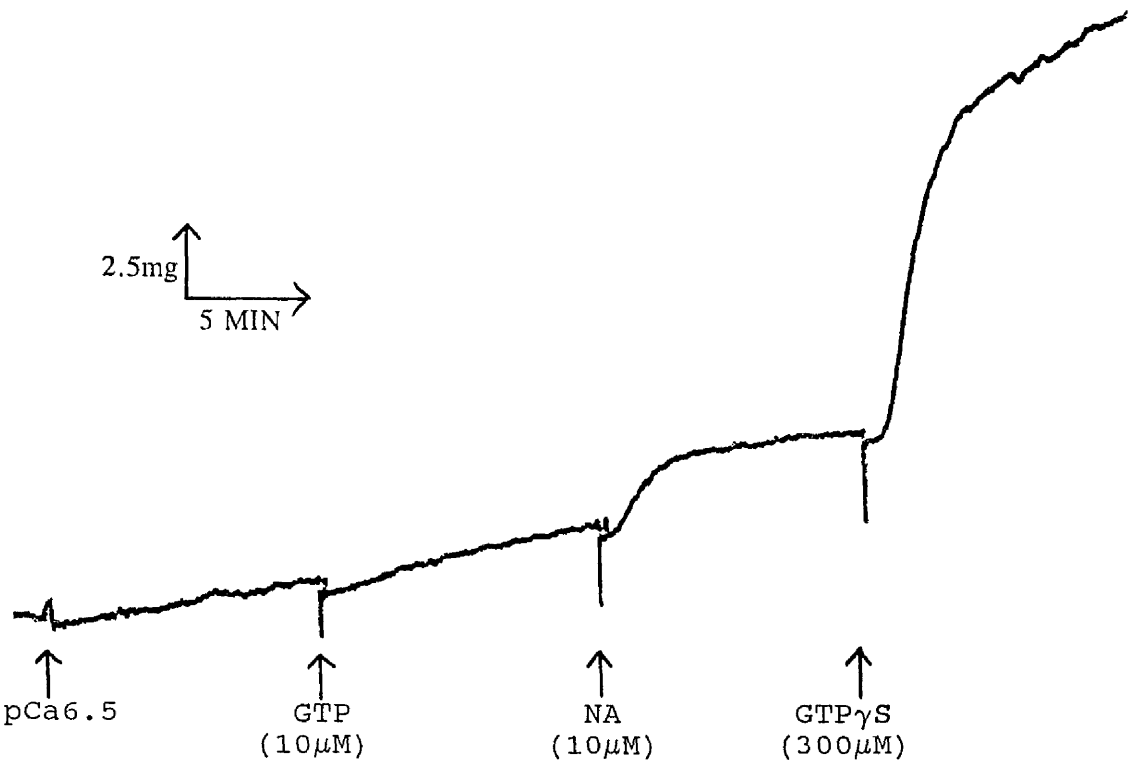


FIGURE 2.11 Representative tracing showing the response of an α-toxin permeabilised rat mesenteric arterial strip to pCa6.5, GTP (10μM), NA (10μM) and GTPγS (300μM).

pCa6.5 = 1.00mg = 10.00%

GTP (10μM) = 2.75mg = 27.50%

NA (10μM) = 5.50mg = 55.00%

GTPγS (300μM) = 20.75mg = 207.50%

In addition the responses to pCa4.5 persisted for up to 3 hours.

7.1c. <u>Third method.</u>

The previous protocol was then tried using rat mesenteric small arteries in the myograph system. It was discovered that

a 10μl droplet could be retained on the surface of the mounted vessel by the creation of surface tension between the two wires. In a separate experiment a 10μl droplet of PSS left on the vessel for a period of one hour had no effect on the viability of the vessel to contractile responses. This discovery meant much smaller amounts of α-toxin were needed for permeabilisation. After a few trial experiments it was found that a 10μl droplet of 20μl of α-toxin stock solution in 100μl pCa6.7 (to give a concentration of 300-800 units/ml α-toxin) brought about a similar development in tension to that observed in the 'bubble' plate system which again reached a plateau after 15-20 minutes. The slightly higher concentration of α-toxin required for permeabilisation in the myograph may be due to the α-toxin being held on the surface of the vessel whereas it was completely bathing the strip in the 'bubble' plate.

Following the removal of α-toxin the vessels were stimulated with pCa6.7 to check permeabilisation had taken place then re-stimulated with pCa6.7 to give a sub-maximum, constant contraction. GTP (10μM) and NA (10μM) added in succession produced large increases in tension to that developed to pCa6.7 (Figure 2.12) demonstrating that the receptor-effector coupling had not been disrupted:

Max KES tension = 1.612mN/mm

pCa6.7 = 0.201mN/mm = 12.50%

GTP (10μM) = 1.780mN/mm = 110.42%

NA (10μM) = 2.519mN/mm = 156.25%

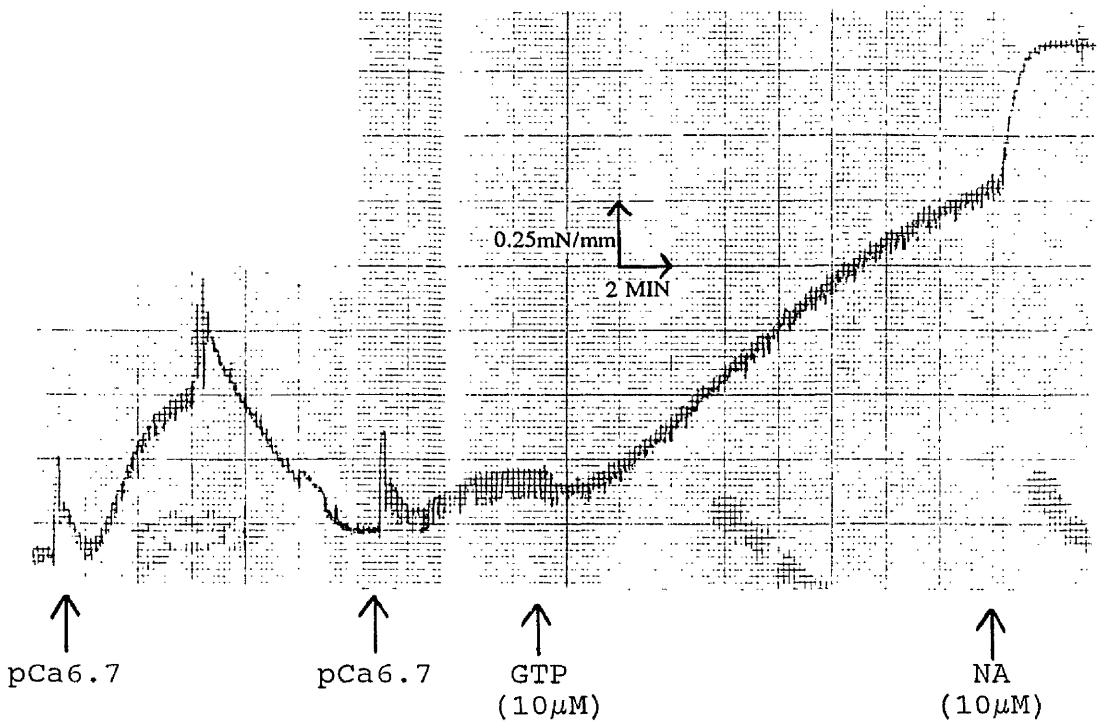


FIGURE 2.12 Representative tracing showing the response of an α-toxin permeabilised rat mesenteric small artery to 2 consecutive activations of pCa6.7, GTP (10μM) and NA (10μM).

As this method yielded the best responses to calcium plus receptor agonists following permeabilisation it was used in the subsequent calcium sensitisation experiments. However, because the responses to pCa6.7 were not entirely reproducible only one sensitisation experiment was performed for each set of vessels.

From Chapter 5 onwards α-toxin was obtained from GIBCO/BRL. The same protocol was used as for the α-toxin from List Laboratories but permeabilisation was achieved with 1,250 units/ml α-toxin in pCa6.7. Using this concentration the increase in tension development also took 15-20 minutes to reach a plateau.

7.2. Beta escin.

This is a saponin ester which, like α-toxin, permeabilises the cell membrane without disrupting the receptor-effector coupling. However, the pores created by ß-escin are bigger than those created by α-toxin therefore making the cell membrane permeable to higher molecular weight molecules such as heparin and calmodulin.

7.2a. Method.

The same protocol was used for permeabilisation with ß-escin as for α-toxin that is vessels were incubated in NES for 10 minutes then activated with KES and 10μM NA for 2 minute periods. After equilibration in G_1 for 15 minutes vessels were permeabilised with ß-escin according to the method of Kobayashi et al (1989b). Briefly, vessels were incubated in pCa7.0 containing 50μM ß-escin for 20 minutes. However, using this method no increase in tension was observed in contrast to permeabilisation with α-toxin.

In the following experiment 50μM ß-escin was again used to permeabilise the vessels, but in conjunction with 1μM leupeptin (a protease inhibitor) and 1μM calmodulin (to replace the calmodulin that may be passing out of the cells via the pores created by ß-escin). The ß-escin was left in for 20 minutes, but again no development of force was observed. After equilibrating in G_1 for 15 minutes vessels were activated with pCa6.7 then pCa6.3, but there was no response to either calcium solution.

The lack of development of tension during permeabilisation

with ß-escin may be due to the pCa of the incubating solution being too low to initiate smooth muscle contraction. Therefore in the next experiment vessels were incubated in pCa6.3 together with 50μM ß-escin, 1μM leupeptin and 1μM calmodulin, but again after 20 minutes there was no increase in tension. The concentration of ß-escin was raised to 200μM, which had no effect, then 400μM. This concentration did cause a small contraction which reached a plateau after 8-10 minutes (Figure 2.13).

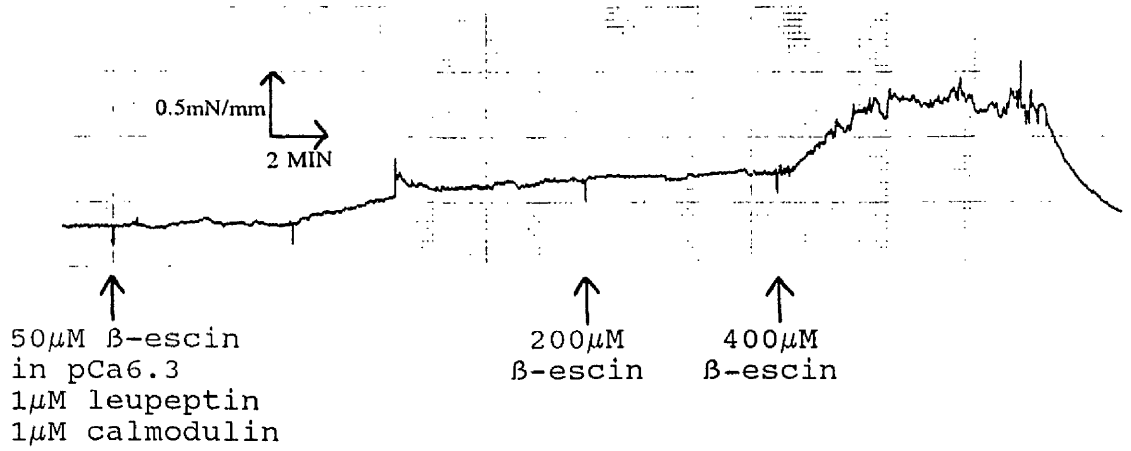


FIGURE 2.13 Representative tracing showing the profile of the response of a rat mesenteric small artery to increasing concentrations of ß-escin.

Vessels were re-equilibrated in G_i for 15 minutes then a dose response curve to pCa6.7, pCa6.3, pCa6.0 and pCa4.5 was performed (Figure 2.14). Again there was no response to the two lower doses but pCa6.0 elicited a small response which was slightly increased by the addition of pCa4.5:

Max KES tension = 2.288mN/mm

pCa6.7 = 0mN/mm = 0%

pCa6.3 = 0.057mN/mm = 2.50%

pCa6.0 = 1.258mN/mm = 55.00%

pCa4.5 = 1.659mN/mm = 72.49%

However, as the results show the final amplitude of the pCa4.5-induced contraction was only about 75% of the KES-induced contraction in intact arteries.

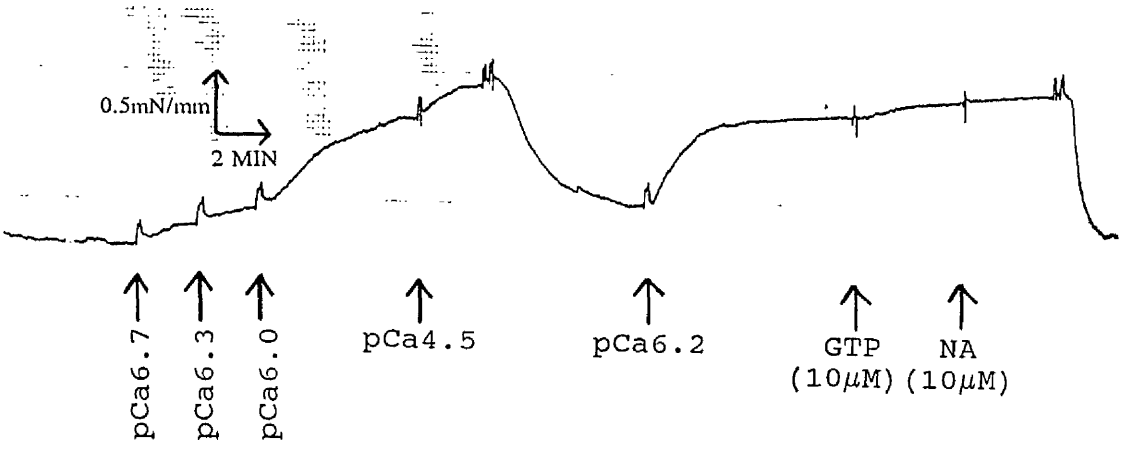


FIGURE 2.14 Representative tracing showing the response of a ß-escin permeabilised rat mesenteric small artery to increasing concentrations of calcium followed by pCa6.2, GTP (10μM) and NA (10μM).

Vessels were equilibrated in G_1 once more then a calcium sensitisation experiment was carried out with NA: vessels were preconstricted with pCa6.2 after which GTP (10μM) was added when the contraction had reached a plateau (Figure 2.14). However, only a slight increase in tension was observed to GTP and none at all following the subsequent addition of NA (10μM).

In the final experiment a concentration of 300μM ß-escin was used in pCa6.3 solution with 1μM leupeptin and a slightly higher concentration of 2.5μM calmodulin. Again there was a small development of force which reached a plateau after about 15 minutes. However, vessels again gave poor responses to pCa6.7 and pCa6.3 and increasingly larger responses to pCa6.0 and pCa4.5 (Figure 2.15). This time the contraction to pCa4.5 was comparable in amplitude to the contraction to KES in intact arteries:

Max KES force = 1.862mN/mm

pCa6.7 = 0.200mN/mm = 10.72%

pCa6.3 = 0.399mN/mm = 21.43%

pCa6.0 = 1.300mN/mm = 71.45%

pCa4.5 = 1.663mN/mm = 89.31%

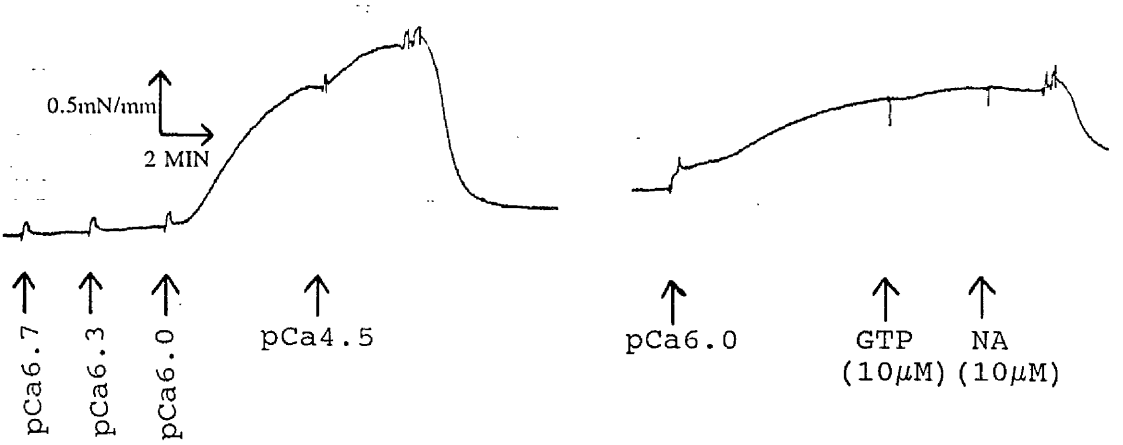


FIGURE 2.15 Representative tracing showing the response of a ß-escin permeabilised rat mesenteric small artery to increasing concentrations of calcium followed by pCa6.0, GTP (10μM) and NA (10μM).

Another sensitisation experiment was performed with pCa6.0, GTP (10μM) and NA (10μM)(Figure 2.15). pCa6.0 caused a large development of tension which was slightly increased by GTP. Once again NA did not cause any enhancement of calcium sensitivity indicating that the mechanism involved in calcium sensitisation was not functioning in ß-escin permeabilised rat mesenteric small arteries. As a result of the poor responses to the low calcium solutions and receptor agonists following permeabilisation ß-escin was not used in any experiments with calcium sensitivity.

8. Statistics.

The results from each treatment group are calculated as the mean value which is the point about which the distribution of measurements of individuals in the group is symmetrical. The standard deviation (STD) is a measure of the variability or spread of the measurements about the mean.

The standard error (SE) and can be calculated as STD/$\sqrt{N}$ where N is the number of measurements in the group.

Student's t-test.

This is one of the most widely used Tests of Significance in biology and compares the means and SEs of two separate groups of data to see if they are significantly different from each other.

Analysis of variance.

Analysis of variance (ANOVA) is a more powerful Test of Significance than the Student's t-test as it allows differences between the means of more than two treatment

groups to be examined. It is used to assess whether there is a significant trend within all the groups being studied.

Least Significant Difference test.

The Least Significant Difference (LSD) test can be used following ANOVA to examine individual differences between treatment means. However, it should only be performed if the preliminary ANOVA has shown there is a significant trend within the groups.

CHAPTER THREE

CALCIUM SENSITISATION EXPERIMENTS

1. Introduction.

Following agonist-receptor coupling the primary mechanism responsible for smooth muscle contraction is a rise in intracellular calcium and subsequent activation of myosin light chain (MLC) kinase (Walsh 1994). However, there is evidence an agonist-induced increase in the calcium sensitivity of the myofilaments may also play a role which appears to be dependent on G-proteins (Nishimura et al 1988, Kitazawa et al 1989). It has been proposed this G-protein-mediated calcium sensitisation is due to an increase in MLC phosphorylation (Fujiwara et al 1989, Kitazawa and Somlyo 1990, Kitazawa et al 1991a, Kitazawa et al 1991b) via the inhibition of MLC phosphatase (Somlyo et al 1989, Kubota et al 1992) by arachidonic acid (Gong et al 1992). Protein kinase C (PKC) has also been implicated in this response (Moreland et al 1992a) via the phosphorylation of the thin filaments calponin (Winder and Walsh 1993) and caldesmon (Walsh 1994) or intermediate filaments (Rasmussen et al 1987). None of these investigations, however, have been carried out using small arteries mounted in a wire myograph despite their importance in regulating peripheral vascular resistance.

Therefore the ability of various agonists to increase myofilament calcium sensitivity in rat mesenteric small arteries mounted in a wire myograph and permeabilised with Staphylococcal aureus α-toxin has been investigated. These included a number of receptor agonists, phorbol esters and

the lipid second messengers arachidonic acid, lysophosphatidylcholine and phosphatidic acid.

2. Protocols.

The full protocol for permeabilisation with Staphylococcal aureus α-toxin (as developed in Materials and Methods) can be seen in Figure 3.1. Briefly vessels were equilibrated in normal external solution (NES) for 10 minutes then activated with potassium external solution (KES) followed by 10μM noradrenaline (NA) in NES. Activations were for 2 minutes and the strips were allowed to fully relax between each stimulation. They were then incubated in relaxing solution (G_1) for approximately 15 minutes before permeabilisation with 300-800 units/ml List Laboratories α-toxin in pCa6.7 with 10μM calcium ionophore A23187 (dissolved in dimethyl sulfoxide (DMSO)). After tension development had reached a plateau (a period of 15-20 minutes) vessels were re-equilibrated in G_1 for 15 minutes then stimulated with pCa6.7 to check permeabilisation had taken place. When this contraction had reached a plateau vessels were equilibrated in G_1 once more for 20-25 minutes before being re-stimulated with pCa6.7 for 8-10 minutes. This gave a sub-maximum, constant contraction and the agonists were then added directly to the myograph bath. Responses were calculated as active tension (as described on Page 56) and expressed as a % of the maximum KES contraction in the intact artery.

2.1. Receptor agonists.

Receptor agonist-induced calcium sensitisation requires the presence of GTP so it was added to the myograph bath prior to

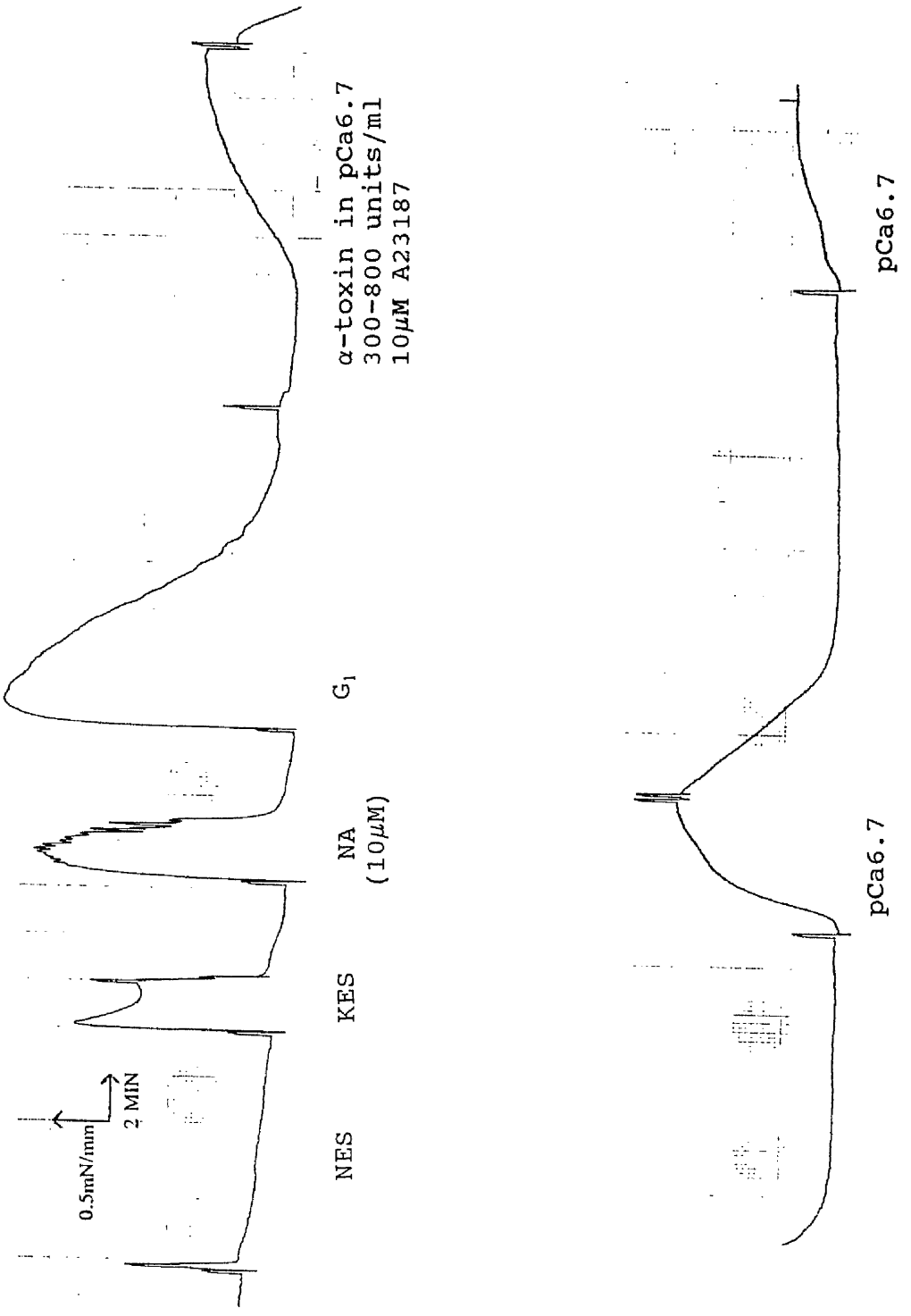


FIGURE 3.1 Representative tracing showing the protocol for permeabilisation of rat mesenteric small arteries using α-toxin. NES - normal external solution; KES - potassium external solution; G_1 - relaxing solution; NA - noradrenaline.

addition of the receptor agonists. However, GTP itself increased myofilament calcium sensitivity (Fujiwara et al 1989) therefore preliminary dose response curves were carried out to find a concentration of GTP that produced a sub-maximum contraction thereby allowing a further contraction to the receptor agonists. The results are summarised below for n=3 (mean±SEM):

Maximum KES tension = 1.822±0.210mN/mm

pCa6.7 = 0.392±0.138mN/mm = 23.06±8.65%

pCa6.7 + 1μM GTP = 0.392±0.138mN/mm = 23.06±8.65%

" + 3μM GTP = 0.392±0.138mN/mm = 23.06±8.65%

" + 5μM GTP = 0.468±0.189mN/mm = 27.15±10.71%

" + 8μM GTP = 0.845±0.207mN/mm = 49.37±14.96%

" + 10μM GTP = 1.116±0.293mN/mm = 64.67±19.23%

10μM GTP (dissolved in distilled water) gave a large increase in tension compared with that developed to pCa6.7, but it was only approximately 65% of the maximum KES contraction in the intact vessels. Therefore 10μM GTP was used in all calcium sensitisation experiments involving receptor agonists. In order to investigate whether the endothelium contributed to the sensitising effect of GTP it was removed by placing a human hair down the lumen of the vessel following normalisation and rubbing it from side to side. The vessel was then preconstricted with 10μM NA and acetylcholine (ACh)(1μM)(dissolved in distilled water) added to see if it induced relaxation. Using this technique no relaxation was observed implying the vessel had been successfully de-endothelialised. It was found that removal of the endothelium

made no difference to the increase in calcium sensitivity elicited by GTP (Figure 3.2) for n=2 (mean±STD):

Maximum KES tension = 2.576±0.232mN/mm

pCa6.7 = 0.720±0.294mN/mm = 27.15±8.97%

pCa6.7 + 10μM GTP = 1.771±0.550mN/mm = 65.87±15.26%

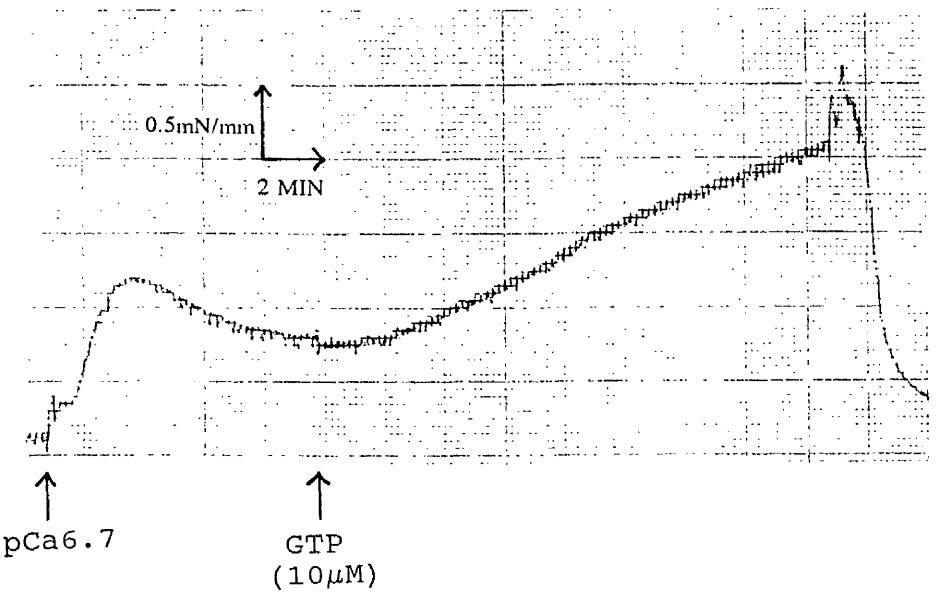


FIGURE 3.2 Representative tracing showing the response of a de-endothelialised rat mesenteric small artery to pCa6.7 and GTP (10μM).

Consequently, the endothelium was left intact in future experiments which also minimalised damage to the arteries as a result of the endothelium removal procedure.

The ability of GTP to potentiate the force developed to the sub-maximal calcium concentration may have been a result of activation of the G-protein or an artifact of the presence of highly charged phosphate groups on the GTP molecules themselves. The compound diphosphoglycerol (DPG) (dissolved in distilled water) also contains highly charged phosphate

groups but it only caused a slight potentiation of force development to pCa6.7 at concentrations considerably higher than those used for GTP (mean±STD for n=2):

Maximum KES tension = 2.939±0.562mN/mm

pCa6.7 = 1.297±0mN/mm = 45.79±8.75%

pCa6.7 + 10μM DPG = 1.297±0mN/mm = 45.79±8.75%

" + 20μM DPG = 1.329±0.033mN/mm = 46.72±7.83%

" + 50μM DPG = 1.639±0.018mN/mm = 57.99±11.69%

" + 100μM DPG = 1.948±0.068mN/mm = 69.27±15.56%

" + 150μM DPG = 2.226±0.039mN/mm = 79.63±10.37%

Therefore the GTP-induced contraction in permeabilised arteries was probably due to the G-protein coupling of the receptor to the effector ie the contractile apparatus.

The receptor agonists NA (10μM), vasopressin (AVP)(4.0mU/ml), endothelin-1 (ET-1)(100nM) and angiotensin II (AII)(100nM) (all dissolved in distilled water) were added to the myograph bath after the contraction to GTP had reached a plateau. The concentration of each agonist was determined from preliminary dose response curves in intact arteries in which they induced maximum contractile responses.

2.2. <u>Phorbol esters.</u>

The ability of two phorbol esters to enhance myofilament calcium sensitivity was investigated: phorbol 12,13-dibutyrate (PdBu) and 12-0-tetradecanoylphorbol-13-acetate (TPA) which were dissolved in DMSO (to give a final maximum DMSO concentration of <0.5% of the volume of the myograph

bath). Unlike receptor agonists phorbol esters do not require GTP to induce calcium sensitisation therefore they were added directly to the contraction elicited by pCa6.7.

2.3. Arachidonic acid.

Arachidonic acid (AA) was originally dissolved in ethanol therefore in order to ensure the ethanol did not contribute to the results the equivalent concentration (3% of the volume of the myograph bath) was added to the pCa6.7-induced contraction. It was left in for the same length of time as AA but caused no potentiation of the contraction to the sub-maximal calcium concentration.

2.4. Lysophosphatidylcholine.

L-α-lysophosphatidylcholine, palmitoyl (LysoPC) was dissolved in distilled water in the first sensitisation experiment. In the following experiment it was dissolved in ethanol.

2.5. Phosphatidic acid.

The effect of 3 different phosphatidic acids (PAs) on calcium sensitivity were studied: dipalmitoyl, dioleoyl and stearate arachidonate. Dipalmitoyl and dioleoyl PA were dissolved in DMSO but on addition to the pCa6.7 solution in the myograph bath the mixture immediately clumped together. Therefore a calculated small volume of each PA solution was added to 0.5ml pCa6.7 and sonicated. This solution was then mixed with 12ml pCa6.7 and added to the myograph bath to give a final concentration of 100μM. Stearate arachidonate PA was dissolved in chloroform which was dried under nitrogen and re-dissolved in DMSO. A calculated small volume of this

solution was again sonicated in 0.5ml pCa6.7 and mixed with 12ml pCa6.7 which was then added to the myograph bath to give a final concentration of 100μM.

3. Results.

3.1. Receptor agonists.

NA, AVP and ET-1 all enhanced the calcium sensitivity of the myofilaments in α-toxin permeabilised rat mesenteric small arteries (Table 3.1). This effect was immediate and the contractions reached a plateau within 5-10 minutes (Figure 3.3a and 3.3b). However, the contraction elicited by AII was only slightly greater than the contraction elicited by GTP in permeabilised vessels with an intact endothelium (Table 3.1). Following the removal of the endothelium (as described on page 83) the results show there was no improvement to the AII-induced calcium sensitisation (Table 3.1).

3.2. Phorbol esters.

PdBu caused a dose dependent increase in the tension developed to pCa6.7 (Table 3.2). The maximum concentration of 2μM PdBu induced an immediate contraction in permeabilised rat mesenteric small arteries, but this was more slowly-developing than the contraction to the receptor agonists and took 15-20 minutes to reach a plateau (Figure 3.3c). TPA, on the other hand, had no calcium-sensitising effect at similar doses (Table 3.2).

TABLE 3.1 Contractile response of α-toxin permeabilised rat mesenteric small arteries to receptor agonists. Mean±STD for n=2.

	NA (10μM)	AVP (4.0mU/ml)	ET-1 (100nM)	AII (100nM)	
				+endo	-endo
Max KES (mN/mm)	2.599 ±0.298	2.439 ±0.111	2.774 ±0.658	3.408 ±0.111	2.257 ±0.557
pCa6.7 (mN/mm)	0.485 ±0.193	0.765 ±0.036	0.374 ±0.028	1.046 ±0.394	0.353 ±0.240
%KES	18.05 ±5.34	31.48 ±2.91	14.02 ±2.33	31.10 ±12.56	13.85 ±7.20
+ 10μM GTP (mN/mm)	1.059 ±0.144	1.424 ±0.261	0.972 ±0.183	1.844 ±0.368	1.137 ±0.284
%KES	41.95 ±10.35	57.46 ±10.39	35.46 ±1.83	54.80 ±11.37	50.36 ±0.18
+ agonist (mN/mm)	1.600 ±0.039	2.544 ±0.308	2.420 ±0.667	1.948 ±0.435	1.332 ±0.296
%KES	62.55 ±8.64	103.97 ±7.94	86.38 ±3.55	57.94 ±13.45	59.31 ±1.63

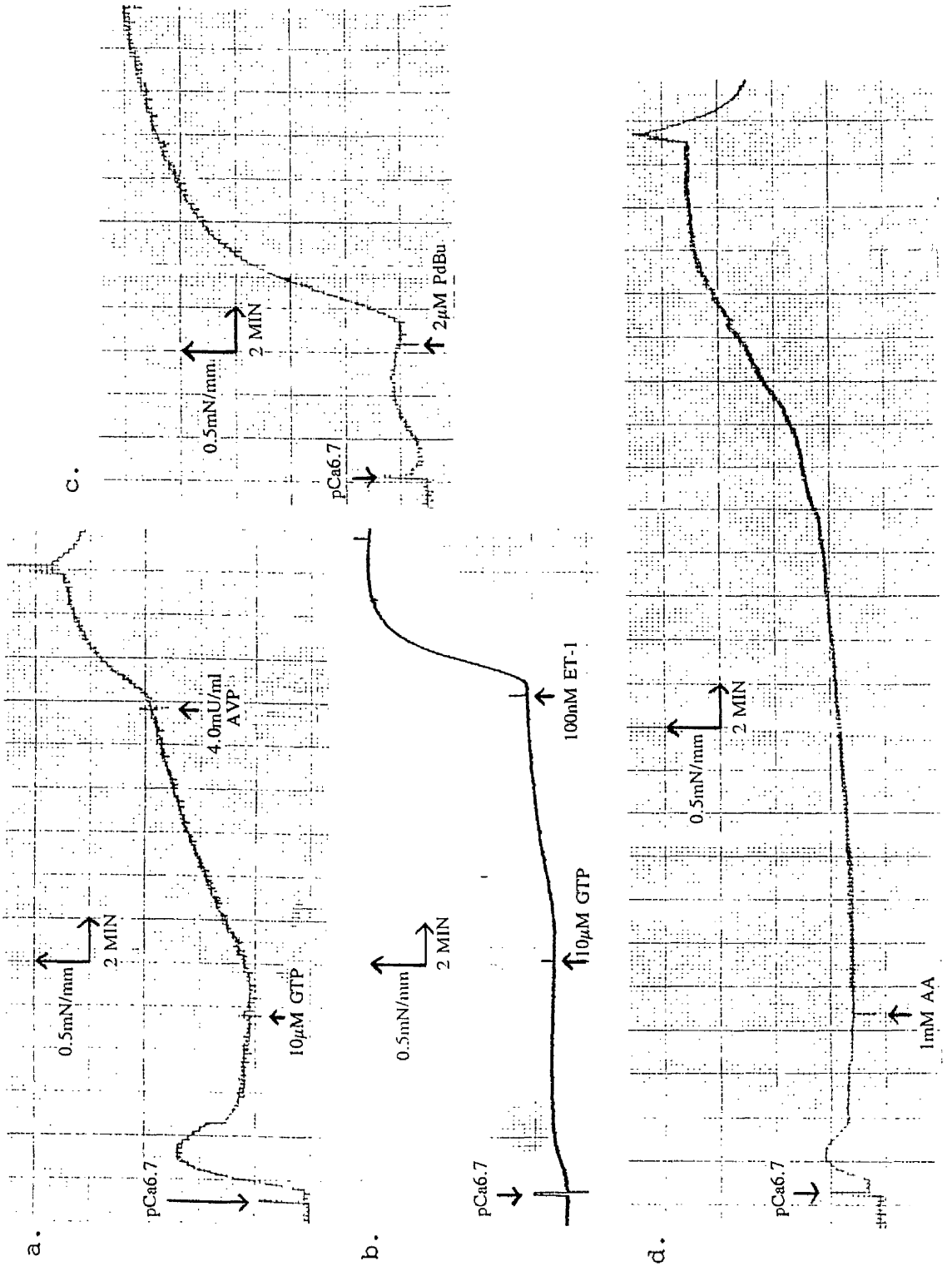


FIGURE 3.3 Representative tracings showing the calcium sensitisation profiles of a. GTP (10μM) + AVP (4.0mU/ml) b. GTP (10μM) + ET-1 (100nM) c. PdBu (2μM) and d. AA (1mM) at pCa6.7 in α-toxin permeabilised rat mesenteric small arteries.

TABLE 3.2 Contractile response of α-toxin permeabilised rat mesenteric small arteries to phorbol esters. Mean±STD for n=2.

	PdBu	TPA
Max KES (mN/mm)	2.504±0.461	1.930±0.157
pCa6.7 (mN/mm)	0.317±0.044	0.437±0.829
% KES	13.09±1.73	21.98±11.27
+ phorbol ester 2nM (mN/mm)	0.317±0.044	0.437±0.829
% KES	13.09±1.73	21.98±11.27
+ " " 20nM (mN/mm)	0.317±0.044	0.437±0.829
% KES	13.09±1.73	21.98±11.27
+ " " 200nM (mN/mm)	1.167±0.416	0.437±0.829
% KES	45.37±4.68	21.98±11.27
+ " " 2μM (mN/mm)	3.489±0.108	0.437±0.829
% KES	150.97±14.16	21.98±11.27

The lack of calcium sensitisation with TPA may have been due to the concentration being too low. However, when higher concentrations were tried over a time period of about an hour they only caused a slight increase in the tension developed to the sub-maximal calcium concentration for n=2 (mean±STD):

Maximum KES tension = 2.395±0.446mN/mm

pCa6.7 = 0.633±0.268mN/mm = 25.23±6.48%

" + 10μM TPA = 0.633±0.268mN/mm = 25.23±6.48%

" + 40μM TPA = 0.702±0.337mN/mm = 27.67±8.92%

" + 70μM TPA = 0.872±0.446mN/mm = 34.11±12.33%

" + 100μM TPA = 0.971±0.484mN/mm = 38.11±13.11%

3.3. Arachidonic acid.

300μM AA was initially added to permeabilised rat mesenteric small arteries stimulated with pCa6.7, but it had no effect. 600μM, however, caused a steady increase in myofilament calcium sensitivity which was further increased with 1mM AA (mean±STD for n=2):

Maximum KES tension = 2.076±0.246mN/mm

pCa6.7 = 0.557±0.175mN/mm = 28.24±11.77%

" + 1mM AA = 2.414±0.468mN/mm = 115.20±8.87%

Figure 3.3d shows this contraction was very slow in developing and took 40-45 minutes to reach a plateau.

3.4. Lysophosphatidylcholine.

100μM LysoPC dissolved in distilled water only caused a slight potentiation of the force developed to the sub-maximal calcium

concentration (Table 3.3). When it was dissolved in ethanol there was no change in this result (Table 3.3).

3.5. Phosphatidic acid.

The PAs dipalmitoyl, dioleoyl or stearate arachidonate (100μM) had no calcium sensitising effect at all (Table 3.4).

Discussion

These results have demonstrated a technique for the successful permeabilisation of rat mesenteric small arteries using Staphylococcal aureus α-toxin without disruption of the membrane receptors or their intracellular signal transduction pathways. This has enabled the ability of a number of agonists to increase the calcium sensitivity of the contractile apparatus to be investigated.

Using this technique GTP (10μM) induced calcium sensitisation in α-toxin permeabilised rat mesenteric small arteries suggesting this effect was mediated by G-proteins. Both GTP and the non-hydrolysable GTP analogue GTPγS increased the force developed to a sub-maximal calcium concentration in permeabilised vascular smooth muscle (Nishimura et al 1988, Fujiwara et al 1989, Nishimura et al 1990, Kitazawa et al 1991a, Kitazawa et al 1991b, Kubota et al 1992, Kawase and van Breeman 1992, Crichton et al 1993). It was reversible in the case of GTP, but irreversible in the case of GTPγS due to the inability of GTPase to hydrolyse GTPγS (Nishimura et al 1988, Fujiwara et al 1989, Nishimura et al 1990, Kubota et al 1992, Kawase and van Breeman 1992, Crichton et al 1993). Nishimura et al (1988), Fujiwara et al (1989), Kitazawa et al (1991a) and Crichton et al (1993) found

TABLE 3.3 Contractile response of α-toxin permeabilised rat mesenteric small arteries to lysophosphatidylcholine (LysoPC). Mean±STD for n=2.

	LysoPC dissolved in water (100μM)	LysoPC dissolved in ethanol (100μM)
Max KES (mN/mm)	2.682±0.529	2.196±0.076
pCa6.7 (mN/mm)	0.290±0.161	0.275±0.068
% KES	12.47±8.47	12.44±2.67
+ 100μM LysoPC (mN/mm)	0.348±0.190	0.356±0.013
% KES	14.93±10.03	16.25±1.14

TABLE 3.4 Contractile response of α-toxin permeabilised rat mesenteric small arteries to phosphatidic acid (PA). Mean±SEM for n=3.

	Dipalmitoyl PA (100μM)	Dioleoyl PA (100μM)	Stearate arachidonate PA (100μM)
Max KES (mN/mm)	2.556±0.242	2.278±0.235	1.728±0.283
pCa6.7 (mN/mm)	0.388±0.002	0.302±0.039	0.238±0.104
% KES	16.14±5.49	13.78±2.88	17.17±9.12
+ 100μM PA (mN/mm)	0.388±0.002	0.302±0.039	0.238±0.104
% KES	16.14±5.49	13.78±2.88	17.17±9.12

the GTPγs-induced enhancement of calcium sensitivity was much greater than that induced by GTP which confirmed the GTP-binding protein was a genuine G-protein with GTPase activity. Furthermore, the response to maximal concentrations of GTPγS was not further increased by the addition of agonists, suggesting GTPγS brought about the full expression of G-protein-mediated calcium sensitisation (Kitazawa et al 1991a, Crichton et al 1993).

In the present study the receptor agonists NA (10μM) and ET-1 (100nM) potentiated the contraction to GTP in α-toxin permeabilised rat mesenteric small arteries which is in agreement with previous studies using these agonists in permeabilised vascular smooth muscle preparations (Nishimura et al 1988, Nishimura et al 1990, Kitazawa et al 1991a, Nishimura et al 1991, Moreland et al 1992a, Nishimura et al 1992, Satoh et al 1994b, Yoshida et al 1994, Fujita et al 1995, Jensen 1996, Jensen et al 1996). Nishimura et al (1990), Kitazawa et al (1991a), Nishimura et al (1991), Nishimura et al (1992), Satoh et al (1994b), Yoshida et al (1994), Fujita et al (1995), Jensen (1996) and Jensen et al (1996) reported GDPβS (a non-hydrolysable GDP analogue) inhibited these potentiated contractions which was corroborated by similar investigations using α-toxin permeabilised arteries stimulated with phenylephrine (PE) (Kitazawa et al 1989, Kitazawa et al 1991a, Masuo et al 1994). Since GDP is responsible for the inactivation of the G-protein and therefore uncoupling of the receptor to the contractile apparatus these investigations (Kitazawa et al 1989, Nishimura et al 1990, Nishimura et al 1991, Kitazawa et al 1991a, Nishimura et al 1992, Satoh et al 1994b, Yoshida et al 1994,

Masuo et al 1994, Fujita et al 1995, Jensen 1996, Jensen et al 1996) reinforced a role for G-proteins in the receptor agonist-induced calcium sensitisation.

Both the ET-1 and NA receptors have been shown to be coupled to a pertussis toxin-insensitive G-protein (Takuwa et al 1990, LaBelle and Murray 1990). Therefore, the similarity in the profile and mechanism involved in the contraction elicited by ET-1 and NA in α-toxin permeabilised rat mesenteric small arteries implied the two vasoconstrictor hormones were acting via a similar signal transduction pathway to bring about calcium sensitisation. Furthermore, these data suggested the mechanism involved in calcium sensitisation was the same between small arteries and other types of vascular smooth muscle.

Vasopressin causes vasoconstriction in intact smooth muscle by a similar mechanism to NA and ET-1 ie binding to its receptor and stimulation of the phosphoinositol cascade (Doyle and Ruegg 1985) therefore it is not surprising that it also increased the calcium sensitivity of the myofilaments like NA and ET-1. However, as yet there have been no similar investigations on the sensitising effect of AVP in permeabilised vascular smooth muscle. On the other hand 100nM AII did not increase myofilament calcium sensitivity in α-toxin permeabilised rat mesenteric small arteries with or without an endothelium despite the fact it is also a receptor-mediated vasoconstrictor (Griendling et al 1986). This is in contrast to a study by Nishimura et al (1991) who reported AII (100nM) significantly increased calcium sensitivity in the presence of GTP. However, they used α-toxin permeabilised rabbit mesenteric arteries therefore the

discrepancy between the two studies could be due to the difference in species used. Indeed, intact rat mesenteric arteries mounted in a myograph have previously been shown to elicit poor responses to AII (Juul et al 1987). This was attributed to a strong tachyphylaxis and only eliminated by inducing sub-maximal tone in the arteries (Juul et al 1987).

In this thesis the phorbol ester PdBu produced a slowly-developing, sustained contraction in permeabilised rat mesenteric small arteries depending on the dose administered which is in agreement with earlier studies in which PdBu produced dose dependent contractions in permeabilised vascular smooth muscle tissue (Chatterjee and Tejada 1986, Nishimura et al 1991, Masuo et al 1994, Yoshida et al 1994). The phorbol ester TPA, on the other hand, caused only a slight enhancement of calcium sensitivity at concentrations up to 100μM. This is surprising as TPA has been shown to induce contractions in intact (Danthuluri and Deth 1984, Rasmussen et al 1984) and permeabilised (Fujiwara et al 1989, Nishimura et al 1990) rabbit vascular smooth muscle at much lower concentrations. It did not induce contractions, however, in permeabilised porcine carotid (Chatterjee and Tejada 1986) or permeabilised rabbit femoral (Masuo et al 1994) arteries. A possible explanation for the unresponsiveness to TPA, but not to PdBu, in these studies and in the present study is that the two phorbol esters were acting via different PKC isotypes to induce contractile responses. In Chapter Four it is demonstrated that rat mesenteric small arteries possess PKCs α, γ, δ, ε and ζ therefore TPA may have been acting via a PKC isotype which was not present in these arteries for example PKCß. Indeed, long-term treatment

with TPA resulted in the activation and down-regulation of PKCß in liver macrophages (Duyster et al 1993). In view of these observations it was decided to use PdBu in preference to TPA in future experiments with phorbol ester-induced calcium sensitisation.

Arachidonic acid caused a slow, steady increase in myofilament calcium sensitivity in α-toxin permeabilised rat mesenteric small arteries which is similar to observations in α-toxin permeabilised large arteries from the rabbit (Gong et al 1992, Parsons et al 1996). This did not appear to be mediated by G-proteins as it occurred in the absence of GTP (Gong et al 1992, Parsons et al 1996) and was not inhibited by GDPßS (Gong et al 1992). However, PKC may have been involved as AA can activate PKC (Nishizuka 1995). Indeed, the PKC inhibitor RO-31-8220 significantly reduced the AA-induced increase in force developed to a sub-maximal calcium concentration by approximately 30% in permeabilised rabbit mesenteric arteries (Parsons et al 1996). In contrast, a peptide pseudosubstrate inhibitor of PKC had no effect on the enhancement of calcium sensitivity elicited by AA in permeabilised rabbit femoral arteries (Gong et al 1992). The potential role for AA as a second messenger in calcium sensitisation was supported by a later study by Gong et al (1995) in which AA and DAG levels were increased following stimulation of permeabilised arteries with a variety of calcium-sensitising agents including GTPγS, PE, carbachol and PdBu. It has been demonstrated that the rise in AA following vasoconstrictor hormone and phorbol ester stimulation was due to activation of phospholipase A_2 (PLA_2) (Ito et al 1993, Lehman et al 1993, Rao et al 1994, Ho and Klein 1987) and indeed the PLA_2 inhibitor

quinacrine completely inhibited the increase in myofilament calcium sensitivity elicited by serotonin (but not PE) (Parsons et al 1996).

The hydrolysis of phospholipids by PLA_2 also results in the production of LysoPC suggesting it too could be a second messenger involved in calcium sensitisation. However, no calcium sensitising effect of 100μM L-α-lysophosphatidylcholine, palmitoyl was observed in α-toxin permeabilised rat mesenteric small arteries. This is in contrast to a recent study by Jensen et al (1996) who found L-α-lysophosphatidylcholine, palmitoyl (and myristoyl) (100μM) potentiated the sub-maximal calcium-induced contraction in α-toxin permeabilised rat mesenteric small arteries through a G-protein independent, but PKC-dependent mechanism. Indeed, LysoPC has been shown to activate PKC (Nishizuka 1995). There is no explanation for the difference in results between the two investigations as exactly the same protocols and conditions were used for both. However, Jensen et al (1996) found LysoPCs containing other fatty acids had no sensitising effect plus during stimulation with NA there was no increase in ^{3}H-myristate labelled LysoPC. Therefore the role of this second messenger in regulating calcium sensitivity is still uncertain.

PLA_2 can also cause the hydrolysis of phosphatidyl-inositol or -ethanolamine to produce lysophosphatidylinositol (LysoPI) and lysophosphatidylethanolamine (LysoPE) respectively leading to a potential role for these second messengers in calcium sensitisation. Indeed, LysoPI increased myofilament calcium sensitivity in α-toxin permeabilised rat mesenteric small

arteries via a PKC-dependent mechanism, whereas LysoPE had no effect (Jensen 1996).

Finally, although PA can increase contractility in intact isolated smooth muscle cells (Salmon and Honeyman 1980) and possibly activate PKC (Nishizuka 1995) it did not enhance the calcium sensitivity in this thesis. This is in agreement with Gong et al (1992) who also observed a lack of calcium sensitisation with 100μM PA (dimyristoyl and dioleoyl PA).

4.1. Summary.

These data show that the vasoconstrictor hormones NA, ET-1 and AVP, the phorbol ester PdBu and the fatty acid arachidonate increased the sensitivity of the contractile apparatus to calcium in α-toxin permeabilised rat mesenteric small arteries. In contrast the vasoconstrictor hormone AII, the phorbol ester TPA and the lipids PA and LysoPC were without effect. The vasoconstrictor hormone-induced increase in calcium sensitivity was dependent on GTP and GTP itself increased calcium sensitivity, indicating that G-proteins were involved in this response. Calcium sensitisation induced by PdBu was independent of GTP suggesting that PKC was involved in the response downstream from G-protein-receptor coupling. Vasoconstrictor hormones and phorbol esters have been shown to increase PKC activity (Haller et al 1990) and potentiate AA release (Ho and Klein 1987, Ito et al 1993, Lehman et al 1993, Rao et al 1994, Gong et al 1995) in intact vascular smooth muscle. However, whether PKC and AA act by parallel and independent or interrelated mechanisms to produce calcium sensitisation has not yet been established.

CHAPTER FOUR

INVOLVEMENT OF PROTEIN KINASE C IN THE CONTRACTILE RESPONSE TO RECEPTOR AGONISTS AND PHORBOL ESTERS

1. Introduction.

In the previous chapter using rat mesenteric small arteries permeabilised with Staphylococcal aureus α-toxin it was demonstrated that the vasoconstrictor hormones noradrenaline (NA), endothelin (ET-1) and vasopressin (AVP) and the phorbol ester phorbol 12,13-dibutyrate (PdBu) increased the calcium sensitivity of the myofilaments. In cultured smooth muscle cells vasoconstrictor hormones can generate 1,2-diacylglycerol (DAG)(Griendling et al 1986, Ohlstein et al 1989) and activate PKC indirectly whereas phorbol esters bind to activate PKC directly (Castagna et al 1982). These results suggest PKC may be involved in the agonist-induced calcium sensitisation in vascular smooth muscle but the evidence for this is controversial. Furthermore, this effect has not been studied using small arteries mounted in a wire myograph despite their crucial role in maintaining tone in the vasculature.

Therefore the contractile response of both intact and α-toxin permeabilised rat mesenteric small arteries mounted in a wire myograph to the receptor agonists NA, ET-1 and AVP and the phorbol ester PdBu following PKC down-regulation by prolonged exposure to PdBu or in the presence of the potent and specific PKC inhibitor RO-31-8330 (Davis et al 1989) has been investigated.

2. Protocols.

2.1. PKC down-regulation studies.

PKC was down-regulated by placing a short section of rat mesenteric small artery in a petri-dish containing 20ml of tissue culture medium M199 composed of (major constituents only)(mM): NaCl 128, KCl 5.4, $MgSO_4.7H_2O$ 0.34, $CaCl_2.2H_2O$ 1.3, $NaHCO_3$ 4.2, KH_2PO_4 0.44, $Fe(NO_3)_3.9H_2O$ 0.002, glucose 5.6, Hepes 25 with 500nM PdBu. This was the minimum concentration of PdBu required to completely abolish the contractile response to the phorbol ester following incubation. A second petri-dish was set up containing 20ml M199 and another section of artery with either 500nM 4-α-phorbol 12,13-didecanoate (4αPdD)(dissolved in dimethyl sulfoxide (DMSO)), which is an inactive phorbol ester, or 0.1% DMSO as a control. The petri-dishes were then placed in a CO_2 incubator at 37°C for 15 hours (a control experiment using vessels mounted in the myograph showed the tension developed to 500nM PdBu reached a plateau after about half an hour then declined slowly to basal values over a period of 8-10 hours therefore 15 hours would have been sufficient for down-regulation of PKC).

Following overnight incubation the vessels were carefully removed from each petri-dish, mounted in a myograph in PSS and warmed to 37°C. After normalisation (as described on Page 56) vessels were stimulated with 3 activations of KPSS, each one was for 2 minutes and vessels were allowed to relax fully between stimulations. After equilibration in PSS for 15 minutes cumulative dose response curves to NA and AVP were performed. In order to investigate the role of PKC in the non-receptor-mediated contraction a dose response curve to

potassium chloride (KCl) was also performed. The α_1 adrenoceptor antagonist prazosin (10μM) (dissolved in DMSO) was included in the KCl solutions to ensure stimulation of the α_1 adrenoceptors by neuronally released catecholamines did not contribute to the contractile response. However, because prazosin may not wash out completely the KCl dose response curve was carried out as the final stimulation. The effects of ET-1 also appeared to be long-lasting therefore this dose response curve was performed in a separate experiment. Responses were calculated as active tension (as described on Page 56) and % of maximum contraction. The sensitivity for each dose response curve was calculated as the ED_{50} defined as the concentration necessary to elicit half-maximum contractions and was estimated by fitting a simple linear regression line to the central part of the individual curves. The tension developed to PdBu (2μM) and AII (100nM), which induced maximum contractions, was also measured. Finally, the effect of overnight incubation on the integrity of the endothelium was investigated by pre-constricting the vessels with 10μM NA and adding acetylcholine (ACh) (10μM) when the contraction had reached a plateau to see if it caused relaxation. The order of stimulation with the different agonists was varied for each experiment and vessels were re-equilibrated in PSS for 15 minutes between each stimulation. Analysis of variance (ANOVA) was used to assess differences between the groups.

2.2. <u>PKC inhibitor studies.</u>

Following overnight incubation with 500nM PdBu to down-regulate PKC the contractile response of the vessels to

potassium external solution (KES) and 10μM NA at room temperature was very poor. It was greatly improved by raising the temperature to 37°C, but successful permeabilisation could not be achieved under these conditions. In view of these difficulties the PKC inhibitor RO-31-8330 was used as an alternative method for abolishing PKC activity in permeabilised rat mesenteric small arteries.

Following permeabilisation (as described on Page 81) vessels were equilibrated in G_1 for 15 minutes then stimulated with pCa6.7. When this contraction had reached a plateau vessels were re-equilibrated in G_1 for a further 20-25 minutes before incubation with $3x10^{-7}$M RO-31-8330 (dissolved in DMSO) for 10 minutes. Preliminary experiments showed this concentration of PKC inhibitor was the lowest concentration required to completely abolish the contractile response to PdBu in permeabilised arteries. pCa6.7 was added for 8-10 minutes to give a constant, sub-maximum contraction after which vessels were stimulated with 2μM PdBu for 20 minutes (to confirm PKC inhibition) followed by 10μM GTP. When this contraction had reached a plateau a dose response curve to 0.1, 1, 3, 10 and 100nM ET-1 was performed. Each contraction was allowed to reach a plateau before the next dose was added. The control group was not incubated with the PKC inhibitor or stimulated with 2μM PdBu and the dose response curve to ET-1 was carried out in the presence of 0.1% DMSO. The effect of $3x10^{-7}$M RO-31-8330 on the calcium-induced contraction was studied by adding it to a fixed, maximum calcium contraction following permeabilisation.

Finally, after normalisation and equilibration in PSS for 15 minutes intact rat mesenteric small arteries were incubated in $3x10^{-7}$M RO-31-8330 for 10 minutes. They were then stimulated with 2μM PdBu for 20 minutes after which a dose response curve to 0.1, 1, 10 and 100nM ET-1 was performed. Again each contraction was allowed to reach a plateau before the next dose was added. In addition, intact arteries were stimulated with increasing concentrations of KCl in the presence of $3x10^{-7}$M RO-31-8330 to investigate the effect of the PKC inhibitor on the KCl-induced contraction. Controls consisted of identical dose response curves in the presence of 0.1% DMSO. Responses were calculated as active tension and differences between dose response curves were calculated using ANOVA plus the Least Significant Difference (LSD) test. Student's t-test was used to assess differences in the contraction to pCa6.7 and pCa6.7 + GTP (10μM) in permeabilised arteries.

3. Results.

3.1. PKC down-regulation studies.

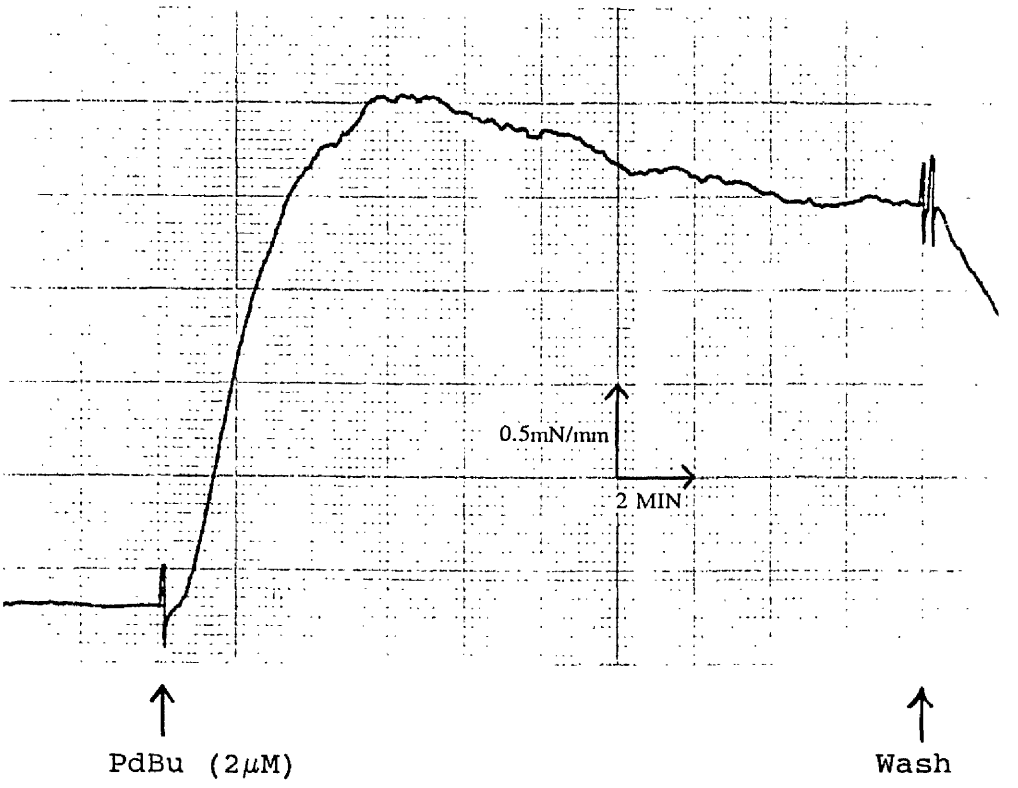
Effect on PdBu-induced contraction.

Vessels incubated in 500nM PdBu overnight failed to contract to PdBu (2μM) whereas those incubated in 500nM 4αPdD and 0.1% DMSO gave a large contraction (Figure 4.1, Table 4.1).

Effect on receptor agonist-induced contraction.

The ED_{50} values and maximum tension developed to NA (10μM), ET-1 (100nM) and AVP (4.0mU/ml) (Table 4.1) plus dose response curves to ET-1 and AVP (Figures 4.2 and 4.3) were not different between the 3 pretreatment groups.

a. Pretreatment: 4αPdD



b. Pretreatment: Pdbu

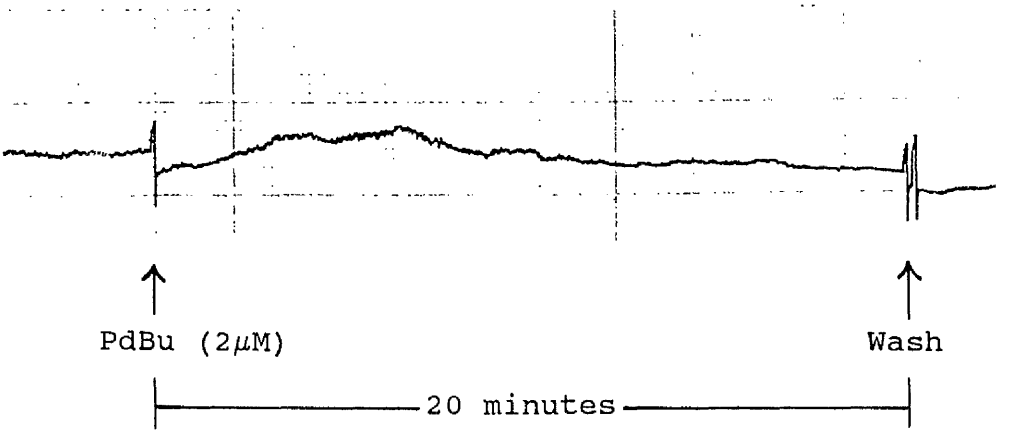


FIGURE 4.1 Representative tracings showing the contractile response of intact rat mesenteric small arteries to PdBu (2μM) following overnight incubation with a. 500nM 4αPdD and b. 500nM PdBu.

TABLE 4.1 Contractile response of intact rat mesenteric small arteries to agonists and phorbol ester following overnight incubation with 500nM PdBu, 500nM 4αPdD and 0.1% DMSO. Data show the ED_{50} values for ET-1, AVP, NA and KCl + prazosin (10μM) dose response curves. The tension developed to maximal doses of ET-1 (100nM), AVP (4.0mU/ml), NA (10μM), KCl (125mM), AII (100nM) and PdBu (2μM) is also shown. Mean±SEM for 10 arterial segments taken from individual animals. *p<0.05 (vs DMSO) by ANOVA.

Pretreatment	Stimulation	Max. Tension	ED_{50}
		mN/mm	nM
PdBu	ET-1	2.78±0.19	23.6±5.56
4αPdD		2.66±0.27	17.2±1.63
DMSO		2.65±0.22	21.3±3.42
			mU/ml
PdBu	AVP	3.63±0.32	0.91±0.20
4αPdD		3.19±0.39	0.89±0.21
DMSO		3.21±0.14	0.71±0.12
			μM
PdBu	NA	3.79±.38	1.38±0.28
4αPdD		3.53±0.26	1.91±0.65
DMSO		3.73±0.24	0.81±0.09
			mM
PdBu	KCl+prazosin	2.53±0.17	31.7±0.90*
4αPdD		2.71±0.08	30.0±1.98*
DMSO		2.36±0.19	24.4±1.42
PdBu	AII	0±0	
4αPdD		0.06±0.04	
DMSO		0.26±0.21	
PdBu	PdBu	0±0*	
4αPdD		2.61±0.41	
DMSO		2.14±0.43	

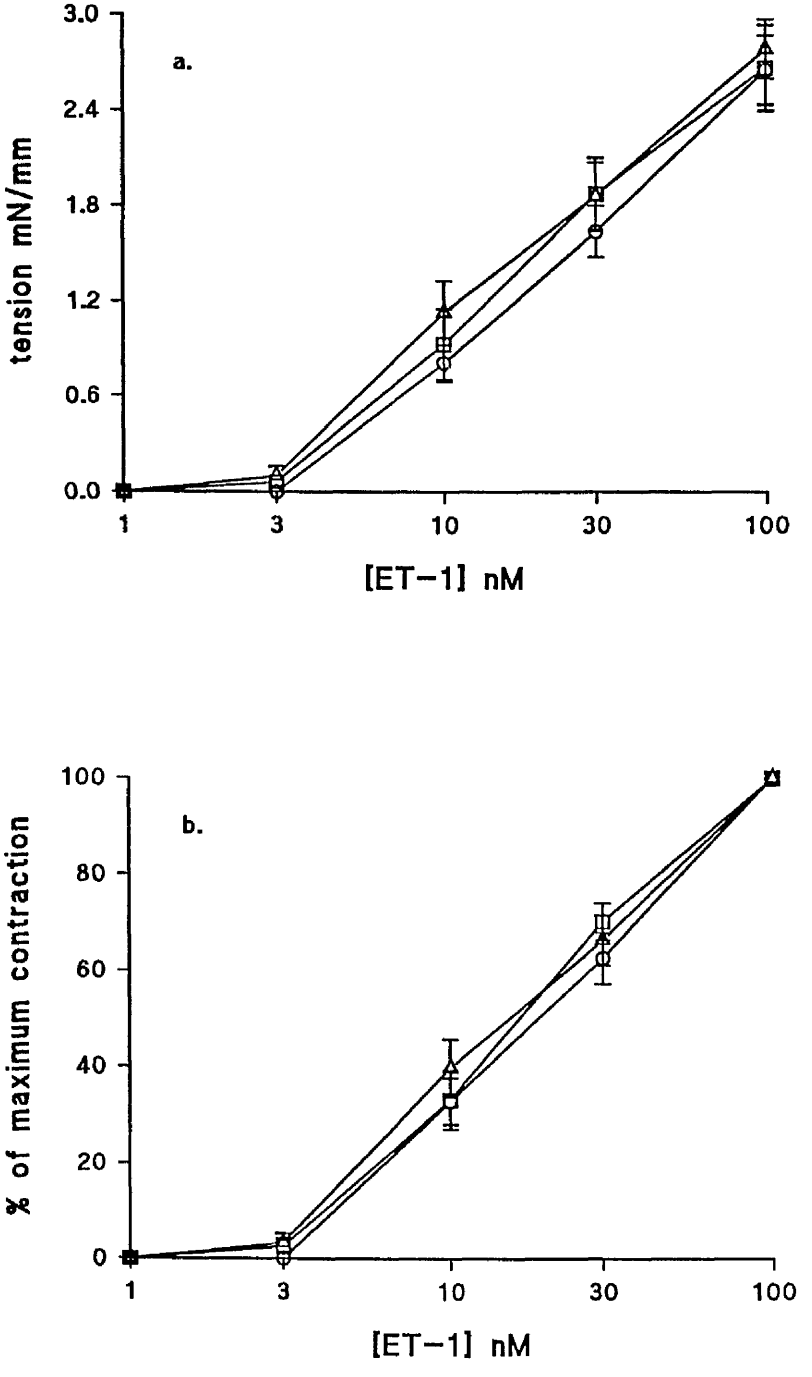


FIGURE 4.2 a. Tension and b. % of maximum contraction dose response curves to ET-1 in intact rat mesenteric small arteries following overnight incubation with 500nM PdBu (Δ), 500nM 4αPdD (□) and 0.1% DMSO (○). Mean±SEM for 10 arterial segments taken from individual animals.

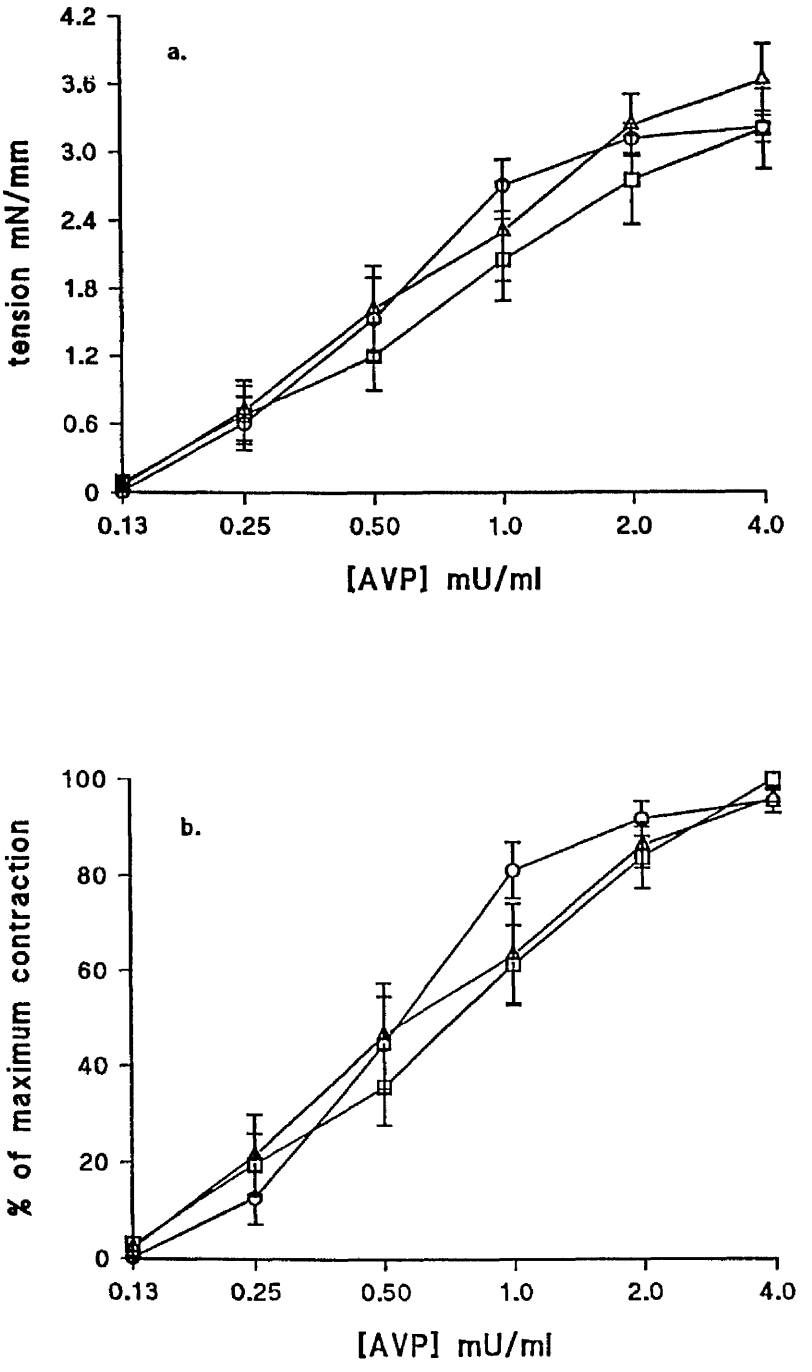


FIGURE 4.3 a. Tension and b. % of maximum contraction dose response curves to AVP in intact rat mesenteric small arteries following overnight incubation with 500nM PdBu (Δ), 500nM 4αPdD (□) and 0.1% DMSO (○). Mean±SEM for 10 arterial segments taken from individual animals.

Statistically there was also no difference in the dose response curve to NA between the 3 pretreatments due to a wide variability in the 4αPdD group. Table 4.1 shows the contractile response to AII (100nM) was equally poor in each of the groups following incubation.

Effect on KCl-induced contraction.

The ED_{50} for KCl-induced contraction was slightly increased following incubation with PdBu and 4αPdD compared to DMSO ($p<0.05$, Table 4.1) but there was no difference in the maximum tension developed to KCl (125mM) between the 3 groups (Table 4.1). The maximum tension developed to sub-maximal doses of 20 and 30mM KCl was significantly reduced in vessels pretreated with PdBu and 4αPdD compared to DMSO ($p<0.05$, Figure 4.5a). The % of maximum contraction at 20, 30 and 40mM KCl was also reduced in the PdBu and 4αPdD groups compared to DMSO (with the exception of 4αPdD at 40mM) and in the PdBu group compared to 4αPdD at 20mM ($p<0.05$, Figure 4.5b).

Effect on ACh-induced relaxation.

The mean±SEM % relaxation induced by 10μM ACh following a maximum contraction to NA (10μM) in the PdBu-, 4αPdD- and DMSO- pretreated groups was 45.37±10.14, 60.91±8.00 and 31.54±3.68 respectively, therefore ACh did not cause complete relaxation in any group. This suggested the endothelium had been partially damaged by the incubation procedure.

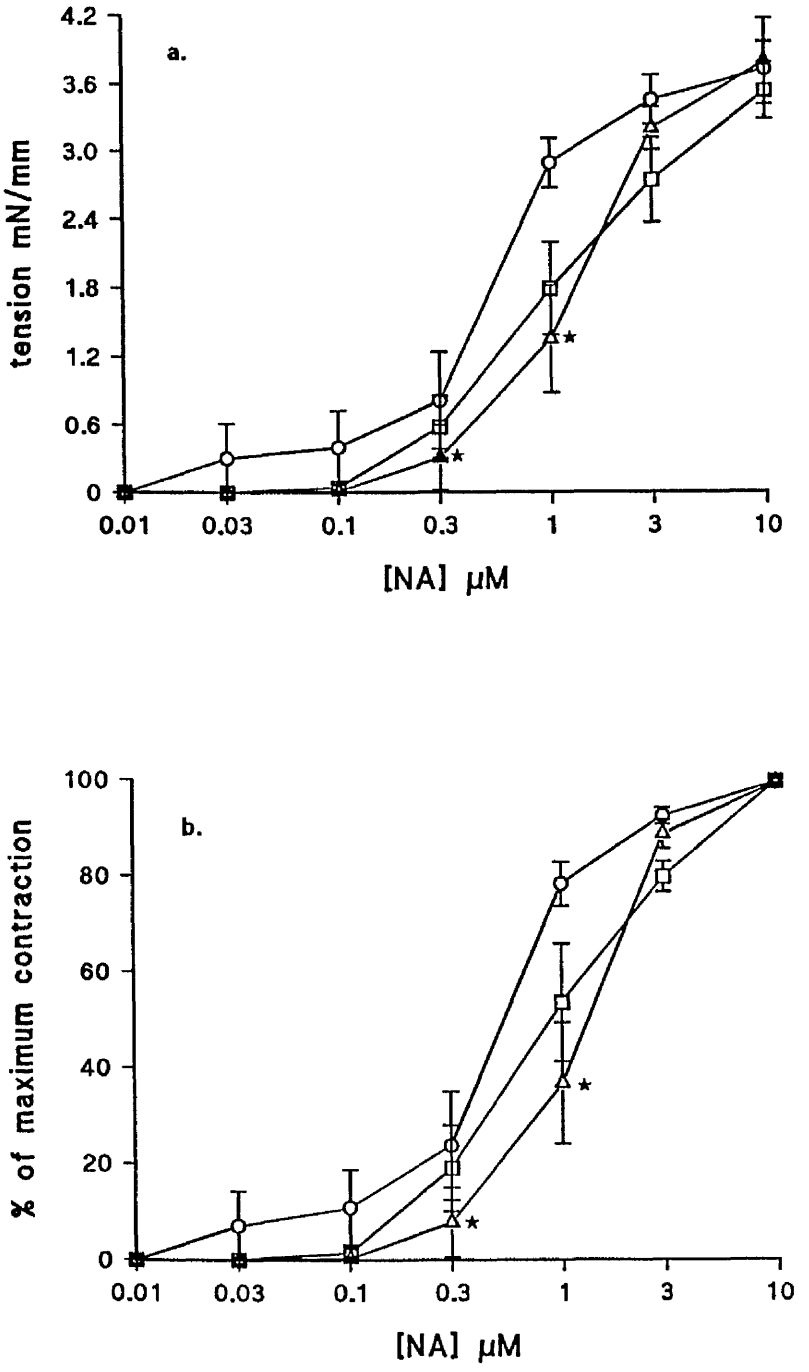


FIGURE 4.4 a. Tension and b. % of maximum contraction dose response curves to NA in intact rat mesenteric small arteries following overnight incubation with 500nM PdBu (Δ), 500nM 4αPdD (□) and 0.1% DMSO (○). Mean±SEM for 10 arterial segments taken from individual animals. *$p<0.05$ for PdBu (vs DMSO) by ANOVA.

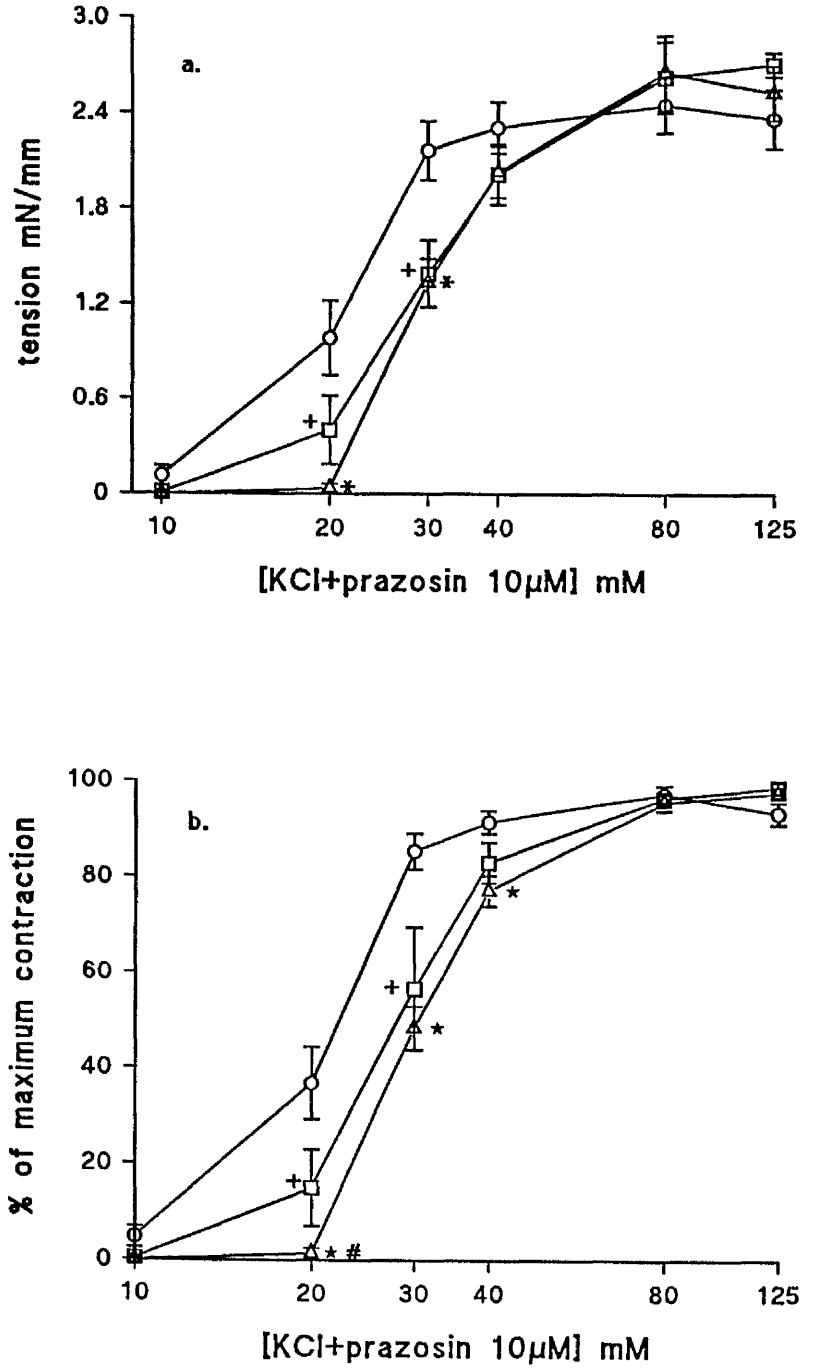


FIGURE 4.5 a. Tension and b. % of maximum contraction dose response curves to KCl + prazosin (10μM) in intact rat mesenteric small arteries following overnight incubation with 500nM PdBu (Δ), 500nM 4αPdD (□) and 0.1% DMSO (○). Mean±SEM for 10 arterial segments taken from individual animals. *$p<0.05$ for PdBu (vs DMSO); +$p<0.05$ for 4αPdD (vs DMSO); #$p<0.05$ for PdBu (vs 4αPdD) by ANOVA.

Identification of PKC isoforms.

Western blot analysis showed the PKC isoforms present in rat mesenteric small arteries included α, γ, δ, ε and ζ. Subcellular fractionation demonstrated that PKC γ was located primarily in the soluble fraction whilst PKC δ was located primarily in the particulate fraction. PKCs α, ε, and ζ were distributed more or less equally between the two fractions. Following overnight incubation with PdBu only PKCs α and δ were down-regulated which was seen as a loss of the immunoreactive signal (Figure 4.6).

3.2. PKC inhibitor studies.

3.2a. Intact arteries.

Effect on PdBu-induced contraction.

Following a 10 minute incubation with $3x10^{-7}$M PKC inhibitor RO-31-8330 the contraction to 2μM PdBu was completely abolished in intact rat mesenteric small arteries.

Effect on ET-1-induced contraction.

The maximum tension developed to ET-1 (100nM) was significantly greater in the DMSO-treated group compared to vessels treated with $3x10^{-7}$M RO-31-8330 ($p<0.05$, Table 4.2). Furthermore, the maximum tension developed to sub-maximal doses of 1 and 10nM ET-1 was also greater in the DMSO group compared to vessels incubated with RO-31-8330 ($p<0.05$, Figure 4.7).

Effect on KCl-induced contraction.

There was no difference in the maximum tension developed to KCl (125mM)(Table 4.2) or dose response curve to KCl (Figure

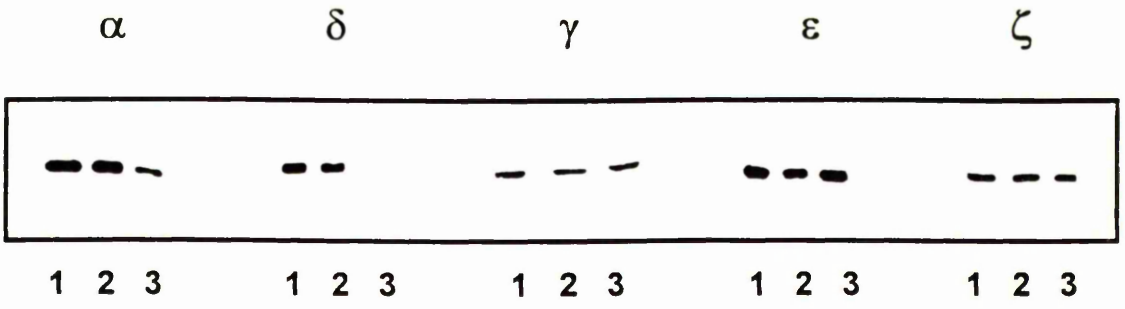


FIGURE 4.6 Immunoblots of PKC isoforms α, δ, γ, ε and ζ following overnight incubation with 1: DMSO (0.1%), 2: 4αPdD (500nM) and 3: PdBu (500nM). Whole vessels were homogenised, run on SDS-PAGE, electrophoretically transferred to membranes and probed with isotype specific primary antibodies. Representative of 3 experiments.

TABLE 4.2 Contractile response of rat mesenteric small arteries to agonists in the presence of $3x10^{-7}M$ RO-31-8330 (PKC inhibitor) and 0.1% DMSO. Data show the tension developed to maximal doses of ET-1 (100nM) and KCl (125mM) in intact vessels. The tension developed to pCa6.7, pCa6.7 + GTP (10μM) and ET-1 (100nM) in permeabilised vessels is also shown. Mean±SEM for n=3. *p<0.05 (vs DMSO) by ANOVA plus the LSD test.

Treatment	Stimulation	Max. Tension
		mN/mm
Intact		
RO-31-8330	ET-1	2.670±0.254*
DMSO		3.809±0.270
RO-31-8330	KCl	2.663±0.283
DMSO		2.350±0.226
Permeabilised		
RO-31-8330	pCa6.7	0.492±0.116
DMSO		0.286±0.136
RO-31-8330	pCa6.7 + GTP	0.603±0.138
DMSO		0.942±0.144
RO-31-8330	ET-1	0.742±0.170*
DMSO		1.135±0.156

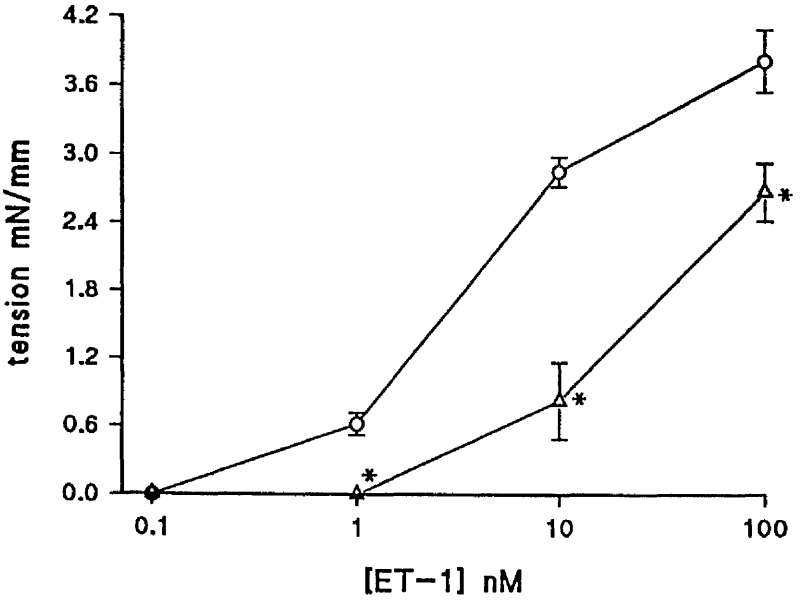


FIGURE 4.7 Tension dose response curve to ET-1 in intact rat mesenteric small arteries in the presence of $3x10^{-7}$M PKC inhibitor RO-31-8330 (Δ) and 0.1% DMSO (O). Mean±SEM for n=3. *p<0.05 (vs DMSO) by ANOVA plus the LSD test.

4.8) in the presence of RO-31-8330 compared to DMSO control.

3.2b. Permeabilised arteries.

Effect on PdBu-induced contraction.

Incubation with $3x10^{-7}$M RO-31-8330 completely inhibited the contraction to a maximal PdBu concentration in permeabilised rat mesenteric small arteries.

The maximum tension developed to pCa6.7 and pCa6.7 + 10μM GTP was not different between vessels treated with RO-31-8330 and DMSO control following permeabilisation (Table 4.2).

Effect on ET-1-induced contraction.

The maximum tension developed to ET-1 (100nM) was significantly reduced in the RO-31-8330-treated group compared to DMSO control ($p<0.05$, Table 4.2). Furthermore, the maximum tension developed to sub-maximal doses of 3 and 10nM ET-1 was reduced in the RO-31-8330 group compared to vessels incubated with DMSO ($p<0.05$, Figure 4.9).

Effect on calcium-induced contraction.

$3x10^{-7}$M RO-31-8330 caused a 34.80±3.74% (mean±STD) decrease in the tension developed to a maximal calcium concentration in permeabilised arteries (for n=2).

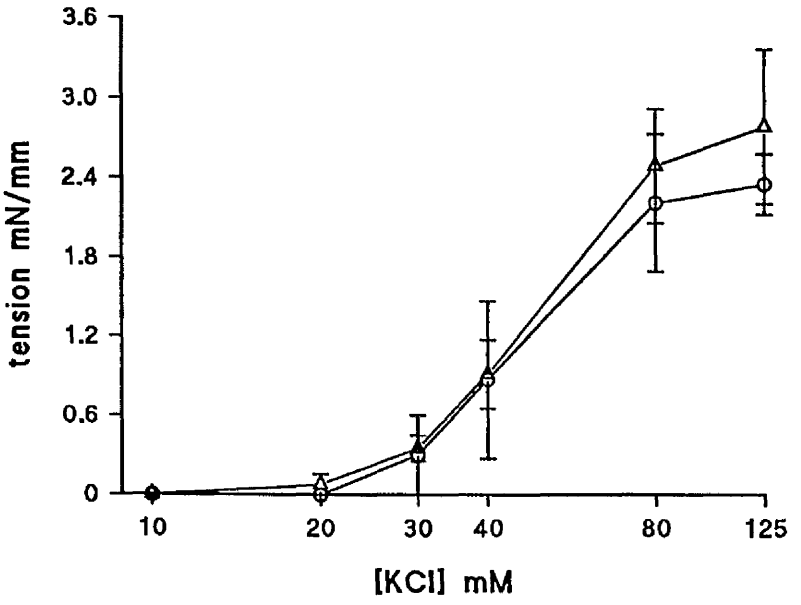


FIGURE 4.8 Tension dose response curve to KCl in intact rat mesenteric small arteries in the presence of $3x10^{-7}$M PKC inhibitor RO-31-8330 (Δ) and 0.1% DMSO (O). Mean±SEM for n=3.

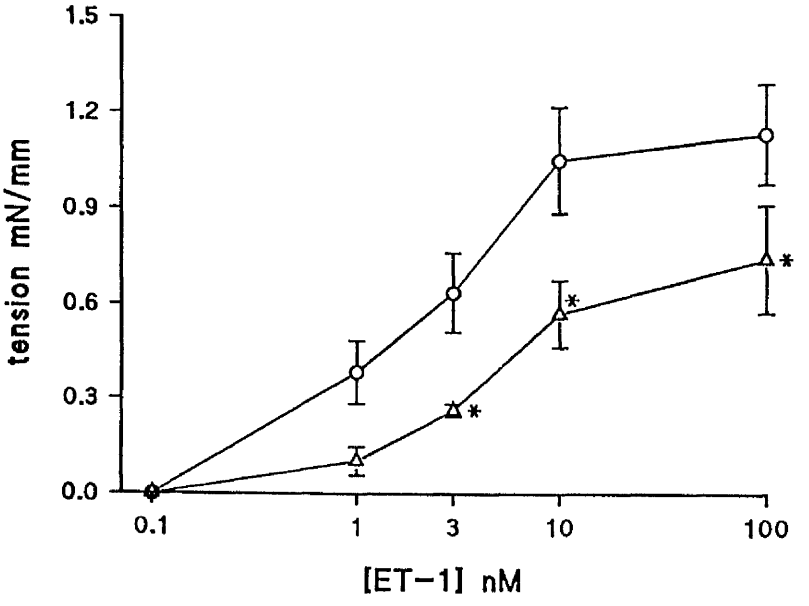


FIGURE 4.9 Tension dose response curve to ET-1 in α-toxin permeabilised rat mesenteric small arteries in the presence of $3x10^{-7}$M PKC inhibitor RO-31-8330 (Δ) and 0.1% DMSO (O). Mean±SEM for n=3. *$p<0.05$ (vs DMSO) by ANOVA plus the LSD test.

4. Discussion.

4.1. PKC down-regulation studies.

The technique of down-regulation of PKC by prolonged exposure to phorbol ester was first used in this study as it appears to be a very 'clean' and specific method of abolishing PKC activity. Overnight incubation with the phorbol ester PdBu (500nM) completely abolished the contractile response to 2μM PdBu in intact rat mesenteric small arteries suggesting PKC had been successfully down-regulated. Overnight incubation with PdBu did not affect the contractile response to the receptor agonists ET-1, AVP and NA. These results suggested the receptor agonist-induced contraction in intact rat mesenteric arteries was not dependent on the activation of PKC.

There is evidence that PKC can modulate the function of voltage-gated calcium channels (Gleason and Flaim 1986, Fish et al 1988) therefore PKC may have been involved in the contractile response of vascular smooth muscle to depolarisation. Down-regulation of PKC did not affect the maximum tension developed to KCl (125mM) although the dose response curve and ED_{50} were both shifted in the KCl-induced contraction in the PdBu pretreated group. This suggested prolonged exposure to PdBu slightly reduced the sensitivity of intact rat mesenteric small arteries to a depolarising

stimulus. However, since the same result was observed in the group pretreated with 4αPdD it was probably due to a non-specific effect of phorbol esters on the cell membrane rather than down-regulation of PKC.

Previous investigations using PKC down-regulation to determine the role of PKC in the agonist-induced vasoconstriction have yielded conflicting results. Hori et al (1993) found the maximum contractile response to KCl, NA and ET-1 (and prostaglandin F2 alpha ($PGF_{2\alpha}$)) in intact rat aortic smooth muscle strips was unaffected by prolonged exposure to phorbol ester which was similar to the observations in the present study in intact rat mesenteric small arteries. However, they assessed enzyme activity as a measure of PKC down-regulation which may not have been sufficiently sensitive. Indeed, a recent study showed although PKC activity was decreased by 95% in rat aorta following 17 hours incubation with PdBu (10μM) there was still a significant contraction to the phorbol ester 12-0-tetradecanoylphorbol-13-acetate (TPA) suggesting PKC was still present (Rapoport et al 1995). In agreement with the findings in this thesis they found the contraction to sub-maximal doses of NA (and $PGF_{2\alpha}$) but not maximum tension was reduced following PKC down-regulation. They also reported there was no change in sensitivity to KCl but the maximum KCl-induced contraction was decreased by approximately 40%. However, Rapoport et al (1995) used a comparatively high concentration of PdBu (10μM) which may have caused a larger amount of down-regulation resulting in a decreased maximum KCl-induced contraction.

In contrast, other investigators found down-regulation of PKC had marked effects on smooth muscle contractility. Merkel et al (1991) reported that incubation of intact canine femoral arterial rings mounted in a tissue bath with 100nM PdBu for 24 hours completely inhibited the contraction to NA and serotonin and partially inhibited the contraction to PdBu and KCl. Furthermore, porcine coronary arterial rings mounted in an organ chamber exposed to similar conditions showed a 70-80% reduction in the force developed to maximal PdBu and ET-1 concentrations (Marala et al 1993). More recently, the contractile response to NA (but not 60mM KCl) was significantly reduced in rat epididymal vas deferens by pretreatment with 30μM PdBu for 2 hours (Burt et al 1996). The difference in these results to those in this thesis could be attributed to differences in tissue or species used or conditions of exposure to PdBu.

Although individual PKC isoforms have been implicated in contractile responses in isolated and cultured vascular smooth muscle cells (Khalil et al 1992, Khalil et al 1994) this is the first time they have been implicated in contractile responses in intact small arteries - at least when stimulated with PdBu. In this study pretreatment with PdBu in rat mesenteric small arteries resulted in the down-regulation of only two PKC isoforms, namely α and δ. This down-regulation was associated with a parallel loss of the contractile response to PdBu suggesting that PKCs α and δ were involved in the PdBu-induced contraction. However, with the possible exception of NA, down-regulation of PKCs α and δ did not appear to affect the receptor agonist-induced

contraction implying phorbol esters and receptor agonists induced contractile responses in intact vascular smooth muscle via different mechanisms and/or PKC isoforms.

Of the five isoforms identified in rat mesenteric small arteries only two (α and γ) belonged to the calcium-dependent group. Since long term exposure to PdBu resulted in the down-regulation of α, and γ is thought to be neuronal in origin, if the contractile response to receptor agonists was utilising a PKC pathway it would have to have been via a calcium-independent isoform(s). There is some evidence for this as Collins et al (1992) found the contraction to phenylephrine (PE) in single saponin-skinned ferret aortic smooth muscle cells occurred at constant $[Ca^{2+}]_i$ and was blocked by a pseudosubstrate peptide PKC inhibitor. They also demonstrated a parallel calcium-independent translocation of PKCε to the plasma membrane and consequently speculated the PE-induced contraction, at least in their system, was associated with PKCε activation (Khalil et al 1992).

4.2. <u>PKC inhibitor studies.</u>

Due to the difficulties in down-regulating PKC in permeabilised rat mesenteric small arteries the potent and specific PKC inhibitor RO-31-8330 (Davis et al 1989) was used as an alternative method for abolishing PKC activity in these and intact arteries.

4.2a. <u>Intact arteries.</u>

Following incubation with $3x10^{-7}M$ PKC inhibitor RO-31-8330 there was no contraction to a maximal concentration of PdBu

in intact rat mesenteric small arteries confirming inhibition of PKC. The maximum ET-1-induced tension was significantly reduced by approximately 30% in the presence of RO-31-8330 consistent with a partial role for PKC in regulating the ET-1-induced vascular smooth muscle contraction. The results in intact vessels incubated with $3x10^{-7}$M RO-31-8330 were different to those following down-regulation of PKC by prolonged exposure to PdBu as the maximum ET-1-induced tension was significantly reduced in the presence of RO-31-8330 whereas it was not affected by PKC down-regulation. This can be explained by the fact the PKC inhibitor may have inhibited the activity of all PKC isoforms whereas overnight incubation with PdBu only down-regulated PKCs α and δ. Indeed, work from this laboratory showed that RO-31-8330 inhibited the activity of PKC in a crude PKC extract from the rat brain which contains at least 8 PKC isotypes. Therefore the PKC-dependent ET-1-induced vasoconstriction in intact rat mesenteric small arteries may have been due to activation of one or more of the non-down-regulated calcium-independent isoform(s), for example PKCε.

Previous studies using the PKC inhibitors staurosporine and H-7 have proposed an important role for PKC in the contractile response to ET-1 (Ohlstein et al 1989, Auguet et al 1989, Sugiura et al 1989, Sasaki et al 1991) and NA (Abraham and Rice 1992, Burt et al 1996) in intact vascular smooth muscle. They also inhibited the contraction to other vasoconstrictors including PE and KCl (Merkel et al 1991, Sasaki et al 1991, Shimamoto et al 1993), $PGF_{2\alpha}$ (Heaslip and Sickels 1989, Morimoto et al 1990, Sasaki et al 1991) and

serotonin (Clark and Garland 1991, Murray et al 1992). However, H-7 and staurosporine are non-specific inhibitors of PKC and these results may have been due to inhibition of myosin light chain (MLC) kinase (Rüegg and Burgess 1989).

It has been reported that low concentrations of staurosporine can selectively inhibit PKC but they failed to alter the contraction to NA (although the response to KCl was reduced)(Boonen and De Mey 1991) or ET-1 and KCl (Moreland et al 1992b) in intact vascular smooth muscle tissue. Furthermore, Shimamoto et al (1992) found the more specific PKC inhibitor calphostin C only decreased the maximum ET-1-induced tension in rat aorta by 13.2% and 25.8% in calcium-containing medium and calcium-free Krebs containing 1mM EGTA respectively. In a subsequent study Shimamoto et al (1993) compared all three PKC inhibitors on contractions to KCl, PE, TPA and PdBu and indeed concluded calphostin C to be the most potent and specific inhibitor of PKC. 10^{-6}M calphostin C completely blocked the contractile response to TPA and PdBu in rat aorta but had no effect on the contractile response to 40mM KCl (Shimamoto et al 1993). More recently, the ET-1-mediated constriction in bovine bronchial smooth muscle tissue was not antagonised by the PKC inhibitor RO-31-8220 (Nally et al 1994). These studies implied that the NA-, ET-1- and KCl-induced contraction in intact smooth muscle was not regulated by PKC.

10^{-6}M calphostin C, however, reduced the maximum tension developed to PE in rat aorta by approximately 35% (Shimamoto et al 1993). Furthermore, Burt et al (1996) found the same

concentration of calphostin C significantly reduced the maximum NA (but not KCl) tension in rat epididymal vas deferens. Both these results implied PKC was involved in the contractile response to receptor agonists in intact smooth muscle which is in agreement with the data in this thesis using the PKC inhibitor RO-31-8330. This may have been via a PKC-mediated increase in the sensitivity of the contractile apparatus to calcium, which can be examined using permeabilised arteries.

4.2b. <u>Permeabilised arteries.</u>

$3x10^{-7}$M RO-31-8330 completely abolished the contractile response to 2μM PdBu in rat mesenteric small arteries permeabilised with Staphylococcal aureus α-toxin and significantly reduced the contractile response to ET-1 by approximately 30%. Similarly, Yoshida et al (1994) found the synthetic peptide inhibitor PKC_{19-36} completely inhibited the PdBu-induced enhancement of the sub-maximal calcium-induced contraction in strips of ß-escin skinned rabbit mesenteric arteries but only partly inhibited the enhancement of the sub-maximal calcium-induced contraction by ET-1. Furthermore, PKC_{19-31} depressed the force for PE activation at pCa7.0 in α-toxin permeabilised smooth muscle cells from the rabbit portal vein by approximately 30% (Brozovich 1995). These results suggested that PKC was involved in the increase in calcium sensitivity of the contractile apparatus elicited by ET-1 and PE, but it was not the only regulator of this response.

Indeed, other studies using PKC_{19-36} and PKC_{19-31} showed

inhibition of PKC had no effect on the GTPγS- (Itoh et al 1994, Fujita et al 1995), NA- (Fujita et al 1995) or aluminum fluoride (AlF_4^-)- (Fujita et al 1995) induced enhancement of myofilament calcium sensitivity. Furthermore, the calcium sensitisation response to ACh in α-toxin permeabilised smooth muscle cells from the guinea pig stomach was not affected by calphostin C (Oishi et al 1992). In contrast, other studies using various PKC inhibitors found the increase in calcium sensitivity elicited by GTPγS (Nishimura et al 1988, Nishimura et al 1991, Kawase and van Breeman 1992, Jensen et al 1996), ET-1 (Nishimura et al 1991, Nishimura et al 1992), NA (Nishimura et al 1991, Satoh et al 1994b), PE (Parsons et al 1996), serotonin (Seager et al 1994, Parsons et al 1996), $PGF_{2\alpha}$ (Katsuyama and Morgan 1993), the thromboxane A_2 analogue (15S)-hydroxy-11α, 9α-(epoxymethano) prosta-5Z, 13E-dienoic acid (U46619)(Jiang et al 1994) and AlF_4^- (Kawase and van Breeman 1992) was predominantly dependent on PKC. The discrepancy between these results and the results in this thesis using rat mesenteric small arteries may have been due to differences in species or tissue used or non-specific effects of the PKC inhibitors.

In the present study $3x10^{-7}$M PKC inhibitor RO-31-8330 relaxed the contraction to a maximal calcium concentration in permeabilised arteries by approximately 30%. In contrast, studies using other PKC inhibitors found they had no effect on the contraction to calcium alone in permeabilised vascular smooth muscle tissue (Nishimura et al 1988, Kawase and van Breeman 1992, Nishimura et al 1992, Satoh et al 1994b, Itoh et al 1994, Yoshida et al 1994, Seager et al 1994, Brozovich

1995, Fujita et al 1995, Jensen et al 1996). The difference in these results to those in the present study again could be attributed to differences in species, tissue or PKC inhibitor used. Alternatively, it has been reported that RO-31-8330 (an isothiourea) interacted with the ATP-binding site of PKC (Davis et al 1989). Therefore, the relaxation of the maximum calcium contraction may have been caused by a non-specific inhibition of MLC kinase by RO-31-8330, but there is no evidence for this (Davis et al 1989).

KCl brings about smooth muscle contraction via depolarisation and the opening of voltage-gated calcium channels to allow the entry of calcium and phosphorylation of MLC therefore $3x10^{-7}$M RO-31-8330 may have also been expected to affect the KCl-induced contraction in intact arteries. However, the maximum tension developed to KCl (125mM) was not different in vessels pretreated with RO-31-8330 compared to DMSO control suggesting it was a relatively specific inhibitor. The difference in the KCl-induced contraction with RO-31-8330 and the calcium-induced contraction with RO-31-8330 may have been caused by the difference in preparations - it may be that the PKC inhibitor had access to MLC kinase or the contractile proteins in permeabilised preparations, but not in intact preparations. Alternatively the difference may have been due to the fact RO-31-8330 was added to a maximum calcium preconstriction and the relaxation observed whereas it was added at the same time as KCl.

4.3. Summary.

Overnight incubation with the phorbol ester PdBu in intact

rat mesenteric small arteries resulted in the down-regulation of PKCs α and δ which was associated with a loss of the contractile response to PdBu. However, down-regulation of PKCs α and δ had no effect on the contractile response to vasoconstrictor hormones indicating that phorbol esters and vasoconstrictor hormones acted via separate pathways and/or PKC isotypes to bring about vascular smooth muscle contraction. Incubation with $3x10^{-7}$M PKC inhibitor RO-31-8330 completely abolished the PdBu-induced contraction in intact rat mesenteric small arteries and significantly reduced the maximum ET-1-induced contraction by approximately 30% consistent with a partial role for PKC in regulating the ET-1-mediated vasoconstriction. RO-31-8330 probably inhibited all PKC isoforms suggesting that activation of one or more of the non-down-regulated, calcium-independent isoform(s) (specifically PKCε) may have been responsible for the part of the ET-1-induced contraction in intact arteries which was dependent on PKC.

In α-toxin permeabilised rat mesenteric small arteries $3x10^{-7}$M RO-31-8330 completely inhibited the contractile response to PdBu and significantly reduced the maximum contractile response to ET-1 by approximately 30%. This implied PKC was partially involved in the enhancement of the calcium sensitivity of the contractile apparatus elicited by ET-1 - a mechanism which may have been responsible for the sustained phase of the ET-1-induced contraction in intact arteries. However, these results suggested that phosphorylation of proteins by PKC was not the sole mechanism responsible for the ET-1-induced calcium sensitisation.

CHAPTER FIVE

INVOLVEMENT OF TYROSINE KINASES IN THE CONTRACTILE RESPONSE TO ENDOTHELIN-1

1. Introduction.

In the previous chapter using rat mesenteric small arteries permeabilised with Staphylococcal aureus α-toxin it was demonstrated that the protein kinase C (PKC) inhibitor RO-31-8330 significantly reduced the increase in myofilament calcium sensitivity elicited by endothelin-1 (ET-1) by approximately 30%. This suggests PKC does play a role in the receptor agonist-induced calcium sensitisation, but another mechanism is additionally involved in this response.

Numerous studies have demonstrated increased phosphotyrosine levels in vascular smooth muscle cells following stimulation with receptor agonists such as angiotensin II (AII), noradrenaline (NA), ET-1 and vasopressin (AVP)(Tsuda et al 1991, Koide et al 1992). The observation that epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) increase vascular tone which is blocked by the tyrosine kinase (TK) inhibitors genistein and tyrphostins (Hollenberg 1994, Hughes 1995) raises the possibility that tyrosine phosphorylation is involved in vascular smooth muscle contraction. However, the precise role of this signalling pathway in the receptor agonist-induced calcium sensitisation is still unclear. Furthermore, few investigators have studied this response using small arteries mounted in a wire myograph. Therefore the contractile response of both intact and α-toxin permeabilised rat mesenteric small arteries mounted in a wire myograph to ET-1 in the presence of the

active tyrosine kinase inhibitors (TKIs) tyrphostin A23 and A47 and the inactive analogue tyrphostin A1 has been investigated.

2. Protocols.

2.1. Intact procedure.

Vessels were mounted in a myograph in PSS and warmed to 37°C. After normalisation (as described on Page 56) vessels were stimulated with 3 activations of KPSS, each one was for 2 minutes and vessels were allowed to relax fully between stimulations.

2.2. Permeabilisation procedure.

After permeabilisation (as described on Page 81) vessels were equilibrated in relaxing solution (G_1) for 15 minutes then stimulated with pCa6.7. When this contraction had reached a plateau vessels were re-equilibrated in G_1 for 20-25 minutes.

2.3. Titration experiment.

2.3a. Intact arteries.

After equilibration in PSS for 15 minutes vessels were stimulated with 100nM ET-1 which gave a large contraction. This was allowed to reach a plateau before 20μM active TKI tyrphostin A47 (dissolved in dimethyl sulfoxide (DMSO)) was added to the myograph bath. This caused a gradual relaxation in the tension developed to 100nM ET-1, but not completely to baseline. 50μM, however, did relax the maximum ET-1-induced tension to baseline therefore 50μM appeared to be the minimum concentration of TKI required to completely inhibit the ET-1-induced contraction in intact rat mesenteric small arteries.

The experiment was repeated using the other active TKI A23 (dissolved in DMSO) and the inactive analogue A1 (dissolved in DMSO). In both cases 50μM tyrphostin caused complete relaxation of the maximum ET-1 preconstriction (for n=1).

2.3b. Permeabilised arteries.

PCa6.7 was added for 8-10 minutes to give a constant, sub-maximum contraction after which vessels were stimulated with 10μM GTP. When this contraction had reached a plateau 100nM ET-1 was added which caused a large increase in tension to that developed to GTP. This contraction was allowed to reach a plateau before the addition of 20μM active TKI tyrphostin A47 which again caused a partial relaxation of the maximum ET-1-induced tension. When the concentration was increased to 50μM there was a further relaxation in tension but still not completely to baseline. 100μM, however, did relax the maximum ET-1-induced tension to baseline therefore 100μM appeared to be the minimum concentration of TKI required to completely inhibit the ET-1-induced contraction in α-toxin permeabilised rat mesenteric small arteries. A similar relaxation dose response curve to 20, 50 and 100μM A23 and A1 following a maximum ET-1 preconstriction was performed.

2.4. Incubation experiment.

2.4a. Intact arteries.

After equilibration in PSS for 15 minutes a dose response curve to 0.1, 1, 10 and 100nM ET-1 in the presence of 100μM tyrphostin was performed. Each contraction was allowed to reach a plateau before the next dose was added. However, in the case of A47 and A23 the contraction to 10 and 100nM ET-1

was transient therefore results were calculated for the maximum and minimum (when the contraction had reached a plateau) value of the ET-1-induced contraction (Figure 5.1a). The value of the ET-1-induced contraction in the presence of DMSO and A1 was taken at the same time point as the minimum, sustained value of the ET-1-induced contraction in the presence of A47 and A23.

These findings suggested that the active TKIs tyrphostin A47 and A23 may have been inhibiting the mechanism responsible for the sustained phase of the contractile response to ET-1. In order to investigate the possible mechanism vessels were incubated in calcium-free PSS containing 0.1mM EGTA for 10 minutes before 100nM ET-1 in the presence of 0.1% DMSO was added. This gave a transient contraction or "spike" which rapidly declined to near baseline (Figure 5.1b). When normal PSS containing 2.5mM calcium was subsequently added a large contraction was observed which declined very gradually (Figure 5.1b). This experiment was repeated with 100μM A23 and A1 added simultaneously with 100nM ET-1. Finally, the effect of 100μM tyrphostin on the potassium chloride (KCl)-induced contraction was studied by stimulating the intact arteries with increasing concentrations of KCl in the presence of 100μM tyrphostin.

2.4b. <u>Permeabilised arteries.</u>

PCa6.7 was added together with 100μM tyrphostin for 8-10 minutes after which vessels were stimulated with 10μM GTP. When this contraction had reached a plateau a dose response curve to 0.1, 1, 3, 10 and 100nM ET-1 was performed. Each

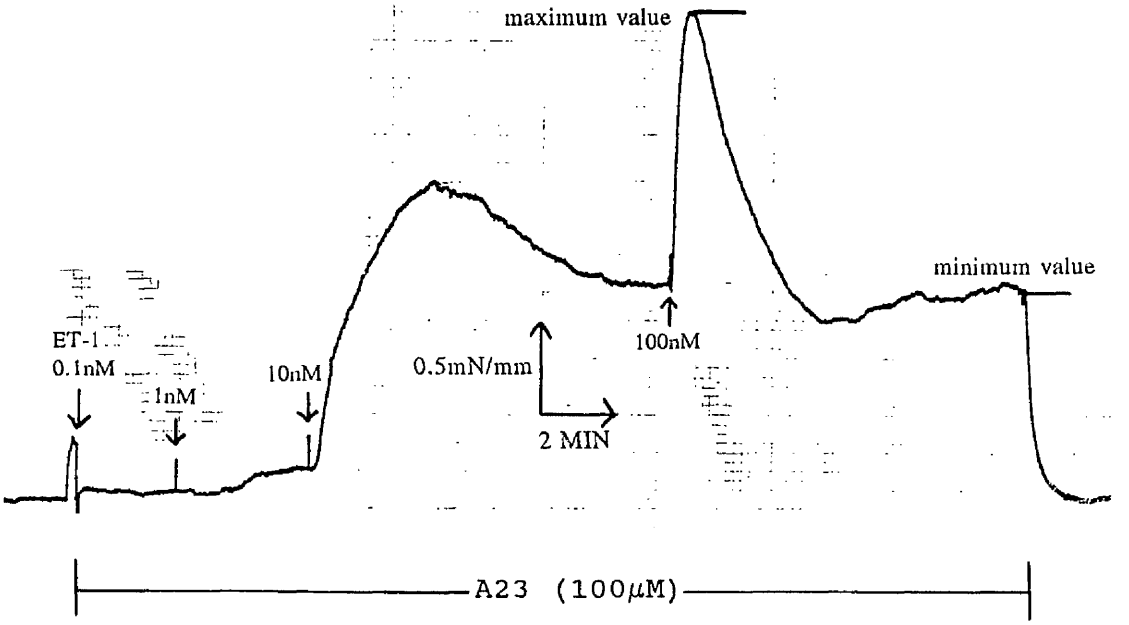
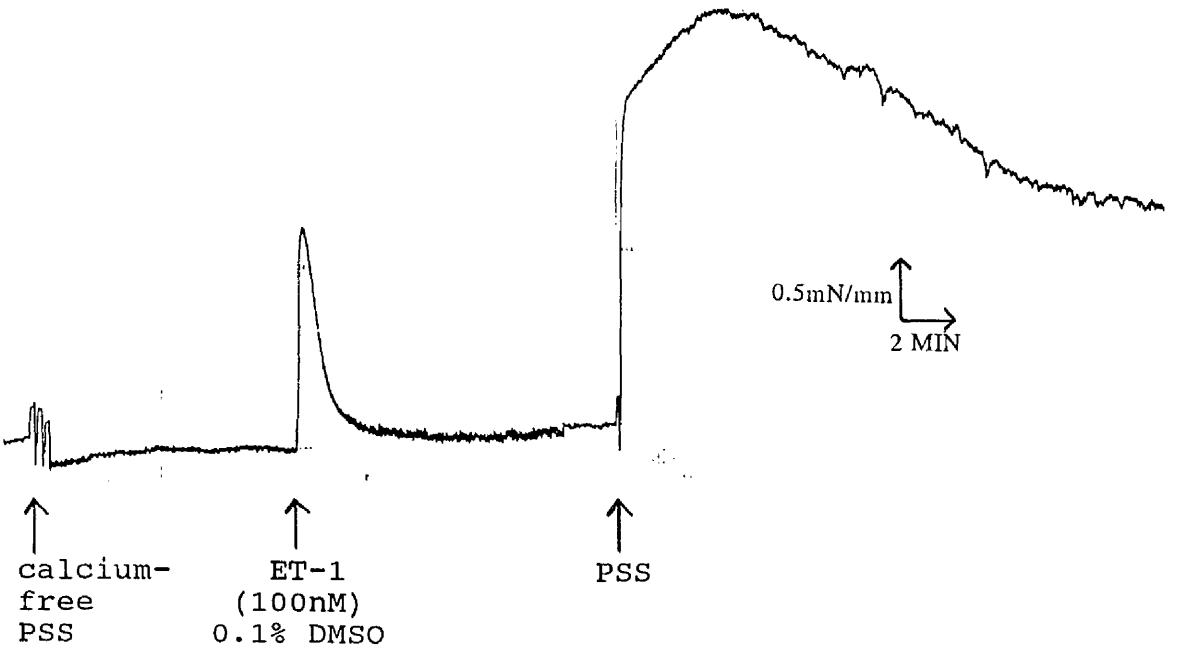


FIGURE 5.1 a. Representative tracing showing the transient contraction to 10 and 100nM ET-1 in the presence of 100μM active TKI tyrphostin A23 in an intact rat mesenteric small artery. Results were calculated for the maximum and minimum value of the contraction as shown for 100nM ET-1.



b. Representative tracing showing the transient "spike" to 100nM ET-1 in the presence of 0.1% DMSO in calcium-free PSS containing 0.1mM EGTA and the large contraction following the addition of normal PSS containing 2.5mM calcium in an intact rat mesenteric small artery.

contraction was allowed to reach a plateau before the next dose was added. The effect of 100μM tyrphostin on the calcium-induced contraction was studied by adding it to a fixed, maximum calcium contraction following permeabilisation.

Controls consisted of identical dose response curves in the presence of 0.1% DMSO. Responses were calculated as active tension (as described on Page 56). Differences between the dose response curves were calculated using analysis of variance (ANOVA) plus the Least Significant Difference (LSD) test. Student's t-test was used to assess differences between the contraction to pCa6.7 and pCa6.7 + GTP (10μM) in permeabilised arteries.

2.5. TKI and PKC inhibitor.

In order to investigate the involvement of both PKC and TKs in the ET-1-induced contraction in α-toxin permeabilised arteries pCa6.7 was added together with 100μM active TKI tyrphostin A23 for 8-10 minutes after which vessels were stimulated with 10μM GTP until the contraction had reached a plateau. 100nM ET-1 was then added and this contraction was allowed to reach a plateau before the addition of $3x10^{-7}$M PKC inhibitor RO-31-8330. The experiment was repeated with 100μM A1 as the control. The maximum tension developed to 100nM ET-1 in the presence of 100μM tyrphostin plus the final tension after the addition of $3x10^{-7}$M RO-31-8330 were calculated.

3. Results.

3.1. Intact arteries.

3.1a. Incubation experiment.

Effect on ET-1-induced contraction.

The maximum and minimum tension developed to ET-1 (100nM) was significantly reduced in the presence of 100μM active TKIs tyrphostin A47 and A23 but was unchanged by 100μM inactive analogue A1 compared to the maximum tension developed to ET-1 in the presence of 0.1% DMSO in intact rat mesenteric small arteries ($p<0.05$, Table 5.1). Furthermore, the minimum tension developed to sub-maximal doses of 1 and 10nM ET-1 was reduced in all 3 tyrphostin groups compared to vessels incubated with DMSO control ($p<0.05$, Figure 5.2).

Effect on ET-1-induced contraction in calcium-free PSS.

There was no difference in the maximum tension of the ET-1 (100nM) "spike" in calcium-free PSS between tyrphostin and DMSO in intact rat mesenteric small arteries (Table 5.1).

Effect on normal PSS-induced contraction.

The maximum tension following the addition of normal PSS containing 2.5mM calcium was not different between the A23-, A1- and DMSO-treated groups (Table 5.1). However, the contraction to PSS in the presence of DMSO took approximately one hour to decline to baseline, whereas the contraction to PSS in the presence of A1 and A23 declined rapidly to baseline within 10 minutes.

Effect on KCl-induced contraction.

The maximum tension developed to KCl (125mM) was

TABLE 5.1 Contractile response of intact rat mesenteric small arteries to agonists on incubation with 100μM tyrphostin A47 (active TKI), 100μM tyrphostin A23 (active TKI) , 100μM tyrphostin A1 (inactive analogue) and 0.1% DMSO. Data show the maximum and minimum tension developed to maximal doses of ET-1 (100nM). The tension developed to ET-1 (100nM) in calcium-free PSS and following the addition of normal PSS plus maximal doses of KCl (125mM) is also shown. Mean±SEM for n=3. *p<0.05 (vs DMSO) by ANOVA plus the LSD test.

Treatment	Stimulation	Tension
Incubation		
Maximum		mN/mm
A47	ET-1	2.856±0.084*
A23		1.630±0.278*
A1		4.033±0.113
DMSO		3.808±0.270
Minimum	ET-1	mN/mm
A47		0.029±0.029*
A23		0.500±0.278*
A1		4.033±0.113
DMSO		3.808±0.270
A23	ET-1	1.055±0.287
A1	in calcium-	1.144±0.264
DMSO	free PSS	1.306±0.232
A23	normal	4.157±0.751
A1	PSS	4.284±0.158
DMSO		3.531±0.119
A47	KCl	1.145±0.148*
A23		1.134±0.197*
A1		1.284±0.242*
DMSO		2.350±0.226

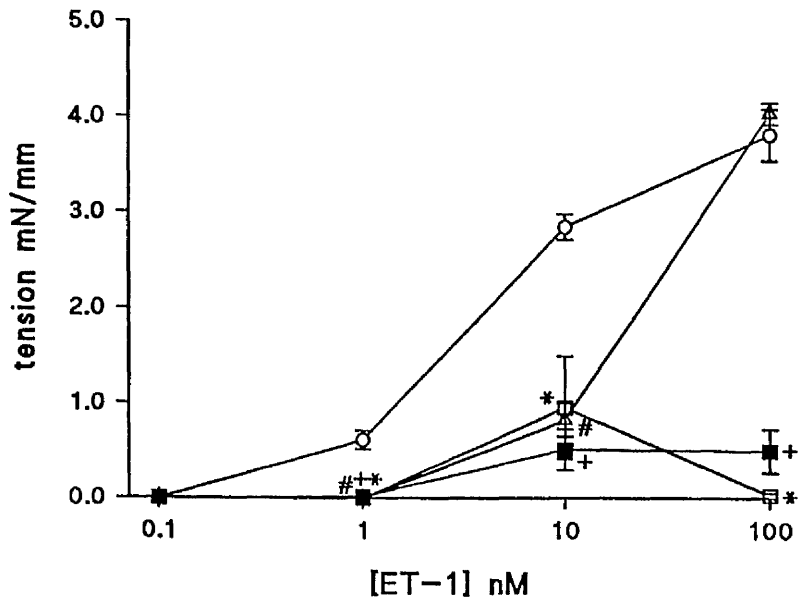


FIGURE 5.2 Tension dose response curve to minimum values of ET-1 in intact rat mesenteric small arteries in the presence of 100μM active TKI tyrphostin A47 (□), 100μM active TKI tyrphostin A23 (■), 100μM inactive analogue tyrphostin A1 (Δ) and 0.1% DMSO (○). Mean±SEM for n=3. *$p<0.05$ for A47 (vs DMSO); +$p<0.05$ for A23 (vs DMSO); #$p<0.05$ for A1 (vs DMSO) by ANOVA plus the LSD test.

significantly greater in the DMSO-treated group compared to vessels treated with all 3 tyrphostins ($p<0.05$, Table 5.1). The maximum tension developed to sub-maximal doses of 40 and 80mM KCl was also greater in the DMSO group compared to all 3 tyrphostin groups ($p<0.05$, Figure 5.3).

Effect on TK activity.

Western blot analysis showed that 100μM A23 completely inhibited the ET-1-induced protein tyrosine phosphorylation of the 75-85 kDa band of proteins over a time period of 10 minutes whereas 100μM A1 had no effect (Figure 5.4).

3.2. Permeabilised arteries.

3.2a. Titration experiment.

100μM active TKIs tyrphostin A47 and A23 significantly relaxed the maximum tension developed to ET-1 (100nM) compared to 100μM inactive analogue A1 in permeabilised rat mesenteric small arteries ($p<0.05$, Table 5.2). 20 and 50μM A47 and A23 also relaxed the maximum ET-1-induced tension ($p<0.05$, Figure 5.5a) and all 3 doses of A47 and A23 caused a significant % relaxation of the maximum ET-1-induced tension ($p<0.05$, Figure 5.5b) compared to A1.

3.2b. Incubation experiment.

Incubation with 100μM tyrphostin did not affect the maximum tension developed to pCa6.7 (Table 5.2), but the maximum tension developed to pCa6.7 + 10μM GTP was significantly greater in the DMSO-treated group compared to all 3 tyrphostin-treated groups ($p<0.05$, Table 5.2).

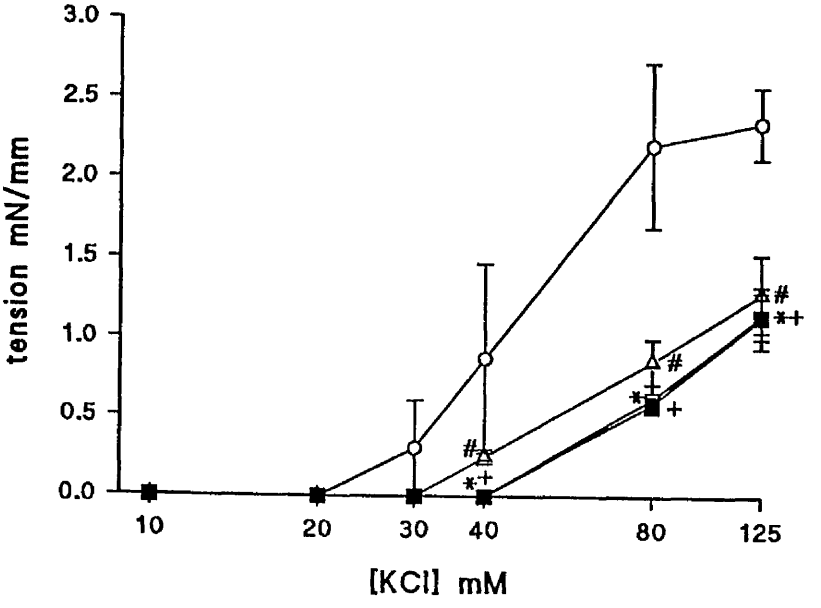


FIGURE 5.3 Tension dose response curve to KCl in intact rat mesenteric small arteries in the presence of 100μM active TKI tyrphostin A47 (□), 100μM active TKI tyrphostin A23 (■), 100μM inactive analogue tyrphostin A1 (Δ) and 0.1% DMSO (o). Mean±SEM for n=3. *$p<0.05$ for A47 (vs DMSO); +$p<0.05$ for A23 (vs DMSO); #$p<0.05$ for A1 (vs DMSO) by ANOVA plus the LSD test.

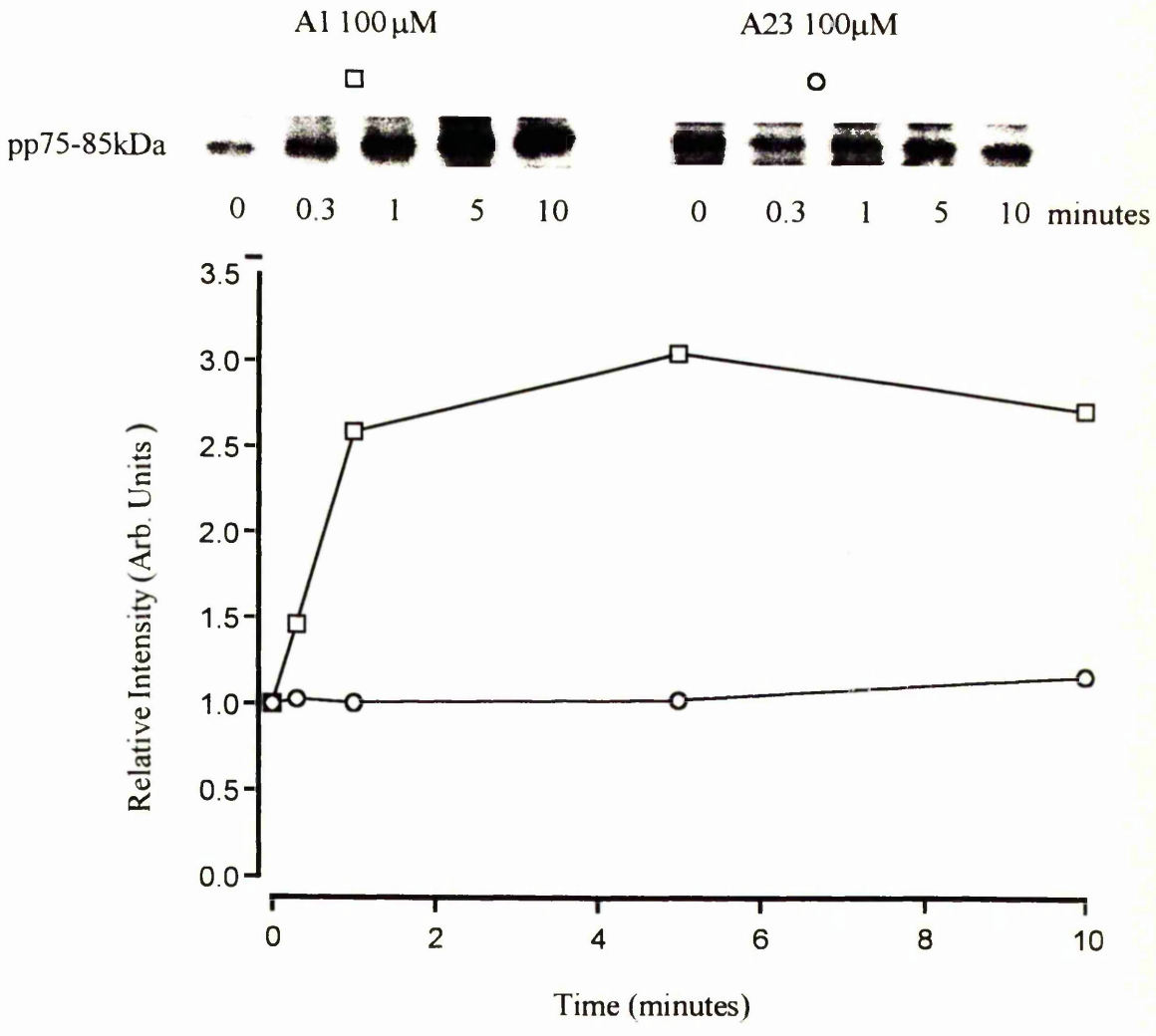


FIGURE 5.4 Immunoblots showing the effect of the active TKI tyrphostin A23 (O) and the inactive analogue tyrphostin A1 (□) on tyrosine phosphorylation of the 75-85 kDa band of proteins. Vessels were incubated with ET-1 (100nM) in the presence of 100μM A1 or A23 for various times as shown. Whole vessels were homogenised, run on SDS-PAGE, electrophoretically transferred to membranes and probed with antiphosphotyrosine antibody RC20. The autoradiogram was scanned by laser densitometry and the relative intensity is presented. The data are representative of 3 experiments.

TABLE 5.2 Contractile response of α-toxin permeabilised rat mesenteric small arteries to agonists on titration or incubation with 100μM tyrphostin A47 (active TKI), 100μM tyrphostin A23 (active TKI) , 100μM tyrphostin A1 (inactive analogue) and 0.1% DMSO. Data show the tension developed to maximal doses of ET-1 (100nM) in the titration experiment. The tension developed to pCa6.7, pCa6.7 + GTP (10μM) and ET-1 (100nM) in the incubation experiment is also shown. Mean±SEM for n=3. +p<0.05 (vs A1); *p<0.05 (vs DMSO) by ANOVA plus the LSD test.

Treatment	Stimulation	Max. Tension
Titration		mN/mm
A47	ET-1	0.044±0.044+
A23		0.475±0.061+
A1		1.931±0.131
Incubation		
A47	pCa6.7	0.014±0.014
A23		0±0
A1		0.150±0.077
DMSO		0.286±0.136
A47	pCa6.7 + GTP	0.037±0.020*
A23		0.015±0.007*
A1		0.224±0.099*
DMSO		0.942±0.144
A47	ET-1	0.403±0.116*
A23		0.543±0.142*
A1		1.136±0.252
DMSO		1.135±0.156

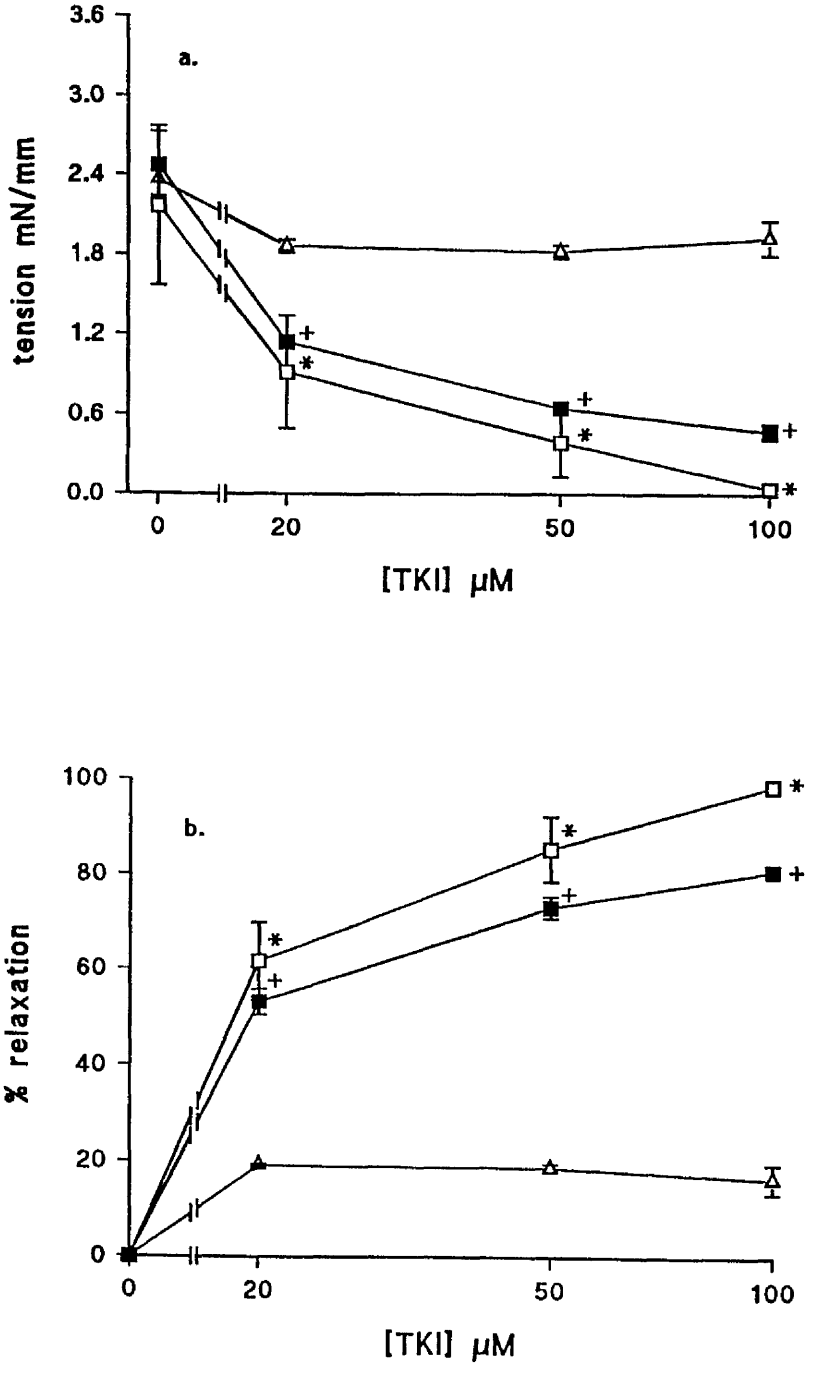


FIGURE 5.5 a. Tension and b. % relaxation dose response curves to active TKI tyrphostin A47 (□), active TKI tyrphostin A23 (■) and inactive analogue tyrphostin A1 (Δ) in α-toxin permeabilised rat mesenteric small arteries following a constant, maximum contraction to 100nM ET-1 . Mean±SEM for n=3. *$p<0.05$ for A47 (vs A1); +$p<0.05$ for A23 (vs A1) by ANOVA plus the LSD test.

Effect on ET-1-induced contraction.

The maximum tension developed to ET-1 (100nM) was significantly reduced in the presence of 100μM active TKIs tyrphostin A47 and A23 compared to DMSO control in permeabilised rat mesenteric small arteries ($p<0.05$, Table 5.2). The maximum tension developed to sub-maximal doses of 3 and 10nM ET-1 was also reduced in the A23- and A47-treated vessels compared to vessels treated with DMSO ($p<0.05$, Figure 5.6).

Effect on calcium-induced contraction.

100μM A1, A47 and A23 caused a 7.68±7.63% (mean±STD), 53.10±1.51% (mean±STD) and 73.38±8.18% (mean±STD) decrease respectively in the tension developed to a maximal calcium concentration in permeabilised arteries (for n=2).

3.3. TKI and PKC inhibitor.

Treatment	Max. tension to 100nM ET-1 mN/mm (n=3)	Final tension after $3x10^{-7}$M RO-31-8330 mN/mm (n=3)
100μM A23	0.498±0.095	0.415±0.075
100μM A1	1.032±0.538	0.373±0.102

The above table shows $3x10^{-7}$M PKC inhibitor RO-31-8330 reduced the tension developed to ET-1 (100nM) in the presence of 100μM inactive analogue tyrphostin A1, but not in the presence of 100μM active TKI A23. The final tension after treatment with both inhibitors was similar in the two groups and was approximately 65% reduced from the maximum tension developed to 100nM ET-1 in the presence of 100μM A1.

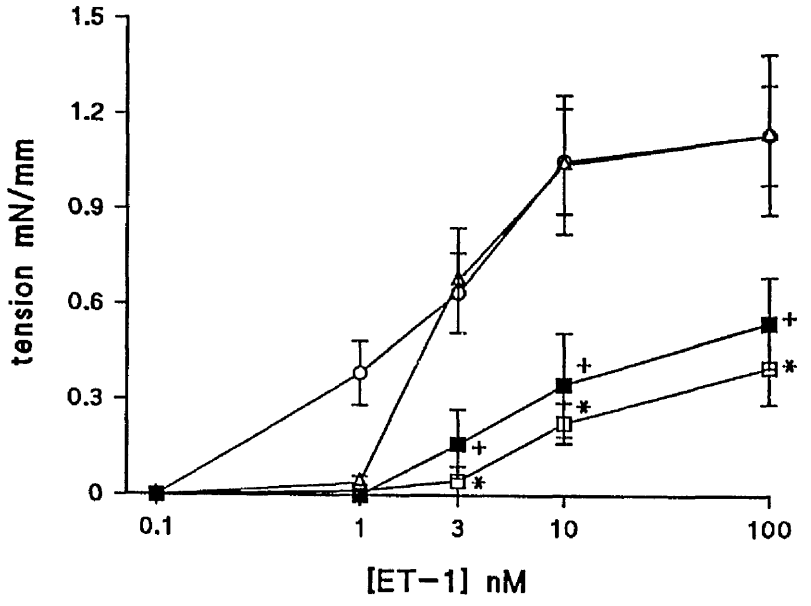


FIGURE 5.6 Tension dose response curve to ET-1 in α-toxin permeabilised rat mesenteric small arteries in the presence of 100μM active TKI tyrphostin A47 (□), 100μM active TKI tyrphostin A23 (■), 100μM inactive analogue tyrphostin A1 (Δ) and 0.1% DMSO (○). Mean±SEM for n=3. *$p<0.05$ for A47 (vs DMSO); +$p<0.05$ for A23 (vs DMSO) by ANOVA plus the LSD test.

4. Discussion

4.1. Intact arteries.

In the present study 100μM active TKIs tyrphostin A23 and A47 significantly inhibited the sustained, minimum contraction to ET-1 (100nM) by approximately 90% and 100% respectively in intact rat mesenteric small arteries whereas 100μM inactive analogue A1 had no effect. These results suggested activation of TKs was predominantly responsible for the contractile response to ET-1 in vascular smooth muscle.

Previous studies using TKIs to investigate the involvement of tyrosine phosphorylation in the receptor agonist-induced vasoconstriction have yielded conflicting results. Yang et al (1992) found that low concentrations of genistein and tyrphostin RG-50864 completely inhibited the EGF-induced contraction in circular and longitudinal preparations of guinea pig gastric smooth muscle. In contrast, these inhibitors had no effect on the carbachol- and bradykinin-induced contraction. However, the contractile response to AII was completely blocked in the longitudinal and partially blocked in the circular smooth muscle preparation in the presence of the TKIs (Yang et al 1992). These results were repeated in later investigations (Yang et al 1993, Hollenberg 1994) therefore Hollenberg (1994) hypothesised that AII (which acts via a G-protein-coupled receptor) and EGF (which acts via the EGF TK receptor) may have activated a common signal transduction pathway involving an unidentified, intermediary non-receptor TK.

Di Salvo et al (1993b) found the contraction elicited by

carbachol, phenylephrine (PE) and NA in vascular smooth muscle preparations was inhibited by higher concentrations of TKIs which was also observed by Filipeanu et al (1995) on the contraction elicited by PE in rat aortic rings. However, in other studies TKIs had no effect on the α adrenoceptor agonist- (Sauro and Thomas 1993a, Merkel et al 1993, Jinsi and Deth 1995, Hughes 1995) and ET-1- (Jinsi and Deth 1995) induced contraction. Furthermore, TKIs were ineffective in suppressing the contractile response to phorbol 12,13-dibutyrate (PdBu) in SHR and WKY (Sauro and Thomas 1993a) and rabbit (Merkel et al 1993) aortic smooth muscle preparations. Laniyonu et al (1994a) showed genistein and tyrphostin AG82 attenuated the contractile actions of AII, AVP, EGF, NA, and prostaglandin F2 alpha ($PGF_{2\alpha}$) in porcine coronary artery helical strips and the contractile action of AII in rat aorta helical strips. Therefore they concluded that TKIs blocked the G-protein-coupled receptor agonist-induced contraction in vascular smooth muscle in a species-dependent and tissue-dependent manner (Laniyonu et al 1994a). This may explain the conflicting results between all these investigations in which a variety of species and tissues were used. Alternatively the confliciting results could be due to differences in TKIs used.

More recently tyrphostin A1, A23, A47, genistein and daidzein caused a dose dependent relaxation of the maximum tension developed to receptor agonists in intact rat mesenteric small arteries mounted in a myograph (Toma et al 1995) which is similar to the findings in this thesis using tyrphostins. However, Toma et al (1995) used NA as the contractile

receptor agonist as opposed to this thesis where ET-1 was used as the contractile receptor agonist.

In the present investigation the profile of the contractile response to high doses of 10 and 100nM ET-1 in intact rat mesenteric small arteries was changed from sustained to transient in the presence of the active TKIs tyrphostin A47 and A23 but not the inactive analogue A1. However, although the response was transient it did not relax completely to baseline. 100μM A23 had no effect on the maximum tension of the ET-1 "spike" in calcium-free PSS indicating TKs did not play a role in the initial release of calcium from the intracellular stores. This is in agreement with recent studies demonstrating the TKI genistein had no effect on the IP_3-mediated store release of calcium following stimulation with ET-1 (Liu and Sturek 1996) and bradykinin (Fleming et al 1995). These results suggested tyrosine phosphorylation was involved in the later sustained phase of the contractile response to ET-1 in intact arteries, although this was not the sole mechanism responsible for this effect.

Following the addition of normal PSS containing 2.5mM calcium in intact rat mesenteric small arteries there was a large contraction which has also been observed in previous studies using rat (Auguet et al 1989) and rabbit (Sugiuri et al 1989, Ohlstein et al 1989) aortas indicating ET-1 remained bound to its receptors. Indeed, it has been reported that the vasoconstrictor effects of ET-1 were long-lasting and very difficult to "wash out" (Yanagisawa et al 1988). This was attributed to an extremely tight association between ET-1 and

its receptor demonstrated by binding studies using cultured rat vascular smooth muscle cells (Hirata et al 1988). The maximum tension developed to PSS was not affected by incubation with 100μM active TKI tyrphostin A23 but the subsequent relaxation in tension occurred much more rapidly compared to vessels incubated with DMSO control. The inactive analogue A1 had the same effect as A23 despite the observation that 100μM A1 did not inhibit TK activity as assessed by protein tyrosine phosphorylation levels (see Figure 5.4). This suggested A1 had effects on the ET-1-induced contraction in intact arteries which were unrelated to inhibition of TKs.

In the dose response curve to ET-1 in intact rat mesenteric small arteries the contractile response to low doses of 1 and 10nM ET-1 was significantly inhibited by the inactive analogue A1 whereas the contractile response to maximal doses of 100nM ET-1 was unaffected by A1 suggesting these non-specific effects were overcome by high doses of ET-1. Furthermore, these non-TK inhibition effects were not restricted solely to receptor-mediated responses as the maximum tension developed to KCl (125mM) was reduced by approximately 65% in the presence of all 3 tyrphostins. Therefore the inhibitory effect of A1 alone was not overcome by the highest concentration of 125mM KCl - in contrast to the effects of the inactive analogue on ET-1. Toma et al (1995) also reported that tyrphostin A1 reversibly relaxed the KCl-induced contraction in intact rat mesenteric small arteries with only slightly less potency than tyrphostin A23. Both these studies question the effective use of these

compounds in contractile studies in intact vascular smooth muscle.

In this thesis the sustained phase of the contractile response to ET-1 in intact rat mesenteric small arteries was predominantly dependent on extracellular calcium confirming previous studies using small arteries (Yanagisawa et al 1988, Moreland et al 1992b, Yoshida et al 1994). The active TKI tyrphostin A23 blocked the sustained ET-1-mediated contraction which suggested TKs may have been involved in the influx of extracellular calcium. This hypothesis is corroborated by recent reports in which the TKI genistein attenuated the increase in $[Ca^{2+}]_i$ in intact vascular smooth muscle in response to ET-1 (Liu and Sturek 1996), NA (Toma et al 1995), PE (Di Salvo et al 1994), bradykinin (Fleming et al 1995) and histamine (Gould et al 1995).

It has been shown that following stimulation with ET-1 in intact vascular smooth muscle the influx of calcium occurred via dihydropyridine-sensitive, voltage-gated calcium channels (Yanagisawa et al 1988, Yoshida et al 1994, Xuan et al 1994). Wijetunge et al (1992) demonstrated that tyrphostin A1, A23 and genistein caused a dose dependent inhibition of the voltage-gated calcium channel currents in smooth muscle cells from the rabbit ear artery. In a later study A23 and genistein blocked the increase in voltage-gated calcium channel currents induced by the non-receptor membrane-associated TK $pp60^{c\text{-}src}$ (Wijetunge and Hughes 1995). Therefore a possible mechanism of the other TK inhibition effects of tyrphostins on the contraction to sub-maximal doses of ET-1

and the contraction to sub-maximal and maximal doses of KCl are inhibition of the voltage-gated calcium channels leading to a decrease in the influx of extracellular calcium. However, in a recent study although genistein and tyrphostin B42 dose dependently inhibited voltage-gated calcium channel currents in rabbit colonic muscularis mucosae tyrphostin A1 had little effect at a concentration of 100μM (Hatakeyama et al 1996).

In contrast ET-1 did not evoke a contraction via calcium influx through dihydropyridine-sensitive, voltage-gated calcium channels in aortic (Sakata et al 1989, Ohlstein et al 1989, Auguet et al 1989, Sasaki et al 1991) and bovine bronchial (Nally et al 1994) smooth muscle. There is evidence that TKs can modulate the activity of membrane channels other than voltage-gated calcium channels including non-selective cation channels (Enoki et al 1995, Minami et al 1994) and K^+ channels (Smirnov and Aaronson 1995, Xiong et al 1995). Enoki et al (1995) found the increase in $[Ca^{2+}]_i$ in response to high concentrations of ET-1 in cultured mouse fibroblast (Ltk^-) cells was due to both the release of calcium from the intracellular stores and the entry of extracellular calcium whereas it was due solely to calcium entry in response to low concentrations of ET-1. These findings explain why the contraction to sub-maximal doses of ET-1 (which was dependent on calcium entry), but not the contraction to maximal doses of ET-1 (which was dependent on both intracellular calcium release and calcium entry) was inhibited by tyrphostins in intact rat mesenteric small arteries in this thesis due to their effects on calcium permeable channels.

4.1a. Summary.

The results from intact rat mesenteric small arteries implied activation of TKs may have been involved in the sustained phase of the ET-1-induced contraction. However, the potent, non-TK inhibition effects of tyrphostins on the contractile response to ET-1, possibly via inhibition of calcium influx, made it difficult to reach a definite conclusion.

An alternative way that TKs may have been responsible for the sustained phase of the contraction to ET-1 is by increasing the sensitivity of the myofilaments to calcium. Therefore, the effects of tyrphostins were investigated in arteries permeabilised with Staphylococcal aureus α-toxin.

4.2. Permeabilised arteries.

100μM active TKIs tyrphostin A47 and A23 relaxed the maximum preconstriction to 100nM ET-1 in α-toxin permeabilised rat mesenteric small arteries by approximately 100% and 80% respectively, implying TKs played a major role in the ET-1-induced enhancement of myofilament calcium sensitivity. In contrast to the results in intact arteries 100μM inactive analogue A1 did not significantly relax the maximum ET-1 preconstriction in permeabilised arteries. This was probably because calcium influx via voltage-gated calcium channels, which have been shown to be inhibited by A1 (Wijetunge et al 1992), did not participate in the contraction to ET-1 in permeabilised arteries where calcium is maintained at a constant level.

In agreement with the findings in this thesis the majority of

evidence from previous investigations using permeabilised vascular smooth muscle preparations suggested TK phosphorylation was involved in the receptor agonist-induced calcium sensitisation. Satoh et al (1993) found the ras p21-induced potentiation of the sub-maximal calcium-induced contraction in ß-escin skinned guinea pig mesenteric microarteries was partially reversed by tyrphostin. Furthermore, genistein inhibited the increase in calcium sensitivity elicited by carbachol in α-toxin permeabilised guinea pig ileal longitudinal smooth muscle (Steusloff et al 1993). More recently, Toma et al (1995) found genistein, daidzein, A47 and A23, but not A1, caused a dose dependent relaxation of the NA-induced contraction in α-toxin permeabilised rat mesenteric small arteries mounted in a myograph. This is very similar to the observations in the present study using tyrphostins but against an ET-1-induced contraction.

When 100μM active TKIs tyrphostin A47 and A23 were given simultaneously with ET-1 they inhibited the tension developed to maximal concentrations by approximately 50% whereas they caused a rapid and complete relaxation when given on top of a maximum ET-1 preconstriction. The reason for this discrepancy is unclear, but it should be noted that tyrphostin also caused a rapid and complete relaxation of the ET-1-induced preconstriction in intact arteries. Therefore the relaxation observed in permeabilised arteries following a maximum ET-1 preconstriction could be attributed to non-TK inhibition effects on the smooth muscle cell membrane.

In this thesis 100μM active TKIs tyrphostin A47 and A23 (but not the inactive analogue A1) caused a substantial relaxation of the contraction to maximal concentrations of calcium in permeabilised arteries. These results are in agreement with Toma et al (1995) who showed A47, A23, genistein and daidzein relaxed the force developed to 1μM calcium in α-toxin permeabilised rat mesenteric small arteries suggesting that TKs may have been involved in the calcium-induced contraction. Indeed, there is evidence that calcium activates TKs in intact vascular smooth muscle as the calcium ionophore ionomycin increased protein tyrosine phosphorylation in cultured rat aortic vascular smooth muscle cells (Tsuda et al 1991). Furthermore, the profile of proteins phosphorylated by ionomycin was similar to that phosphorylated by vasoconstrictor hormones including NA, AII, AVP and serotonin (Tsuda et al 1991). In contrast to the above findings Di Salvo et al (1993b) and Satoh et al (1993) found tyrphostin had no effect on the calcium-activation of the contractile apparatus in ß-escin skinned guinea pig mesenteric microvessels mounted in a muscle chamber. Therefore the difference in results between these studies and the present study could be attributed to the difference in species used.

4.3. TKI and PKC inhibitor.

Following permeabilisation the maximum tension developed to 100nM ET-1 in the presence of 100μM active tyrphostin A23 was approximately the same as the final tension after the addition of $3x10^{-7}$M PKC inhibitor RO-31-8330. This suggested PKC did not phosphorylate any additional proteins over and above those phosphorylated by TKs to bring about calcium

sensitisation in response to ET-1. Inhibition of both PKC and TK activity in α-toxin permeabilised rat mesenteric arteries reduced the maximum tension developed to 100nM ET-1 by approximately 65% implying these two mechanisms were predominantly responsible for the ET-1-induced increase in myofilament calcium sensitivity. However, a residual contraction remained which could have been caused by an arachidonic acid (AA)-related mechanism, which was presumably inhibition of myosin light chain (MLC) phosphatase (Gong et al 1992). This hypothesis is supported by studies demonstrating that binding of ET-1 to its receptor stimulated the release of AA in cultured vascular smooth muscle cells (Resink et al 1989, Reynolds et al 1989).

4.4. Summary.

The results in this thesis using α-toxin permeabilised rat mesenteric small arteries suggested TKs played a substantial role in the ET-1-induced enhancement of calcium sensitivity of the myofilaments which may have been responsible for the sustained phase of the contractile response to ET-1 in intact arteries. Activation of both PKC and TKs was predominantly responsible for the ET-1-induced calcium sensitisation, but another mechanism may have also been involved in this effect.

CHAPTER SIX

CALCIUM SENSITIVITY AND AGONIST-INDUCED CALCIUM SENSITISATION IN YOUNG AND ADULT SPONTANEOUSLY HYPERTENSIVE RATS.

1. Introduction.

Hypertension is associated with an increased total peripheral resistance leading to a role for small arteries in the causes and consequences of this disease. The increased peripheral resistance may be due to an enhanced reactivity of these arteries to vasoconstrictor agonists which has been observed in the adult SHR in some (Mulvany et al 1980, Tomobe et al 1988), but not other (Bund et al 1991, Mulvany and Aalkjaer 1990, Dohi and Lüscher 1991) studies. In view of this controversy the vascular responsiveness of intact small arteries from young and adult SHRs and WKYs to vasoconstrictor agonists was investigated in this thesis.

An enhanced reactivity in response to vasoconstrictor agonists in small arteries may be caused by an elevated level of $[Ca^{2+}]_i$ available to the myofilaments as a result of abnormal calcium handling (Kwan and Daniel 1981). This has indeed been demonstrated in freshly isolated aortic smooth muscle cells from the SHR compared to WKY using ion selective electrodes (Losse et al 1994). Alternatively it may be caused by an augmented agonist-induced calcium sensitisation which may result from an increased sensitivity of the myofilaments themselves to calcium. However, both these factors have been rarely studied in permeabilised small arteries.

It has been observed that different vascular beds contribute unequally to the elevated total peripheral resistance in the

SHR (Ferrone et al 1979) therefore they may respond differently to calcium and/or vasoconstrictor agonists. Furthermore, the increased peripheral resistance in hypertension has been associated with morphological changes in small arteries (Heagerty et al 1993) which may also differ in individual vascular beds. Therefore, the myofilament calcium sensitivity and the calcium sensitisation induced by various agonists including the phorbol ester phorbol 12,13-dibutyrate (PdBu) and the receptor agonists noradrenaline (NA) and endothelin-1 (ET-1) and morphological parameters were studied in SHRs and WKYs at 5 and 20 weeks using α-toxin permeabilised mesenteric, renal and femoral small arteries.

2. Protocols.

2.1. Animals.

For this study male spontaneously hypertensive rats (SHRs) and their control strain Wistar-kyoto rats (WKYs) were used. They were obtained from Charles River Ltd. (Margate, Kent).

2.2. Blood pressure measurement.

At 5 or 20 weeks of age femoral artery cannulae were implanted under anaesthesia to permit direct measurement of blood pressure. Anaesthesia was induced by a 3.3ml per kg body weight i.p. injection of a 1:1:2 mixture of Hypnorm, Hypnovel and water. Therefore rats received 0.26mg Fentanyl Citrate, 8.25mg Fluorisone and 4.1mg Medazolam per kg body weight. Occasionally supplemental doses were administered as required. Polyethylene catheters (portex tubing 0.61mm outside diameter, 0.28mm inside diameter) were inserted into the left femoral artery and the proximal tip advanced into

the abdominal aorta. The distal region of the catheter was exteriorised between the scapulae, flushed with saline containing 100 units/ml heparin and blocked with a stainless steel spigot. The catheter was secured to the femoral artery with 4/0 silk suture. 0.3 mg/kg body weight burenorphrine was used to provide analgesia. Animals were then housed in separate cages. 24 hours after catheter implantation unrestrained, conscious blood pressure recordings were made under quiet resting conditions with a Viggo-Spectramed TXX-R pressure transducer connected to a chart recorder. Mean aortic blood pressure was calculated as diastolic blood pressure plus one-third pulse pressure.

2.3. Morphology.

Following blood pressure recording rats were killed and small arteries from various vascular beds were dissected out and cleaned of extraneous fat and connective tissue. These included femoral small arteries (third order branch vessels originating distal to the femoral artery were used in 20 week rats, but due to size limitations second order vessels were used in 5 week rats), mesenteric small arteries (third order branches off the superior mesenteric artery) and renal small arteries (first order branches off the main renal artery).

Vessels were mounted in a myograph in PSS and warmed to 37°C as this was the correct physiological temperature for the accurate measurement of vascular structure. Measurements of vascular morphology were made (as described on Page 54) after which vessels were normalised (as described on Page 56). The myograph was then cooled to room temperature (which took 1-

1.5 hours) so that vessels could be permeabilised (as described on Page 81). Following permeabilisation vessels were equilibrated in relaxing solution (G_1) for 20-25 minutes then stimulated by a series of solutions of increasing calcium concentration. These were made from mixing G_{10} and CaG in the following quantities to give 12ml of solution:

	CaG (ml)	G_{10} (ml)
pCa7.5	1.22	10.78
pCa7.0	3.18	8.82
pCa6.7	5.03	6.97
pCa6.3	7.74	4.26
pCa6.0	9.41	2.59
pCa5.5	11.04	0.96
pCa5.0	11.70	0.30

A pCa-tension curve was obtained by normalising the tension for each sub-maximal calcium-induced contraction with respect to maximum tension and plotting as a function of pCa. Responses to potassium external solution (KES) and 10μM NA in intact arteries plus the maximal calcium concentration in permeabilised arteries were calculated as active tension (as described on Page 56) and active media stress. Media stress is defined as the active tension divided by media thickness at L_0 which has been described previously (Mulvany et al 1978). Vessels were equilibrated in G_1 for a further 20-25 minutes before stimulation with pCa6.7 for 8-10 minutes to give a constant, sub-maximum contraction. Various agonists were then added including 1. 0.05μM PdBu 2. GTP + 100nM ET-1 and 3. GTP + 10μM NA. In order to investigate the mechanisms involved in the calcium sensitisation response the effect of a combination of GTP + ET-1 + NA + PdBu (Figure 6.1) and PdBu + GTP + ET-1 + Na at pCa6.7 was studied. 100nM ET-1 and 10μM

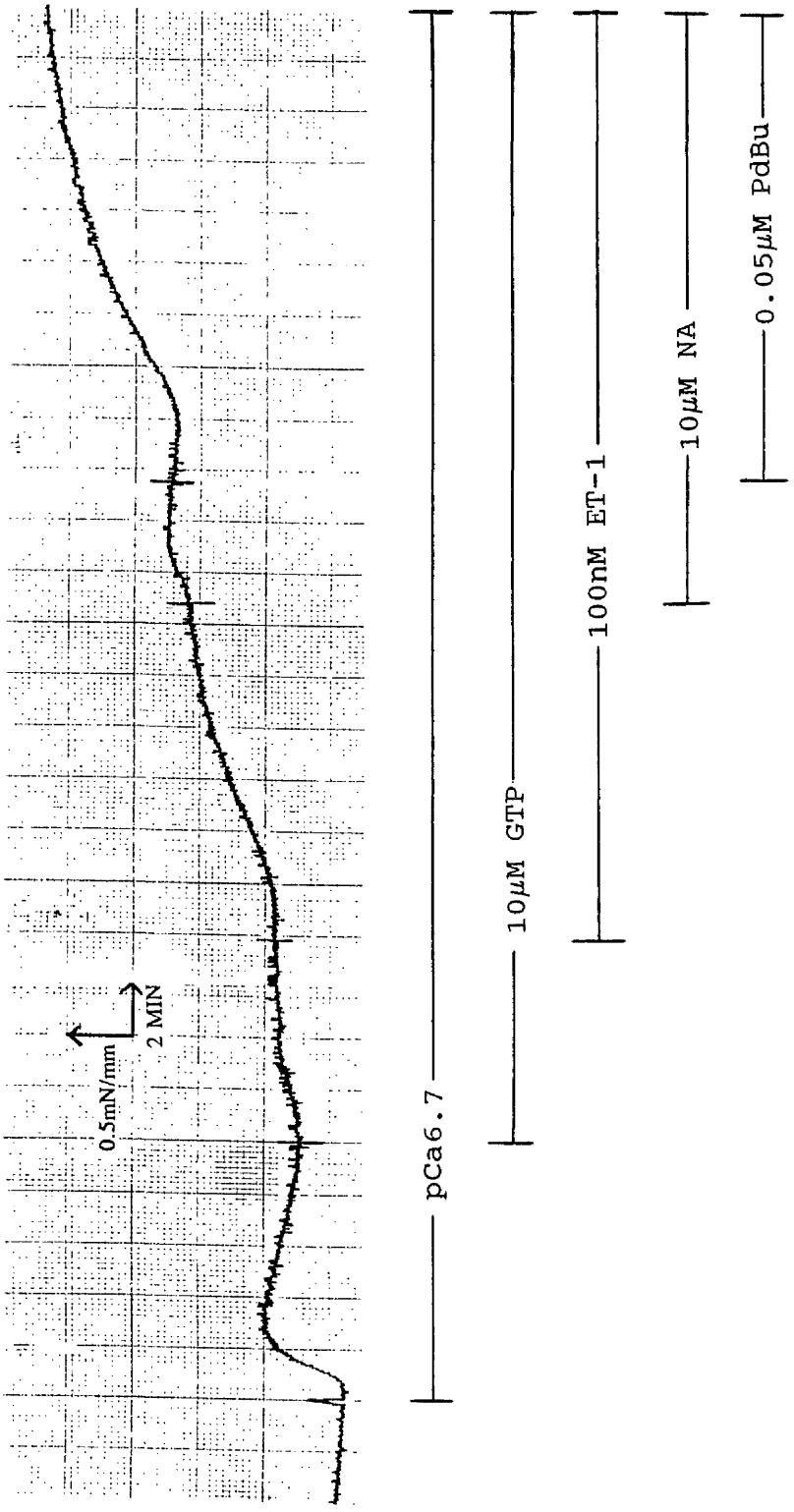


FIGURE 6.1 Representative tracing showing the effect of GTP (10μM), ET-1 (100nM), NA (10μM) and PdBu (0.05μM) on a contraction to pCa6.7 in an α-toxin permeabilised femoral small artery from a 5 week Wistar-kyoto rat (WKY).

NA produced the maximum contractile response to those agonists, but this was not the maximum contraction capable of the permeabilised artery. The maximally effective concentration of PdBu was 2μM and maximum contraction of the artery was also achieved at this dose. Therefore, in this study 0.05μM PdBu was used to produce a sub-maximum contraction of the artery allowing the further addition of the receptor agonists ET-1 or NA. After each addition the contraction elicited by that particular agonist was allowed to reach a plateau before the next was added.

2.4. Data calculation and statistical analysis.

Each contraction was measured from the baseline and calculated as active tension (as described on Page 56). Repeated analysis of variance (ANOVA) was used to determine differences between SHR and WKY pCa-tension dose response curves. ANOVA plus the Least Significant Difference (LSD) test were used to assess differences between strains and ages in blood pressure and in mesenteric and renal arteries. Because femoral arteries from the two age groups were taken from different locations it was not strictly permissable to compare them statistically. Therefore differences between strains in femoral arteries plus all other comparisons were made using Student's t-test.

3. Results.

3.1. Blood Pressure.

Systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean blood pressure (MBP) were significantly increased in 5 week SHRs compared to WKYs by approximately 25% ($p<0.05$,

Table 6.1). SBP, DBP and MBP were also increased in 20 week SHRs compared to WKYs (approximately 45%) ($p<0.05$, Table 6.1). All 3 measurements of blood pressure were approximately 50% greater in older SHRs compared to younger SHRs ($p<0.05$, Table 6.1) and approximately 25% greater in older WKYs compared to younger WKYs ($p<0.05$, Table 6.1).

3.2. Morphology.

3.2a. 5 week animals.

Media thickness.

Media thickness (M_0) was significantly increased in femoral arteries from the SHR compared to WKY by approximately 30% at 5 weeks ($p<0.05$, Table 6.2a). There was no difference in M_0 in mesenteric or renal arteries (Table 6.2a).

Media cross-sectional area.

Media cross-sectional area (media CSA) was increased in the SHR compared to WKY but again only in femoral arteries (approximately 25%)($p<0.05$, Table 6.2b, Figures 6.2a, 6.4a and 6.6a).

Media to lumen ratio.

At 5 weeks media to lumen ratio (M_0/l_0) was approximately 35% greater in the SHR compared to WKY in mesenteric and femoral arteries ($p<0.05$, Table 6.2c, Figures 6.3a, 6.5a and 6.7a), but there was no difference in M_0/l_0 between the two strains in renal arteries (Table 6.2c, Figures 6.3a, 6.5a and 6.7a).

TABLE 6.1 Systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean blood pressure (MBP) for 5 and 20 week spontaneously hypertensive rats (SHRs) and Wistar-kyoto rats (WKYs). Mean±SEM. Numbers of animals are given in parentheses. *p<0.05 (vs SHR); # p<0.05 (vs 5 week SHR); +p<0.05 (vs 5 week WKY) by ANOVA plus the LSD test.

	SBP(mmHg)	DBP(mmHg)	MBP(mmHg)
5 weeks			
SHR	131.3±2.2(18)	109.9±1.8(18)	117.0±1.8(18)
WKY	109.8±2.3(17)*	88.0±2.5(17)*	95.2±2.2(17)*
20 weeks			
SHR	200.3±5.4(16)#	157.6±4.6(16)#	172.2±4.4(16)#
WKY	139.2±3.4(16)*+	107.8±2.4(16)*+	118.3±2.3(16)*+

TABLE 6.2 Morphological characteristics of small arteries from three vascular beds for 5 and 20 week spontaneously hypertensive rats (SHRs) and Wistar-kyoto rats (WKYs). Mean±SEM. Numbers of animals are given in parentheses. *p<0.05 (vs SHR); #p<0.05 (vs 5 week SHR); +p<0.05 (vs 5 week WKY) by Student's t-test for differences between strains in femoral arteries and by ANOVA plus the LSD test for differences between strains and ages in mesenteric and renal arteries.

a. Media thickness (μm)

	Mesenterics	Renals	Femorals
5 weeks			
SHR	18.19±1.28(9)	17.89±2.21(5)	29.70±1.25(5)
WKY	16.57±0.59(12)	16.98±0.80(9)	22.84±1.07(8)*
20 weeks			
SHR	23.76±0.86(16)#	22.87±0.94(12)#	25.52±1.13(12)
WKY	16.97±0.30(12)*	19.05±1.59(9)*	19.40±1.29(8)*

b. Media cross-sectional area ($10^3\mu m^2$)

	Mesenterics	Renals	Femorals
5 weeks			
SHR	12.26±1.53(9)	11.61±1.78(5)	26.63±0.69(5)
WKY	13.07±3.46(12)	12.29±0.88(9)	21.25±1.26(8)*
20 weeks			
SHR	20.23±0.88(16)#	21.83±1.43(12)#	21.28±1.12(12)
WKY	16.19±0.54(12)*+	18.70±1.22(9)+	15.08±0.91(8)*

c. Media to lumen ratio (%)

	Mesenterics	Renals	Femorals
5 weeks			
SHR	9.75±0.46(9)	9.49±1.32(5)	11.78±1.16(5)
WKY	7.21±0.49(12)*	7.86±0.64(9)	8.42±0.40(8)*
20 weeks			
SHR	9.77±0.51(16)	8.81±0.77(12)	11.82±0.84(12)
WKY	6.20±0.32(12)*	6.48±0.87(9)	8.65±0.89(8)*

d. Lumen diameter (μm)

	Mesenterics	Renals	Femorals
5 weeks			
SHR	191.02±13.6(9)	191.7±11.6(5)	257.6±14.7(5)
WKY	238.6±13.2(12)*	231.0±11.2(9)	272.3±8.2(8)
20 weeks			
SHR	247.4±7.1(16)#	277.1±17.2(12)#	222.2±9.1(12)
WKY	280.3±12.0(12)*+	312.8±17.9(9)+	229.1±8.9(8)

Lumen diameter.

Lumen diameter (l_0) was significantly reduced in the SHR compared to WKY in mesenteric arteries by approximately 25% ($p<0.05$, Table 6.2d). l_0 was not different between SHRs and WKYs in renal or femoral arteries (Table 6.2d).

3.2b. 20 week animals.

Media thickness.

M_0 was significantly increased in all three vascular beds in the SHR compared to WKY ($p<0.05$, Table 6.2a).

Media cross-sectional area.

At 20 weeks media CSA was increased in mesenteric and femoral arteries in the SHR compared to WKY by approximately 30% ($p<0.05$, Table 6.2b, Figures 6.2b, 6.4b and 6.6b), but there was no difference in media CSA in renal arteries (Table 6.2b, Figures 6.2b, 6.4b and 6.6b).

Media to lumen ratio

M_0/l_0 was approximately 50% greater in the SHR compared to WKY in mesenteric and femoral arteries ($p<0.05$, Table 6.2c, Figures 6.3b, 6.5b and 6.7b). There was no difference in M_0/l_0 between the two strains in renal arteries (Table 6.2c, Figures 6.3b, 6.5b and 6.7b).

Lumen diameter.

l_0 was significantly reduced in the SHR compared to WKY in mesenteric arteries by approximately 15% at 20 weeks ($p<0.05$, Table 6.2d), but there was no difference in l_0 in renal and femoral arteries (Table 6.2d).

3.2c. 20 week animals compared to 5 week animals.

M_0 was significantly increased in mesenteric and renal arteries in 20 week SHRs compared to 5 week SHRs by approximately 30% ($p<0.05$, Table 6.2a). Media CSA was also increased in mesenteric and renal arteries at 20 weeks compared to 5 weeks but in both SHRs and WKYs ($p<0.05$, Table 6.2b). There was no difference in M_0/l_0 between the two age groups in any vascular bed (Table 6.2c). l_0 was significantly increased in older animals compared to younger animals in mesenteric and renal arteries ($p<0.05$, Table 6.2d).

3.3. KES-induced contraction.

3.3a. 5 week animals.

There was no difference in the maximum tension or media stress in response to KES between strains in any vascular bed at 5 weeks (Table 6.3, Figures 6.2a and 6.3a).

3.3b. 20 week animals.

Mesenteric and femoral arteries developed approximately 40% greater force to KES in the SHR compared to WKY ($p<0.05$, Table 6.3a, Figures 6.2b and 6.3b), but there was no difference in the maximum KES-induced tension in renal arteries (Table 6.3a, Figures 6.2b and 6.3b). Media stress in response to KES was not different between SHRs and WKYs in any vascular bed (Table 6.3b).

3.3c. 20 week animals compared to 5 week animals.

The maximum tension developed to KES was significantly increased by approximately 100% in 20 week animals compared to 5 week animals in mesenteric arteries ($p<0.05$, Table

TABLE 6.3 a. Maximum tension (mN/mm) and b. Media stress (kPa) in response to KES in intact small arteries from three vascular beds for 5 and 20 week spontaneously hypertensive rats (SHRs) and Wistar-kyoto rats (WKYs). Mean±SEM. Numbers of animals are given in parentheses. *p<0.05 (vs SHR); #p<0.05 (vs 5 week SHR);+ p<0.05 (vs 5 week WKY) by Student's t-test for differences between strains in femoral arteries and by ANOVA plus the LSD test for differences between strains and ages in mesenteric and renal arteries.

a.	Mesenterics	Renals	Femorals
5 weeks			
SHR	1.68±0.10(16)	1.79±0.17(12)	3.53±0.42(12)
WKY	1.39±0.11(15)	1.54±0.12(12)	3.77±0.40(12)
20 weeks			
SHR	3.72±0.25(16)#	1.93±0.27(11)	3.60±0.28(12)
WKY	2.73±0.16(16)*+	1.86±0.32(12)	2.60±0.23(12)*

b.	Mesenterics	Renals	Femorals
5 weeks			
SHR	96.4±10.1(9)	114.7±8.6(5)	151.1±14.2(5)
WKY	84.8±7.5(12)	90.9±7.8(9)	191.1±18.4(8)
20 weeks			
SHR	156.0±9.0(16)#	86.2±12.9(11)	142.2±10.8(12)
WKY	170.1±11.7(12)+	109.3±22.3(9)	152.4±20.6(8)

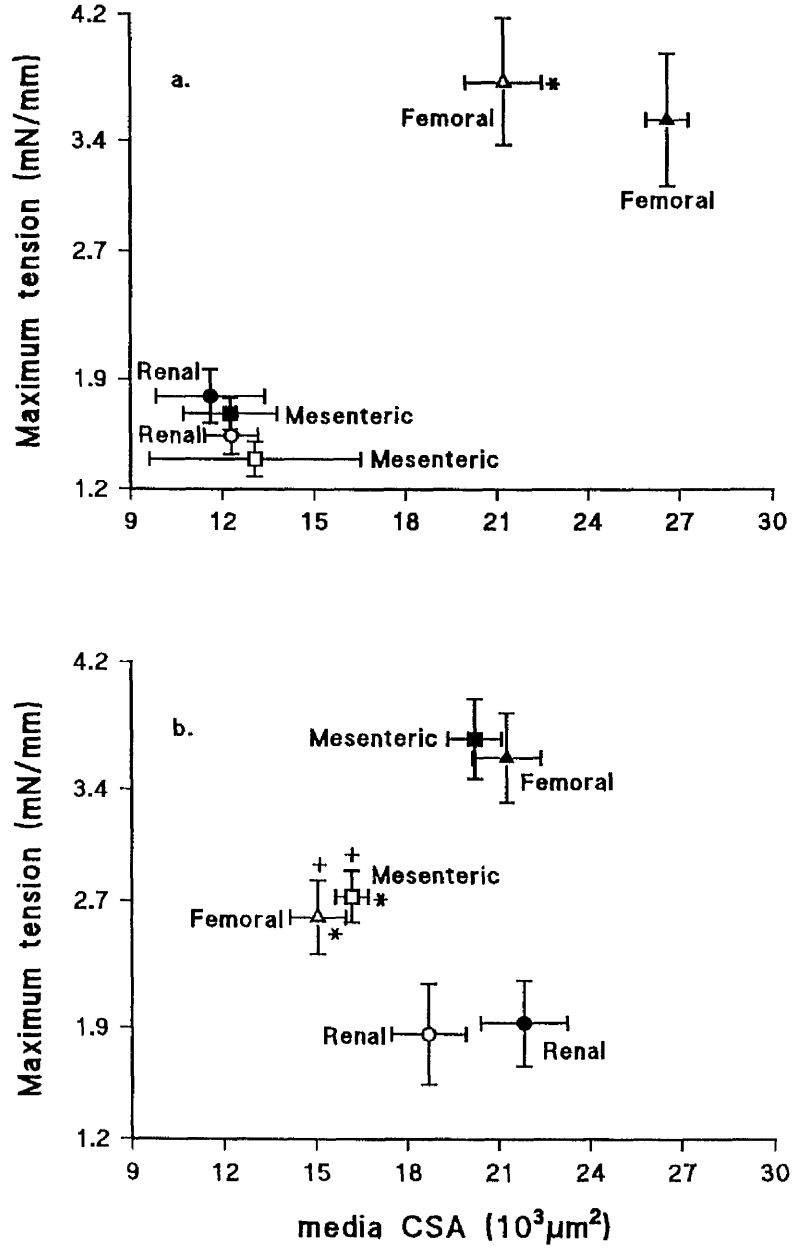


FIGURE 6.2 Relationship between the maximum tension developed to KES and media cross-sectional area (media CSA) in α-toxin permeabilised small arteries from three vascular beds for spontaneously hypertensive rats (SHRs, closed symbols) and Wistar-kyoto rats (WKYs, open symbols) at a. 5 and b. 20 weeks. Mean±SEM from a minimum of 5 and maximum of 16 individual experiments. Media CSA *$p<0.05$ (vs SHR); KES tension +$p<0.05$ (vs SHR) by Student's t-test for differences between strains in femoral arteries and ANOVA plus the LSD test for differences between strains in mesenteric and renal arteries.

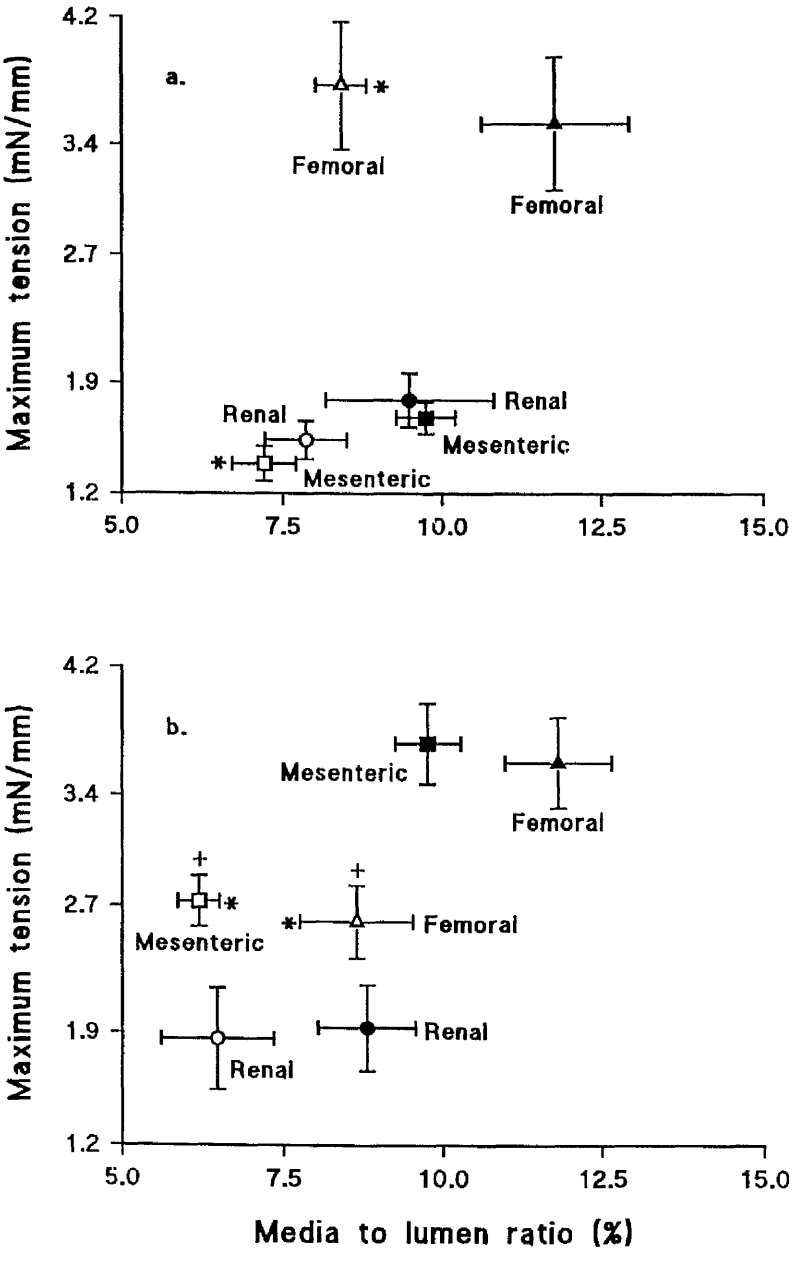


FIGURE 6.3 Relationship between the maximum tension developed to KES and media to lumen ratio in α-toxin permeabilised small arteries from three vascular beds for spontaneously hypertensive rats (SHRs, closed symbols) and Wistar-kyoto rats (WKYs, open symbols) at a. 5 and b. 20 weeks. Mean±SEM from a minimum of 5 and maximum of 16 individual experiments. Media to lumen ratio *$p<0.05$ (vs SHR); KES tension +$p<0.05$ (vs SHR) by Student's t-test for differences between strains in femoral arteries and ANOVA plus the LSD test for differences between strains in mesenteric and renal arteries.

6.3a). Media stress was also increased in older animals compared to younger animals in mesenteric arteries ($p<0.05$, Table 6.3b). However, there was no difference in the maximum tension or media stress in response to KES between the two age groups in renal arteries (Table 6.3).

3.4. NA-induced contraction.

3.4a. 5 week animals.

The maximum tension developed to 10μM NA was approximately 55% greater in the SHR compared to WKY in mesenteric and femoral arteries at 5 weeks ($p<0.05$, Table 6.4a, Figures 6.4a and 6.5a). There was no difference in the maximum NA-induced tension between the two strains in renal arteries (Table 6.4a, Figures 6.4a and 6.5a). Media stress was not different between SHRs and WKYs in any vascular bed (Table 6.4b).

3.4b. 20 week animals.

Mesenteric and femoral arteries developed approximately 50% greater force to 10μM NA in the SHR compared to WKY ($p<0.05$, Table 6.4a, Figures 6.4b and 6.5b), but there was no difference in the maximum NA-induced tension in renal arteries (Table 6.4a, Figures 6.4b and 6.5b). Media stress was again not different between the two strains in any vascular bed (Table 6.4b).

3.4c. 20 week animals compared to 5 week animals.

The maximum tension developed to 10μM NA was significantly increased in 20 week animals compared to 5 week animals by approximately 140% in mesenteric arteries ($p<0.05$, Table 6.4a). Media stress was also increased in older animals

TABLE 6.4 a. Maximum tension (mN/mm) and b. Media stress (kPa) in response to NA (10μM) in intact small arteries from three vascular beds for 5 and 20 week spontaneously hypertensive rats (SHRs) and Wistar-kyoto rats (WKYs). Mean±SEM. Numbers of animals are given in parentheses. *p<0.05 (vs SHR); #p<0.05 (vs 5 week SHR); +p<0.05 (vs 5 week WKY) by Student's t-test for differences between strains in femoral arteries and by ANOVA plus the LSD test for differences between strains and ages in mesenteric and renal arteries.

a.	Mesenterics	Renals	Femorals
5 weeks			
SHR	1.89±0.16(16)	1.91±0.19(12)	3.64±0.26(12)
WKY	1.18±0.15(15)*	1.87±0.15(12)	2.45±0.32(12)*
20 weeks			
SHR	4.40±0.25(16)#	2.18±0.28(11)	3.63±0.26(12)
WKY	2.84±0.15(16)*+	2.33±0.31(12)	2.45±0.32(12)*

b.	Mesenterics	Renals	Femorals
5 weeks			
SHR	95.4±12.0(9)	112.3±7.3(5)	103.6±25.0(5)
WKY	70.7±11.3(12)	116.4±10.2(9)	155.8±19.6(8)
20 weeks			
SHR	186.7±8.6(16)#	97.5±13.1(11)	146.2±12.8(12)
WKY	179.6±9.8(12)+	129.7±19.1(9)	153.9±19.6(8)

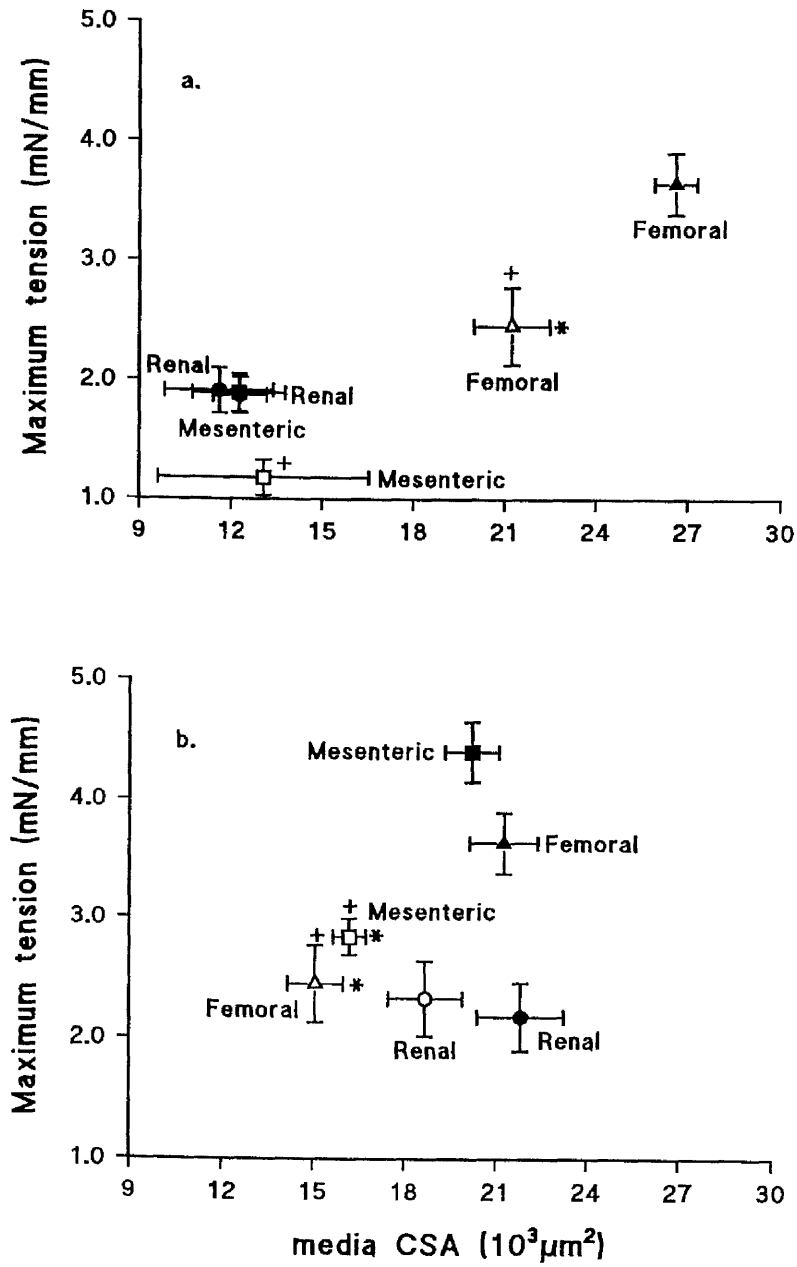


FIGURE 6.4 Relationship between the maximum tension developed to NA (10μM) and media cross-sectional area (media CSA) in α-toxin permeabilised small arteries from three vascular beds for spontaneously hypertensive rats (SHRs, closed symbols) and Wistar-kyoto rats (WKYs, open symbols) at a. 5 and b. 20 weeks. Mean±SEM from a minimum of 5 and maximum of 16 individual experiments. Media CSA *$p<0.05$ (vs SHR); NA tension +$p<0.05$ (vs SHR) by Student's t-test for differences between strains in femoral arteries and ANOVA plus the LSD test for differences between strains in mesenteric and renal arteries.

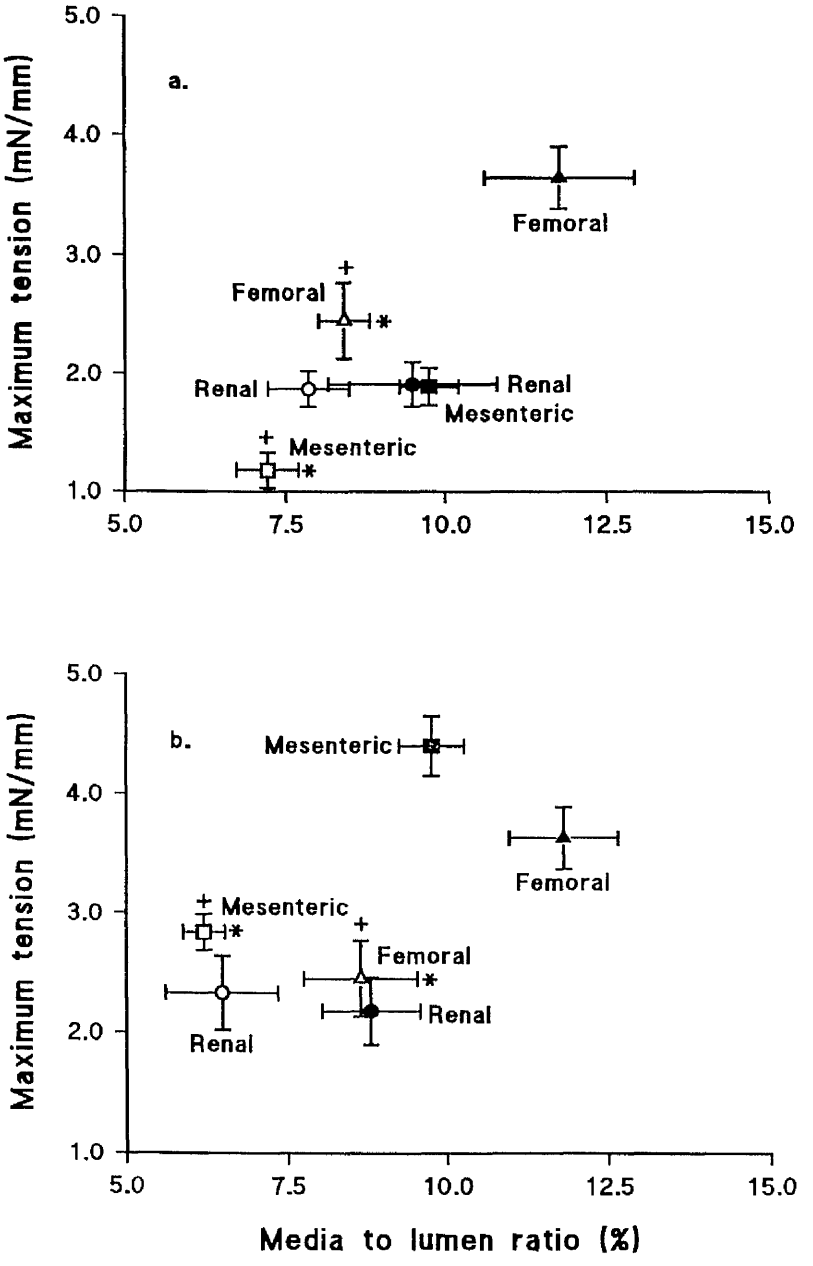


FIGURE 6.5 Relationship between the maximum tension developed to NA (10μM) and media to lumen ratio in α-toxin permeabilised small arteries from three vascular beds for spontaneously hypertensive rats (SHRs, closed symbols) and Wistar-kyoto rats (WKYs, open symbols) at a. 5 and b. 20 weeks. Mean±SEM from a minimum of 5 and maximum of 16 individual experiments. Media to lumen ratio *$p<0.05$ (vs SHR); NA tension +$p<0.05$ (vs SHR) by Student's t-test for differences between strains in femoral arteries and ANOVA plus the LSD test for differences between strains in mesenteric and renal arteries.

compared to younger animals in mesenteric arteries ($p<0.05$, Table 6.4b). However, there was no difference in maximum tension or media stress in response to 10μM NA between the two age groups in renal arteries (Table 6.4).

3.5. Calcium-induced contraction.

3.5a. 5 week animals.

There was no difference in the maximum tension or media stress in response to pCa5.0 in permeabilised arteries between SHRs and WKYs in any vascular bed (Table 6.5, Figures 6.6a and 6.7a).

3.5b. 20 week animals.

The maximum tension developed to pCa5.0 was approximately 25% greater in the SHR compared to WKY in mesenteric arteries at 20 weeks ($p<0.05$, Table 6.5a, Figures 6.6b and 6.7b). There was no difference in the maximum pCa5.0-induced tension in renal or femoral arteries (Table 6.5a, Figures 6.6b and 6.7b). Media stress was not different between the two strains for any vascular bed (Table 6.5b).

3.5c. 20 week animals compared to 5 week animals.

20 week animals developed approximately 60% greater force to the maximal calcium concentration compared to 5 week animals in mesenteric arteries ($p<0.05$, Table 6.5a). Media stress was also increased in older animals compared to younger animals in mesenteric arteries but only in the WKYs ($p<0.05$, Table 6.5b). There was no difference in maximum tension or media stress in response to pCa5.0 between 5 and 20 week animals in renal arteries (Table 6.5).

TABLE 6.5 a. Maximum tension (mN/mm) and b. Media stress (kPa) in response to pCa5.0 in α-toxin permeabilised small arteries from three vascular beds for 5 and 20 week spontaneously hypertensive rats (SHRs) and Wistar-kyoto rats (WKYs). Mean±SEM. Numbers of animals are given in parentheses. *p<0.05 (vs SHR); # p<0.05 (vs 5 week SHR); +p<0.05 (vs 5 week WKY) by Student's t-test for differences between strains in femoral arteries and by ANOVA plus the LSD test for differences between strains and ages in mesenteric and renal arteries.

a.	Mesenterics	Renals	Femorals
5 weeks			
SHR	1.43±0.06(16)	2.06±0.16(12)	2.75±0.27(12)
WKY	1.34±0.08(16)	1.84±0.11(12)	2.84±0.20(12)
20 weeks			
SHR	2.40±0.15(16)#	2.22±0.26(12)	3.63±0.23(12)
WKY	1.89±0.14(16)*+	2.32±0.20(12)	3.14±0.17(12)

b.	Mesenterics	Renals	Femorals
5 weeks			
SHR	90.5±9.7(9)	104.7±10.2(5)	102.5±5.0(5)
WKY	79.3±3.5(12)	102.5±6.4(9)	122.0±13.1(8)
20 weeks			
SHR	107.3±7.5(16)	93.8±11.5(11)	146.1±12.3(12)
WKY	111.1±9.9(12)+	123.3±15.9(9)	183.2±17.9(8)

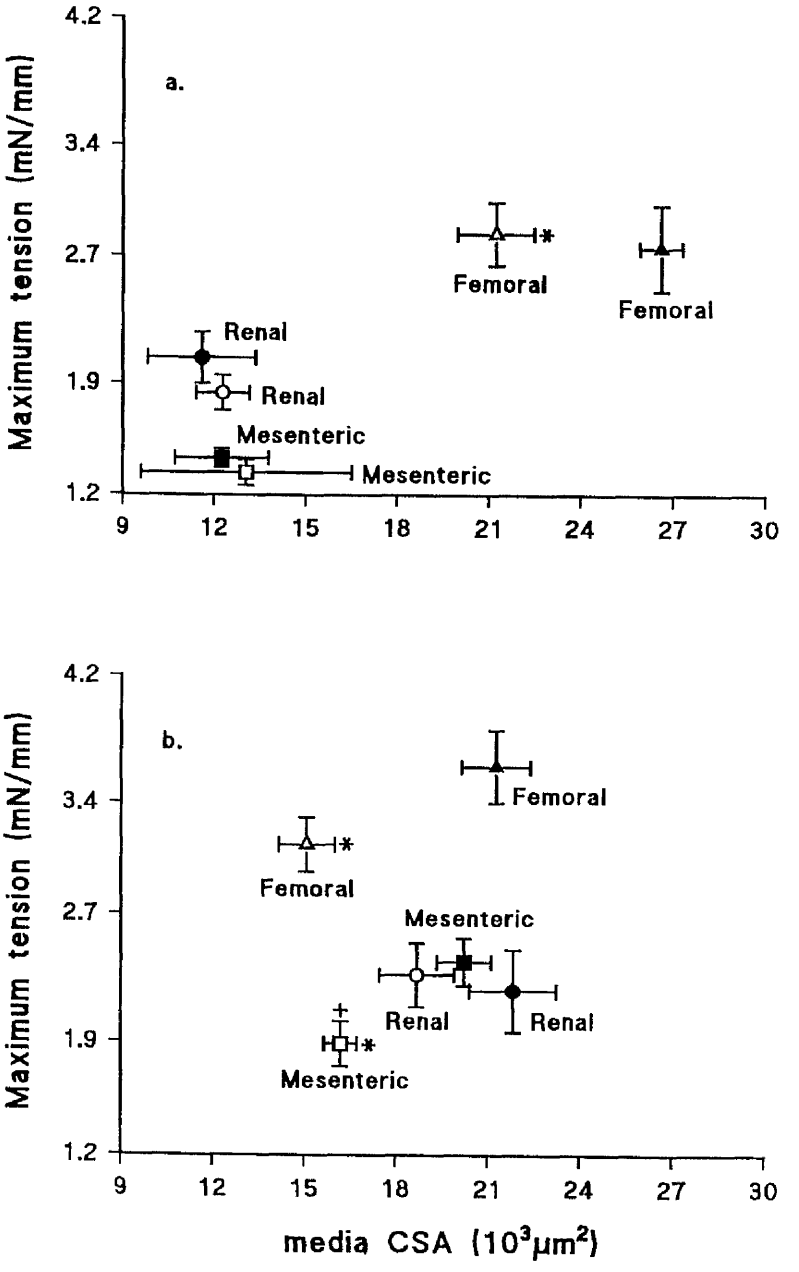


FIGURE 6.6 Relationship between the maximum tension developed to pCa5.0 and media cross-sectional area (media CSA) in α-toxin permeabilised small arteries from three vascular beds for spontaneously hypertensive rats (SHRs, closed symbols) and Wistar-kyoto rats (WKYs, open symbols) at a. 5 and b. 20 weeks. Mean±SEM from a minimum of 5 and maximum of 16 individual experiments. Media CSA *$p<0.05$ (vs SHR); pCa5.0 tension +$p<0.05$ (vs SHR) by Student's t-test for differences between strains in femoral arteries and ANOVA plus the LSD test for differences between strains in mesenteric and renal arteries.

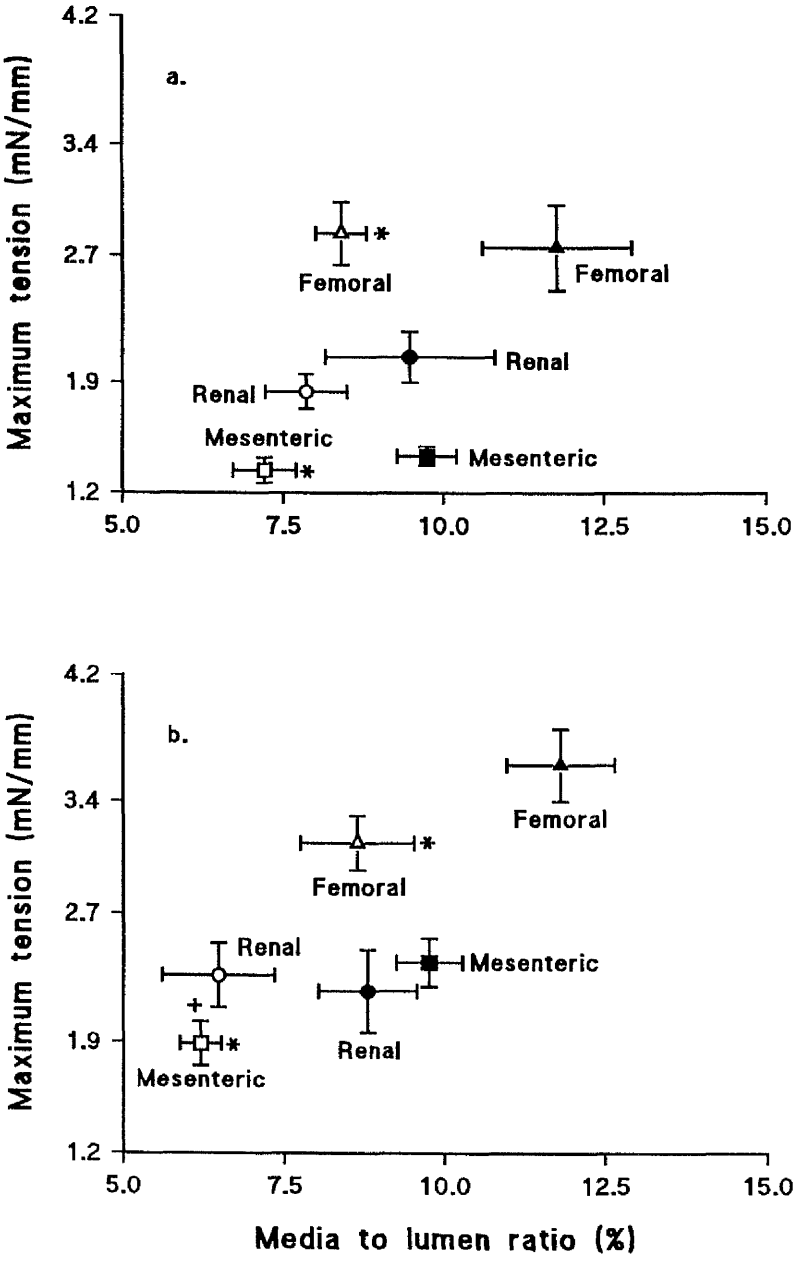


FIGURE 6.7 Relationship between the maximum tension developed to pCa5.0 and media to lumen ratio in α-toxin permeabilised small arteries from three vascular beds for spontaneously hypertensive rats (SHRs, closed symbols) and Wistar-kyoto rats (WKYs, open symbols) at a. 5 and b. 20 weeks. Mean±SEM from a minimum of 5 and maximum of 16 individual experiments. Media to lumen ratio *$p<0.05$ (vs SHR); pCa5.0 tension +$p<0.05$ (vs SHR) by Student's t-test for differences between strains in femoral arteries and ANOVA plus the LSD test for differences between strains in mesenteric and renal arteries.

3.6. Calcium sensitivity.

3.6a. 5 week animals.

There was no difference in calcium sensitivity between SHRs and WKYs in any vascular bed at 5 weeks (Figure 6.8).

3.6b. 20 week animals.

The pCa-tension curves were not different between the two strains in mesenteric (Figure 6.9a) and renal (Figure 6.9b) arteries. However, in femoral arteries there was a greater sensitivity to calcium in SHRs compared to WKYs (Figure 6.9c)(the pCa_{50} values for SHRs and WKYs were 6.35 and 6.07 respectively ($p<0.05$)).

3.6c. 20 week animals compared to 5 week animals.

There was no difference in calcium sensitivity between the two age groups in mesenteric and renal arteries (Figures 6.8 and 6.9).

3.6d. Comparison between vascular beds.

The tension developed to the sub-maximal (pCa6.7) calcium concentration was significantly greater in femoral arteries compared to mesenteric arteries in both strains at 5 and 20 weeks ($p<0.05$, Table 6.6). Renal arteries developed greater force to pCa6.7 compared to mesenteric arteries in the SHR at 20 weeks and in the WKY at both 5 and 20 weeks ($p<0.05$, Table 6.6).

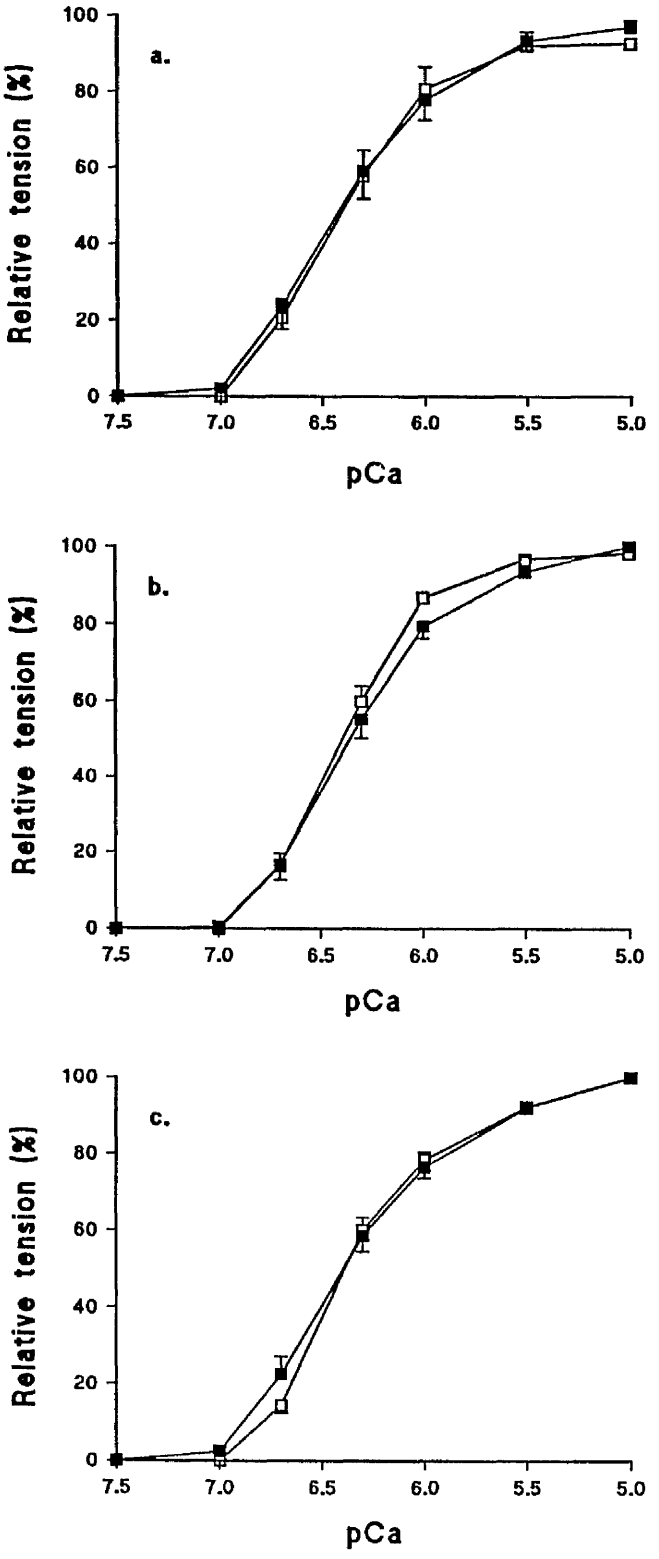


FIGURE 6.8 pCa-tension relationship in α-toxin permeabilised a. mesenteric b. renal and c. femoral small arteries from 5 week spontaneously hypertensive rats (SHRs, ■) and Wistar-kyoto rats (WKYs, □). Responses were normalised to tension achieved at pCa5.0. Mean±SEM for n=12 in renal and femoral arteries and n=16 in mesenteric arteries.

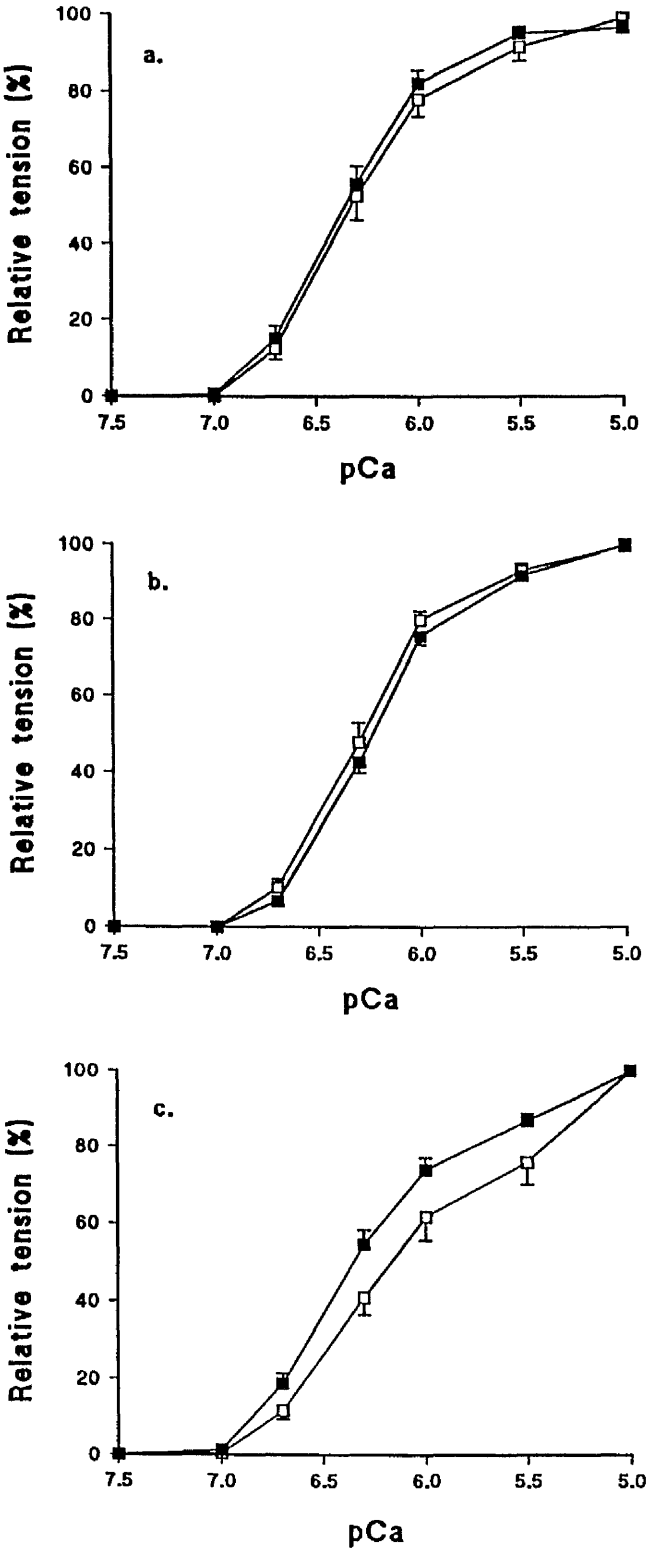


FIGURE 6.9 pCa-tension relationship in α-toxin permeabilised a. mesenteric b. renal and c. femoral small arteries from 20 week spontaneously hypertensive rats (SHRs, ■) and Wistar-kyoto rats (WKYs, □). Responses were normalised to tension achieved at pCa5.0. Mean±SEM for n=12 in renal and femoral arteries and n=16 in mesenteric arteries.

TABLE 6.6 Maximum tension (mN/mm) in response to pCa6.7 in α-toxin permeabilised small arteries from three vascular beds for 5 and 20 week spontaneously hypertensive rats (SHRs) and Wistar-kyoto rats (WKYs). Mean±SEM. Numbers of animals are given in parentheses. * $p<0.05$ (vs mesenteric arteries) by Student's t-test.

	Mesenterics	Renals	Femorals
5 weeks			
SHR	0.29±0.04(16)	0.66±0.15(12)	1.12±0.19(12)*
WKY	0.20±0.02(16)	0.46±0.08(12)*	0.78±0.09(12)*
20 weeks			
SHR	0.21±0.04(16)	0.66±0.09(12)*	0.76±0.18(12)*
WKY	0.15±0.03(16)	0.64±0.11(12)*	0.53±0.20(12)*

3.7. Agonist-induced calcium sensitisation.

An example of the calcium sensitisation response to GTP (10μM), ET-1 (100nM), NA (10μM) and PdBu (0.05μM) in a femoral artery from a 5 week WKY can be seen in Figure 6.1. It shows that GTP induced a small increase in tension to that developed to pCa6.7 followed by a larger increase in tension when ET-1 was added. NA did not significantly potentiate the ET-1-induced contraction in the permeabilised artery in contrast to PdBu which caused a large potentiation of the contraction.

3.7a. Mesenteric arteries.

The addition of ET-1 (100nM) and NA (10μM) in the presence of GTP (10μM) and PdBu (0.05μM) significantly potentiated the force developed to the sub-maximal (pCa6.7) calcium concentration in mesenteric arteries ($p<0.05$, Figure 6.10), with the exception of NA in the 20 week SHR (Figure 6.10b). There was no difference in the calcium sensitisation response to the receptor agonists or PdBu between SHRs and WKYs at either 5 or 20 weeks (Figure 6.10) or between the two age groups (Figure 6.10).

3.7b. Renal arteries.

ET-1 (100nM) and NA (10μM) in the presence of GTP (10μM) did not potentiate the sub-maximal calcium-induced contraction in renal arteries (Figure 6.11), with the exception of ET-1 in the 20 week WKY ($p<0.05$, Figure 6.11b). PdBu (0.05μM) did induce a significant increase in calcium sensitivity in the SHR at 20 weeks and in the WKY at both 5 and 20 weeks ($p<0.05$, Figure 6.11). The calcium sensitisation response to

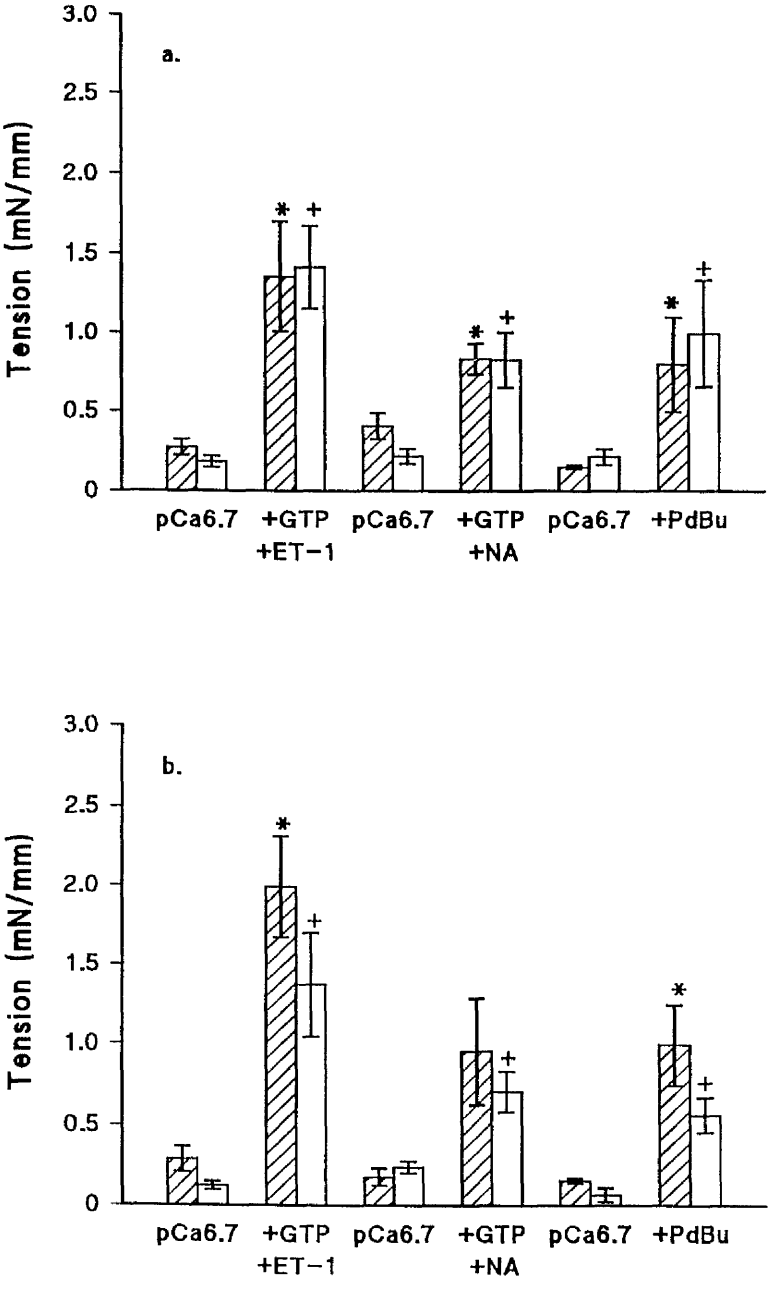


FIGURE 6.10 Bar graphs showing the calcium sensitisation response of α-toxin permeabilised mesenteric small arteries to ET-1 (100nM) and NA (10μM) in the presence of pCa6.7 + GTP (10μM) and PdBu (0.05μM) in the presence of pCa6.7 in spontaneously hypertensive rats (SHRs, hatched bars) and Wistar-kyoto rats (WKYs, open bars) at a. 5 and b. 20 weeks. Mean±SEM from a minimum of 4 individual experiments. *$p<0.05$ (vs pCa6.7 SHR); +$p<0.05$ (vs pCa6.7 WKY) by Student's t-test.

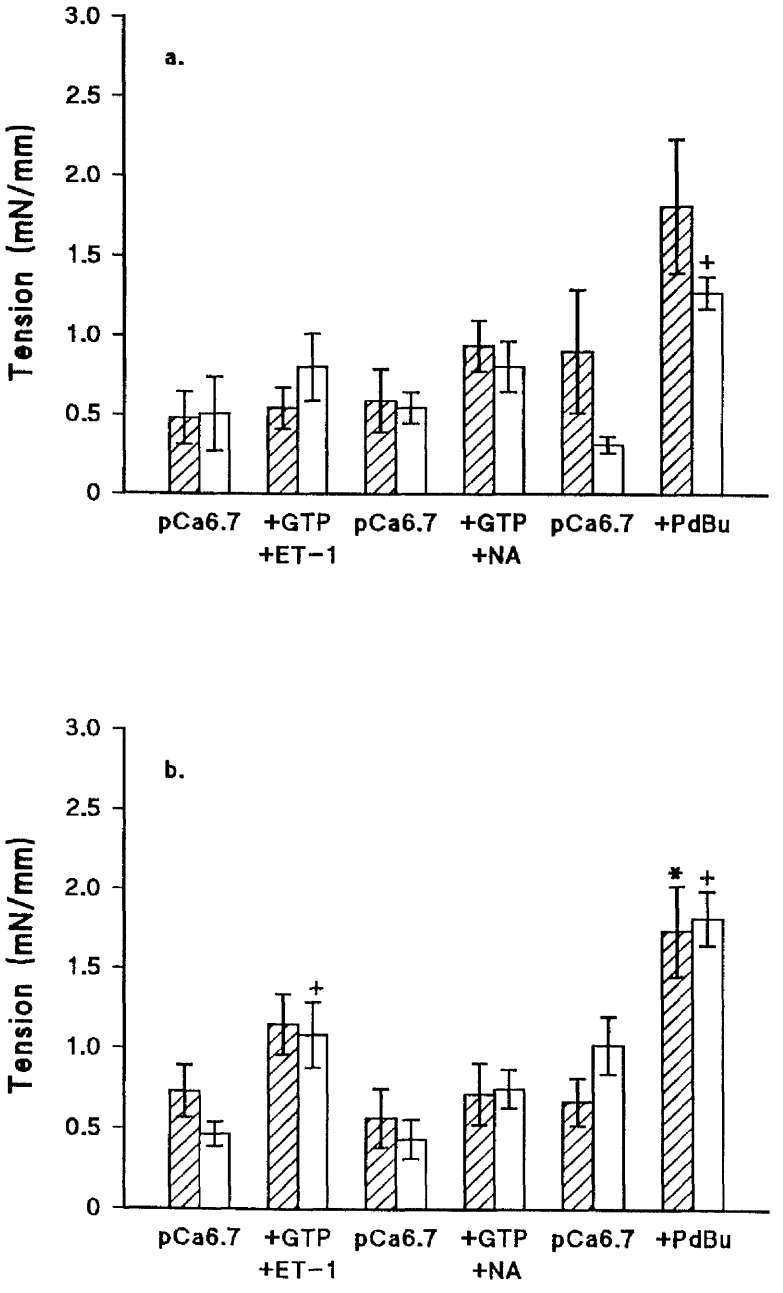


FIGURE 6.11 Bar graphs showing the calcium sensitisation response of α-toxin permeabilised renal small arteries to ET-1 (100nM) and NA (10μM) in the presence of pCa6.7 + GTP (10μM) and PdBu (0.05μM) in the presence of pCa6.7 in spontaneously hypertensive rats (SHRs, hatched bars) and Wistar-kyoto rats (WKYs, open bars) at a. 5 and b. 20 weeks. Mean±SEM from a minimum of 4 individual experiments. *p<0.05 (vs pCa6.7 SHR); +p<0.05 (vs pCa6.7 WKY) by Student's t-test.

the receptor agonists and PdBu was not different between the two strains at either 5 or 20 weeks (Figure 6.11) or between the two age groups (Figure 6.11) in renal arteries.

3.7c. Femoral arteries.

The addition of ET-1 (100nM) and NA (10μM) in the presence of GTP (10μM) or PdBu (0.05μM) did not significantly increase the pCa6.7-induced contraction in femoral arteries (Figure 6.12), with the exception of ET-1 in the 20 week SHR and PdBu in the 20 week WKY ($p<0.05$, Figure 6.12b). There was no difference in the calcium sensitisation response to the receptor agonists or PdBu between SHRs and WKYs at both ages (Figure 6.12) or between 5 and 20 week animals (Figure 6.12).

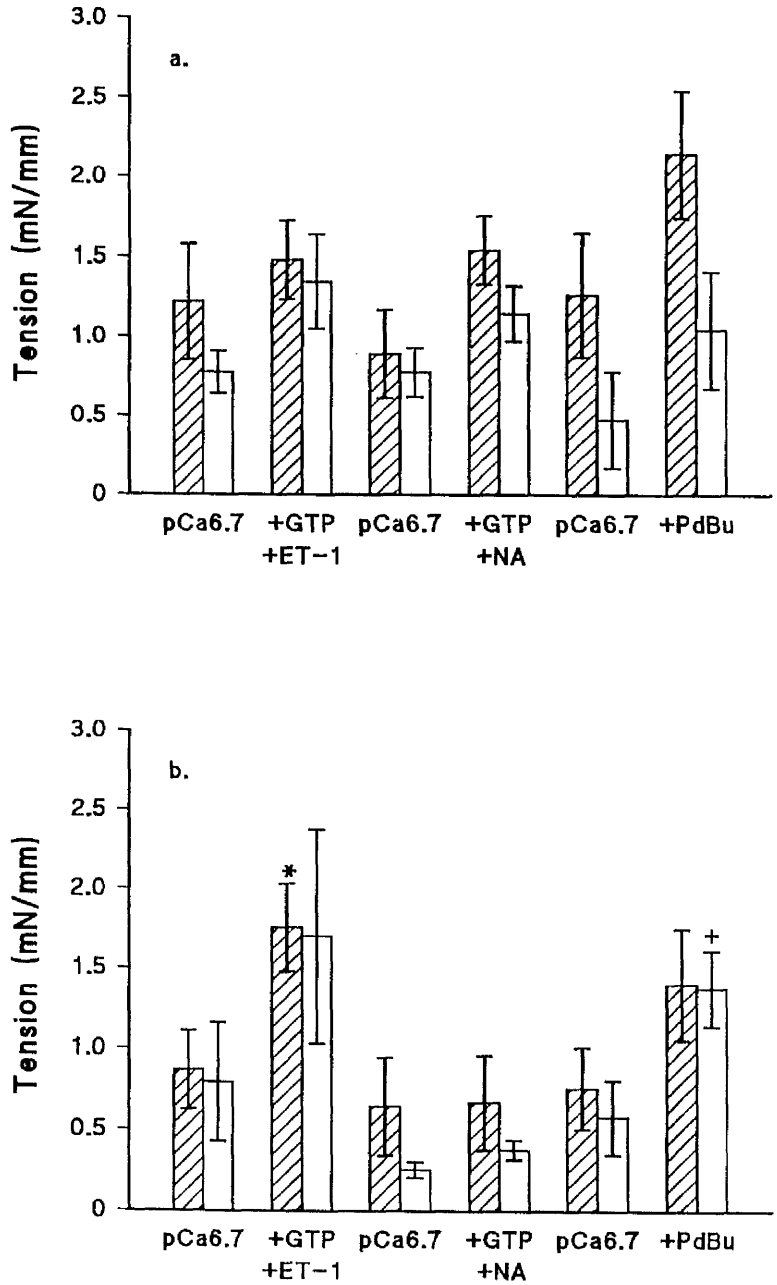


FIGURE 6.12 Bar graphs showing the calcium sensitisation response of α-toxin permeabilised femoral small arteries to ET-1 (100nM) and NA (10μM) in the presence of pCa6.7 + GTP (10μM) and PdBu (0.05μM) in the presence of pCa6.7 in spontaneously hypertensive rats (SHRs, hatched bars) and Wistar-kyoto rats (WKYs, open bars) at a. 5 and b. 20 weeks. Mean±SEM from a minimum of 4 individual experiments. *p<0.05 (vs pCa6.7 SHR); +p<0.05 (vs pCa6.7 WKY) by Student's t-test.

4. Discussion.

4.1. Morphology.

In this thesis mean blood pressure was elevated in SHRs compared to WKYs at five weeks and there were also morphological changes in femoral and mesenteric arteries at this age. Arteries from both vascular beds had an increased media to lumen ratio in hypertensive compared to normotensive animals, but only in femoral arteries was media cross-sectional area increased. This suggested that remodelling may have occurred in the mesenteric arteries whilst hyperplasia and/or hypertrophy may have occurred in femoral arteries. Therefore, the contribution of remodelling and hyperplasia and/or hypertrophy to the structural changes occurring in the vascular wall of small arteries in the SHR appears to vary with the vascular bed studied, confirming an earlier review by Heagerty et al (1993).

At 20 weeks the elevated mean blood pressure in the SHR compared to WKY was accompanied by an increased media to lumen ratio in mesenteric and femoral arteries confirming previous studies (Mulvany et al 1980, Mulvany et al 1982, Lee et al 1983, Bund et al 1991, Izzard et al 1996). This may have may been a result of hyperplasia and/or hypertrophy as media cross-sectional area was also increased in these vascular beds. However, it has been concluded from histological studies the increased media to lumen ratio in femoral (Bund et al 1991) and mesenteric (Lee et al 1983) arteries of the adult SHR was due to smooth muscle cell hyperplasia. In this thesis media to lumen ratio was not increased in renal arteries in the SHR compared to WKY at 20

weeks in contrast to previous investigations in which structural changes had occurred in renal arteries from the SHR at 4 and 21 weeks (Smeda et al 1988) and at 20 weeks (Shaw et al 1995) of age. The conflicting results between the studies cannot be attributed to differences in blood pressure as this was similar in all three studies, with the exception of Smeda et al (1988) who detected no elevation in blood pressure between SHRs and WKYs at 4 weeks. However, there was a difference in methodology as morphological parameters were measured in ring preparations mounted in a myograph in this study as opposed to Smeda et al (1988) where they were measured following fixing the vessels *in situ*. In this study there was an increase in renal artery media thickness at 20 weeks and a tendency for media to lumen ratio increases: +21% at 5 weeks and +36% at 20 weeks which suggested structural changes were occurring in this vascular bed although they did not reach statistical significance.

4.2. KES- and NA-induced contractions.

In small arteries an increase in active tension induced by vasoconstrictor agonists could be due to thickening of the media or an increased responsiveness of the smooth muscle cells. Since media stress takes into account changes in media thickness between different arteries it can be used to assess changes in vascular responsiveness. Although the maximum tension developed to KES was increased in the SHR compared to WKY in mesenteric and femoral arteries at 20 weeks there was no difference in media stress in response to KES between hypertensive and normotensive animals. The maximum tension developed to 10μM NA was increased in mesenteric and femoral

arteries at both 5 and 20 weeks in the SHR compared to WKY, but again there was no change in media stress. This has been previously observed in mesenteric (Mulvany et al 1978, Mulvany et al 1980) and femoral (Mulvany et al 1982, Bund et al 1991) arteries and suggested the vascular responsiveness to KES and NA was similar between the two strains in these vascular beds.

In the present investigation both the maximum tension and media stress in response to KES and 10μM NA was greater in 20 week animals compared to 5 week animals in mesenteric arteries, suggesting that older animals showed an enhanced reactivity to these agonists compared to younger animals in this vascular bed. Similarly, the active media stress in response to 10μM NA in mesenteric arteries increased from the age of 6 to 24 weeks in both SHRs and WKYs (Mulvany et al 1980). Furthermore, Tschudi and Lüscher (1995) found the active wall tension developed to 100mM KCl in coronary arteries was greater in WKYs at 72 weeks of age compared to WKYs at 12 weeks of age. Therefore, they postulated the intracellular excitation-contraction mechanism and/or the contractile machinery became more efficient with age in this strain (Tschudi and Lüscher 1995).

4.3. Calcium-induced contraction.

There was no difference in the maximum tension developed to pCa5.0 between SHRs and WKYs following permeabilisation except for mesenteric arteries at 20 weeks. However, when media thickness was taken into account the effect on the maximal calcium-induced tension was lost. There was a

tendency for a reduced media stress in the femoral vascular bed but this was not significant. The active media stress in response to a maximal calcium concentration was also not different between aldosterone-hypertensive rats (ADRs) and control rats (McMahon and Paul 1985) or SHRs and WKYs (Soloviev and Bershtein 1992). Therefore these results suggested the vascular responsiveness to calcium in permeabilised vascular smooth muscle was similar between hypertensive and normotensive animals.

It may have been expected that the response to calcium on its own in α-toxin permeabilised arteries would be similar to the response to KES in intact arteries as this was essentially the same mechanism to KES but without the membrane effects. Indeed, the vascular responsiveness to pCa5.0 was not increased in the SHR compared to WKY, but was increased in older animals compared to younger animals in mesenteric arteries, which was similar to the results for KES. The only difference was that an enhanced reactivity in 20 week animals compared to 5 week animals in mesenteric arteries was observed in both strains in response to KES, but only in WKYs in response to pCa5.0.

4.4. Calcium sensitivity.

There was no difference in myofilament calcium sensitivity between the two strains at 5 weeks despite an elevated blood pressure and increased media to lumen ratio in femoral and mesenteric arteries. This suggested that an increased calcium sensitivity is unlikely to be involved in the developing stage of hypertension in the SHR. Later, when hypertension

had become established, only femoral arteries showed an enhanced sensitivity to calcium in SHRs compared to WKYs.

The earliest studies on calcium sensitivity were performed in intact vascular smooth muscle in response to NA stimulation or potassium (K^+) depolarisation. Such studies yielded conflicting results depending on the agonist, tissue or type of animal used. A reduced sensitivity to calcium was reported in aortas in response to both NA and potassium chloride (KCl) in the SHR (Pederson et al 1978) and KCl alone in ADRs (McMahon and Paul 1985). Calcium sensitivity was also reduced in deoxycorticosterone-salt hypertensive rats in the presence of NA alone in large mesenteric arteries (Moreland et al 1982) and in SHRs in the presence of KCl alone in cerebral arteries (Winquist and Bohr 1983).

Investigations using mesenteric (Mulvany and Nyborg 1980, Boonen and De Mey 1990b, Bian and Bukoski 1995) and femoral (Mulvany et al 1982) small arteries have demonstrated an increased calcium sensitivity in adult SHRs when NA was used as the stimulant. In contrast, femoral arteries from 20 week stroke-prone spontaneously hypertensive rats (SHRSPs) showed no difference in myofilament calcium sensitivity in the presence of NA compared to WKY (Soltis and Bohr 1987). However, the interpretation of the studies investigating calcium sensitivity in intact arteries using NA to facilitate calcium entry is difficult as these agonists themselves can increase the sensitivity of the myofilaments to calcium. Indeed, when membrane depolarisation by K^+ was used to facilitate calcium entry no difference in calcium sensitivity

was observed between normotensive and hypertensive animals in intact small arteries from the mesenteric (Mulvany et al 1980, Boonen and De Mey 1990b, Bian and Bukoski 1995) or femoral (Soltis and Bohr 1987) vascular beds.

Investigations into differences in calcium sensitivity can only be accurately measured, however, in permeabilised preparations in which the ability of the plasmalemma and intracellular organelles to buffer cytoplasmic calcium has been removed. Therefore, the responses to increases in extracellular calcium reflected the calcium sensitivity of the contractile apparatus itself. However, such investigations are relatively few in number and have also produced different results. Soloviev and Bershtein (1992) found aortic and portal vein smooth muscle cells skinned with saponin showed an increased affinity to calcium in the SHR compared to WKY. Since the protein kinase C (PKC) inhibitor H-7 caused a marked rightward shift of the pCa-tension curve in the SHR it was concluded the increased calcium affinity may have resulted from an enhanced activity of PKC (Soloviev and Bershtein 1992).

However, earlier studies reported no difference in sensitivity of the myofilaments to calcium in SHRs compared to Dahl salt-resistant rats (Nghiem and Rapp 1983) and ADRs compared to control rats (McMahon and Paul 1985) in Triton X-100 skinned vascular smooth muscle. Furthermore, Mrwa et al (1985) demonstrated the calcium requirement for half-maximal activation, relaxation half-time and extent of myosin light chain (MLC) phosphorylation were not different between SHRSPs

and WKYs at 6-7 months in Triton X-100 skinned tail arteries. Therefore they concluded that changes at the level of the contractile proteins were not involved in the increased vascular tone of SHRSP essential hypertension (Mrwa et al 1985). More recently, the pCa-tension relationship was not altered in saponin-skinned spastic coronary arteries of miniature pigs which showed a hyperresponsiveness to histamine (Satoh et al 1990) or in ß-escin-skinned coronary arteries of the SHRSP which showed a hyperresponsiveness to GTPγS and serotonin (Satoh et al 1994a).

Therefore, the majority of previous studies using permeabilised arteries suggested that the calcium sensitivity of the myofilaments was not increased in hypertensive compared to normotensive animals. This is in agreement to the findings in this thesis where marked changes in myofilament calcium sensitivity did not occur in the vascular beds and size of artery which were studied either at the onset or in established hypertension in the SHR compared to WKY. As yet the calcium sensitivity in the same animals from two different age groups has not been investigated.

4.5. Agonist-induced calcium sensitisation.

The present investigation showed no difference in the maximum contractile response to maximal concentrations of ET-1 and NA or sub-maximal concentrations of calcium and PdBu in α-toxin permeabilised small arteries in SHRs compared to WKYs or older animals compared to younger animals in any of the vascular beds studied. This is an interesting observation as an increased sensitivity to NA (Mulvany et al 1980) and

decreased sensitivity to ET-1 (Dohi and Lüscher 1991) has been reported in intact mesenteric small arteries from the SHR compared to WKY. These studies suggested that the differences in reactivity observed in intact mesenteric arteries to NA and ET-1 in the SHR was not caused by an abnormality in agonist-induced calcium sensitisation but may have been due to an altered calcium handling which has been reported previously (Kwan and Daniel 1981). Indeed, the use of ion selective electrodes has demonstrated an increased level of $[Ca^{2+}]_i$ in freshly isolated aortic smooth muscle cells from the SHR compared to WKY (Losse et al 1984). This would be expected to result in an increased basal vascular tone in the SHR which has recently been demonstrated during the development of hypertension, but not after it had become established (Izzard et al 1996).

The results in this thesis are in contrast to a recent investigation in which ß-escin skinned coronary arteries showed an increased reactivity to serotonin and GTPγS in 12 week SHRSPs compared to WKYs (Satoh et al 1994a). The reactivity to serotonin was also increased in intact coronary arteries in the SHRSP compared to WKY which Satoh et al (1994a) concluded may in part have been due to the augmented calcium sensitisation response to serotonin in permeabilised arteries. The reason for the difference in the results from this study to those of our own is probably due to differences in the mechanisms coupling agonists to contraction in the smooth muscle cell and/or differences in vascular beds studied. However, Satoh et al (1994a) also found no difference in myofilament calcium sensitivity between

normotensive and hypertensive animals and consequently postulated the augmented agonist-induced calcium sensitisation was due to events upstream of the contractile machinery and its regulatory proteins: MLC kinase and MLC phosphatase (Satoh et al 1994a). These authors also reported a desensitisation of contraction in response to calcium in coronary arteries from the SHRSP compared to WKY (Satoh et al 1994a) implying these two mechanisms may be disturbed in hypertension and this may have been responsible for the increase in agonist-induced calcium sensitisation.

In general the NA- and ET-1-induced calcium sensitisation was poor in α-toxin permeabilised renal and femoral arteries compared to mesenteric arteries, which was surprising as intact renal and femoral arteries constricted to NA and ET-1 *in vitro* (Tomobe et al 1988, Bund et al 1991). This discrepancy may have been due to an inefficiency in α-toxin permeabilisation in the thicker-walled arteries due to a restricted diffusion of α-toxin. However, in this thesis the tension developed to the maximal calcium concentration showed a positive correlation with the mass of the arterial wall (see Figures 6.2, 6.4 and 6.6) so that the force developed by the thicker-walled femoral arteries was greater than that of the thinner-walled mesenteric arteries, implying that α-toxin had efficiently permeabilised the arteries from all vascular beds. The tension developed to sub-maximal calcium concentrations was greater in α-toxin permeabilised renal and femoral arteries compared to mesenteric arteries. These results suggested that the NA- and ET-1-induced contraction in these vessels was predominantly dependent on calcium

whereas in mesenteric arteries modulation of calcium sensitivity of the myofilaments by NA and ET-1 played a more substantial role. Therefore, there appears to be a heterogeneity in the contribution of pharmaco- and electro-mechanical coupling in response to contractile agonists between arteries from different vascular beds.

The mechanism underlying agonist-induced calcium sensitisation was investigated by adding the receptor agonists ET-1 and NA and the phorbol ester PdBu in succession and comparing their ability to increase the calcium sensitivity of the contractile apparatus. Since NA did not significantly increase the ET-1-induced potentiation of force in this study reinforced the earlier suggestion the two receptor agonists may have been acting via a common pathway. PdBu caused an increase in calcium sensitivity of the myofilaments additive to that induced by a maximal ET-1 concentration. Hori et al (1993) found down-regulation of PKC activity in α-toxin permeabilised rat aortic smooth muscle strips by pretreatment with the phorbol ester 12-0-tetradecanoylphorbol-13-acetate (TPA) for 24 hours blocked the 12-deoxyphorbol 13-isobutyrate (DPB)-induced potentiation of the calcium-induced contraction, but not the prostaglandin F2 alpha ($PGF_{2\alpha}$)-induced potentiation of the calcium-induced contraction. Both these findings suggested phorbol esters and receptor agonists were acting via different mechanisms to bring about calcium sensitisation. PdBu activates PKC therefore it was probably acting via a PKC dependent pathway to increase myofilament calcium sensitivity, whereas it has been previously demonstrated in Chapters Four and Five the

receptor agonist-induced increase in calcium sensitivity probably involved the activation of both PKC and tyrosine kinases. Alternatively, phorbol esters and receptor agonists could be acting via different PKC isotypes to induce contractions in permeabilised rat mesenteric small arteries, which has been previously postulated for the contractions in intact arteries in Chapter Four.

4.6. Summary.

This is one of the first studies investigating the calcium sensitivity of the contractile apparatus and calcium sensitisation induced by vasoconstrictor agonists at two stages of hypertension and in three different vascular beds. The major findings of the study were there were no marked differences in the calcium sensitivity or agonist-induced calcium sensitisation between SHRs and WKYs either at the onset or after hypertension had become established. This suggested these two mechanisms were not responsible for the hyperresponsiveness to contractile agonists which has been previously reported in intact vascular smooth muscle in hypertensive compared to normotensive and older compared to younger animals. In this thesis although the vascular responsiveness to NA and KCl in intact arteries plus calcium in permeabilised arteries was not enhanced in the SHR compared to WKY it was enhanced in 20 week animals compared to 5 week animals but only in the mesenteric vascular bed.

Therefore, there appeared to be a difference in the contractile response of intact small arteries to vasoconstrictor agonists between the three vascular beds,

which was also observed in α-toxin permeabilised arteries. Furthermore, the elevated blood pressure at 5 and 20 weeks in the SHR compared to WKY was associated with an increased media to lumen ratio but only in mesenteric and femoral arteries. These findings suggested there was a heterogeneity in the mechanisms regulating vascular tone between the different vascular beds which may underlie the unequal contribution of organ resistance to the elevated total peripheral resistance observed in various forms of chronic hypertension.

CHAPTER SEVEN

SUMMARY AND CONCLUSIONS

There is growing evidence from studies using permeabilised preparations that calcium sensitisation is an important component of the receptor agonist-induced contraction in vascular smooth muscle. Arachidonic acid (AA), protein kinase C (PKC) and more recently tyrosine kinases (TKs) have been implicated in calcium sensitisation, but such investigations have only concentrated on one pathway at a time. It has been suggested that AA inhibits myosin light chain (MLC) phosphatase leading to an increase in the levels of MLC phosphorylation and force in permeabilised arteries (Gong et al 1992). However, Moreland et al (1992a) found the MLC phosphorylation levels were only transient despite a monotonic increase in force in permeabilised arteries and consequently proposed PKC was additionally involved in the receptor agonist-induced calcium sensitisation. Steusloff et al (1993) and Toma et al (1995) used TK inhibitors (TKIs) in permeabilised vascular smooth muscle preparations to demonstrate that TKs played a role in the increased calcium sensitivity of the contractile apparatus in response to receptor agonists.

The data in this thesis demonstrated both PKC and TKs were involved in the receptor agonist-induced contraction in permeabilsed vascular smooth muscle which is in agreement with the above investigations. However, an additional mechanism also appeared to be responsible for this effect which may have been mediated by AA.

$3x10^{-7}$M PKC inhibitor RO-31-8330 completely blocked the contractile response to the phorbol ester phorbol 12,13-dibutyrate (PdBu) in rat mesenteric small arteries permeabilised with Staphylococcal aureus α-toxin and significantly reduced the maximum contractile response to endothelin-1 (ET-1) by approximately 30%. This is consistent with a partial role for PKC in the enhancement of myofilament calcium sensitivity elicited by ET-1. Identification of the specific PKC isotypes down-regulated by overnight incubation with PdBu implicated a role for one or more of the calcium-independent isotype(s)(specifically PKCε) in the PKC-mediated contractile response to ET-1. Furthermore, the data suggested that phorbol esters and receptor agonists utilised different mechanisms and/or PKC isotypes to bring about calcium sensitisation in vascular smooth muscle.

100μM active TKIs tyrphostin A23 and A47 completely relaxed the maximum ET-1 preconstriction in permeabilised rat mesenteric small arteries implying TKs played a substantial role in the ET-1-induced calcium sensitisation. Interestingly, 100μM A23 and A47 added simultaneously with a maximal ET-1 concentration only reduced the maximum development of tension by approximately 50%.

$3x10^{-7}$M RO-31-8330 together with 100μM A23 inhibited the ET-1-induced potentiation of the sub-maximal calcium-induced contraction in permeabilised arteries by approximately 65%. This suggested mechanisms involving PKC and TKs were predominantly responsible for the ET-1-induced increase in calcium sensitivity. However, the PKC inhibitor did not

significantly inhibit the contraction to ET-1 over and above that inhibited by the active TKI suggesting that phosphorylation of proteins by TKs was sufficient to induce calcium sensitisation in response to ET-1. The next step therefore would be to identify the specific protein substrates which become phosphorylated on tyrosine residues during the ET-1-mediated contraction in rat mesenteric small arteries plus the specific TKs responsible for this effect.

A residual contraction remained in the presence of both the PKC inhibitor RO-31-8330 and the active TKI tyrphostin A23 in α-toxin permeabilised rat mesenteric small arteries which may have been due to an AA-related mechanism. This could be proved by examining the effect of an inhibitor of phospholipase A_2 (PLA_2) such as mepacrine or quinacrine on the increase in calcium sensitivity elicited by ET-1 as PLA_2 has been reported to be predominantly responsible for the increase in AA production following ET-1 stimulation in cultured vascular smooth muscle cells (Resink et al 1989, Reynolds et al 1989).

Finally, the data in this thesis demonstrated that the reactivity to vasoconstrictor agonists was not enhanced in the SHR compared to the WKY in small arteries either during the developmental or established phases of hypertension which is in agreement with the study by Mulvany and Aalkjaer (1990). Furthermore, the data did not support a role for abnormalities in myofilament calcium sensitivity or vasoconstrictor agonist-induced calcium sensitisation as contributing factors to the hypertensive state of the SHR.

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APPENDIX

Composition of solutions for permeabilisation.

Normal external solution (NES).

	mM	g/L
NaCl	150	8.77
KCl	4	0.298
$CaMs_2$	2	20ml of 0.1M stock
$MgMs_2$	1	10ml of 0.1M stock
Hepes	5	1.192
Glucose	5.6	1

pH 7.4 with Tris-base at room temperature.

Potassium external solution (KES).

	mM	g/L
KMs	150	150ml of 1M stock
KCl	4	0.298
$CaMs_2$	2	20ml of 0.1M stock
Hepes	5	1.192
Glucose	5.6	1
$MgMs_2$	1	10ml of 0.1M stock

pH 7.4 with Tris-base at room temperature.

Relaxing solution (G_1).

	mM	g/L
PIPES	30	9.072
Na_2CP	10	2.551
Na_2ATP	5.16	2.844
$MgMs_2$	7.31	73.1ml of 0.1M stock
KMs	74.1	74.1ml of 1M stock
K_2EGTA	1	10ml of 0.1M stock

pH 7.1 with KOH at room temperature.

Calcium-activating solutions.

G_{10}	mM	g/L
PIPES	30	9.072
Na_2CP	10	2.551
Na_2ATP	5.16	2.844
$MgMs_2$	7.92	79.2ml of 0.1M stock
KMs	46.6	46.6ml of 1M stock
K_2EGTA	10	100ml of 0.1M stock

pH 7.1 with KOH at room temperature.

CaG	mM	g/L
PIPES	30	9.072
Na_2CP	10	2.551
Na_2ATP	5.16	2.844
$MgMs_2$	7.25	72.5ml of 0.1M stock
KMs	47.1	47.1ml of 1M stock
Ca_2EGTA	10	100ml of 0.1M stock

pH 7.1 with KOH at room temperature.

All solutions in the myograph were gassed with 100% O_2.

Ms = methanesulfonate.

Chemicals.

Calcium ionophore A23187	Sigma
Dimethyl sulfoxide (DMSO)	Sigma
Guanosine 5′-triphosphate (GTP)	Sigma
Noradrenaline (NA)	Sigma
Acetylcholine (ACh)	Sigma
Diphosphoglycerol (DPG)	Sigma
Vasopressin (AVP)	Sigma
Endothelin-1 (ET-1)	Sigma
Angiotensin II (AII)	Sigma
Phorbol 12,13-dibutyrate (PdBu)	Sigma
12-0-tetradecanoylphorbol-13-acetate (TPA)	Sigma
Arachidonic acid (AA)	Sigma
Ethanol	Sigma
L-α-lysophosphatidylcholine, palmitoyl (LysoPC)	Sigma
Phosphatidic acid (PA):	
Dipalmitoyl PA	Sigma
Dioleoyl PA	Sigma
Stearate arachidonate PA	Sigma
Chloroform	Sigma
4-α-phorbol 12,13-didecanoate (4αPdD)	Sigma
Prazosin	Sigma
PKC inhibitor RO-31-8330	Roche Products Ltd.
TKI tyrphostin A47, A23, A1	Calbiochem/Novachem