Role of the sarcoplasmic reticulum in nitric oxide-induced modulation of cytoplasmic calcium in rabbit aortic smooth muscle cells

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by

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ABSTRACT

Nitric oxide (NO) relaxes vascular smooth muscle in part by reducing cytoplasmic calcium concentration ($[Ca^{2+}]_i$). This has been proposed to be achieved by several mechanisms, including modulation of Ca^{2+} movements from the sarcoplasmic reticulum (SR) and activation of K^+ channels. This study investigated the roles of these mechanisms in the vasodilator effect of NO in rabbit aortic smooth muscle.

Caffeine and noradrenaline evoked $[Ca^{2+}]_i$ transients due to SR Ca^{2+} release. These were recorded with the whole-cell, patch-clamp technique or fura-2 fluorescence. The NO donor, sodium nitroprusside (SNP) did not affect voltage-activated K^+ currents, but enhanced caffeine-activated K^+ currents at 1 μM, while inhibiting them at 10 μM. SNP, glyceryl trinitrate and 8-bromo-cyclic guanosine monophosphate, at concentrations causing maximal vasodilation, had no consistent effect on global $[Ca^{2+}]_i$ or the rate of Ca^{2+} removal from the cell, which largely reflected Ca^{2+} sequestration by the SR Ca^{2+}-ATPase. The augmentation of caffeine-induced Ca^{2+} release by SNP reflected enhanced sequestration, but only in the vicinity of the plasma membrane. The reduction of the caffeine-activated $[Ca^{2+}]_i$ transient at higher concentrations appeared to reflect inhibition of SR Ca^{2+} release via the ryanodine receptor. Two complementary actions of SNP on SR Ca^{2+} handling, which would promote vasodilation, were therefore identified. The inhibitory effect on Ca^{2+} release may be mediated by guanosine 3':5'-cyclic monophosphate (cGMP), because it was blocked by the cytoplasmic guanylate cyclase

inhibitor, 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, and mimicked by a cGMP-specific (zaprinast), and a non-specific (3-isobutyl-1-methyl xanthine; IBMX) phosphodiesterase inhibitor. IBMX unexpectedly reversed the inhibitory effect of SNP, possibly by increasing levels of adenosine 3':5'-cyclic monophosphate, which could enhance SR Ca^{2+} release and uptake. Since maximal vasodilation is produced below 10 μM SNP, the role of reduced Ca^{2+} release is unclear. However, my results support enhanced SR Ca^{2+} sequestration as a mechanism of nitrovasodilator action in rabbit aorta.

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Abbreviations

AM ester	acetoxymethyl ester
cAMP	adenosine 3':5'-cyclic monophosphate
ATP	adenosine 5-trisphosphate
4-AP	4-aminopyridine
BSA	bovine serum albumin
8-Br-cGMP	8-bromo-cyclic guanosine monophosphate
Cl_{Ca}	calcium-activated chloride channel
Ca^{2+}-ATPase	calcium-adenosinetriphosphatase
CICR	calcium-induced calcium release
CIF	calcium influx factor
I_{CRAC}	calcium release activated calcium current
CTX	charybdotoxin
cADPR	cyclic adenosine diphosphate ribose
PKA	cyclic adenosine monophosphate dependent protein kinase
PKG	cyclic guanosine monophosphate dependent protein kinase
CPA	cyclopiazonic acid
cGC	cytosolic guanylate cyclase
K_v	delayed rectifier K^+ channel
DAG	diacyl glycerol
DMSO	dimethylsulfoxide
K_d	dissociation constant

DM	dissociation medium
DTT	dithiothreitol
EDHF	endothelium-derived hyperpolarising factor
EDRF	endothelium-derived relaxing factor
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ester) N, N, N', N',-tetraacetic acid
ECS	extracellular space
GTN	glyceryl trinitrate
cGMP	guanosine 3':5'-cyclic monophosphate
GTP	guanosine-5'-triphosphate
IP_3	inositol 1,4,5-trisphosphate
IICR	inositol 1,4,5-trisphosphate -induced calcium release
$[Ca^{2+}]_i$	intracellular calcium concentration
IBMX	3-isobutyl-1-methyl xanthine
BK_{Ca}	large conductance calcium-activated potassium channels
MB	methylene blue
SIN-1	3-morpholinosydnonimine
MLC_{20}	myosin light chain
MLCK	myosin light chain kinase
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethane-sulfonic acid]

NO	nitric oxide
NOS	nitric oxide synthase
NO^+	nitrosonium ion
NA	noradrenaline
ODQ	1*H*-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one
$OONO^-$	peroxinitrite
PE	phenylephrine
PIP_2	phosphatidylinositol 4,5-bisphosphate
PDE	phosphodiesterase
PLC	phospholipase C
PGI_2	prostacyclin
PKC	protein kinase C
$R_{340/380}$	ratio of fluorescence at 340 nm and 380 nm
ROC	receptor-operated channel
SR	sarcoplasmic reticulum
SMC	smooth muscle cell
SNAP	S-nitroso-*N*-acetylpenicillamine
SNP	sodium nitroprusside
STIC	spontaneous transient inward current
STOC	spontaneous transient outward current
$[Ca^{2+}]_s$	submembrane calcium concentration
SBB	superficial buffer barrier
I_{sus}	sustained current component
SOC	store-operated channel

TEA	tetraethylammonium chloride
TG	thapsigargin
I_t	transient current component
VSM	vascular smooth muscle
VSMC	vascular smooth muscle cell
VOC	voltage-operated channel

Chapter 1

Introduction

1.1 The endothelium and vascular tone

The endothelial cells lining the inside of the blood vessel wall release vasoactive factors that are obligatory for the regulation of vascular smooth muscle (VSM) tone. The best studied of these, the so called endothelium-derived relaxing factor (EDRF), was first demonstrated by Furchgott and Zawadzki (1980) as the relaxant substance released by the endothelial lining of the rabbit aorta. In 1987, the biological actions of EDRF were attributed to nitric oxide (NO) (Furchgott et al., 1987; Khan and Furchgott, 1987; Ignarro et al., 1987; Moncada et al., 1987; Palmer et al., 1987), the continuous release of which maintains a basal dilator tone (Griffith et al., 1984; Martin et al., 1985; Martin et al., 1986; Vanhoutte et al., 1986; Moore et al., 1990).

NO is a chemically unstable molecule with a half-life of between 3-50s (Fostermann et al., 1984; Griffith et al., 1984; Gryglewski et al., 1986; Ignarro et al., 1987), depending upon the experimental conditions and the species. It is synthesised upon demand from the amino acid precursor, L-arginine, via a Ca^{2+}/calmodulin-dependent enzyme, NO synthase (NOS) (Palmer et al., 1988; Moncada et al., 1991; Schini and Vanhoutte, 1993; Schulz and Triggle, 1994). The constitutive isoform of NOS requires multiple co-factors, including nicotinamide adenine dinucleotide phosphate, flavin adenine dinucleotide, flavin mononucleotide and tetrahydrobiopterin for full activity (Murad, 1994; Cosentino and Katusic, 1995). NO diffuses through the endothelium to the adjacent smooth muscle cells (SMCs) where it relaxes VSM through the haeme-dependent activation of smooth muscle cytosolic guanylate cyclase (cGC), responsible for the enzymatic conversion of guanosine-5'-triphosphate (GTP) to the intracellular second messenger guanosine 3':5'-cyclic

monophosphate (cGMP) (Arnold et al., 1977; Katsuki et al., 1977; Griffith et al 1985; Ignarro et al., 1986).

Endothelium-dependent vasorelaxation is considered to be produced by at least three endothelium-derived vasodilator factors. The role of prostacyclin (PGI_2) in mediating endothelium-dependent vasorelaxation is well-established (Dembinska-Kiee et al., 1980; Beetens et al., 1983; Lamontagne et al., 1992; Dong et al., 1998). A number of agonists that produce endothelium-dependent relaxation of VSM can also cause endothelium-dependent hyperpolarisation of the membrane, an action which is distinct from that of NO and PGI_2 (Chen et al., 1988; Huang et al., 1988; Garland and McPherson, 1992). The fact that acetylcholine-induced hyperpolarisation in various blood vessels remained even after combined inhibition of NO and PGI_2 synthesis led Taylor and Weston (1988) to propose that the endothelium releases a third relaxing factor, which hyperpolarises the membrane by opening K^+ channels. This was termed endothelium-derived hyperpolarising factor (EDHF). The exact chemical identity of this endothelium-derived factor remains obscure, although it may be an epoxyeicosatrienoic acid (Hu and Kim, 1993), K ions (Edwards et al., 1998) or a cannabinoid (Randall et al., 1996), though the latter is less likely (White & Hiley, 1997; Chataigneau et al., 1998). However, there is almost certainly a variation in sensitivity of SMCs to EDRF and EDHF in different blood vessels. NO appears to be the primary EDRF in mediating relaxation of large conducting vessels, like aorta, but EDHF may have a greater functional importance than NO as artery size decreases (Taylor and Weston, 1988; Garland et al., 1995). However, Tare et al.

(1990) and Bolotina et al. (1994) have reported relaxation and hyperpolarisation to NO in uterine and aortic arteries.

1.2 Regulation of vascular tone

The cytoplasmic calcium concentration ($[Ca^{2+}]_i$) is critical in regulating VSM tone. The elevation of $[Ca^{2+}]_i$ leads to the formation of the Ca^{2+}/calmodulin complex, which subsequently binds to the catalytic subunit of the inactive myosin light chain kinase (MLCK), the enzyme responsible for the phosphorylation of myosin. Ca^{2+}-dependent stimulation of MLCK phosphorylates serine at position 19 on the 20 kilodalton myosin light chain (MLC_{20}) (Hartshorne et al., 1992; Somlyo and Somlyo, 1994). This is followed by the actin and (phosphorylated) myosin interaction, which results in the initiation of contraction (Hartshorne et al., 1992; Gurney, 1994). The extent of crossbridge interaction between actin and myosin filaments determines the degree of contraction. Contrary to the belief that muscle contraction correlates well with $[Ca^{2+}]_i$, in the continued presence of an agonist, tension can be maintained despite a fall in $[Ca^{2+}]_i$ to a lower level than that required to initiate contraction (Karaki, 1989; van Breemen and Saida, 1989). Moreover, receptor agonists cause greater contraction than high K^+-depolarisation at a given $[Ca^{2+}]_i$ (Karaki, 1989; Hori et al., 1993). This suggests that agonist-induced contraction of VSM may be regulated not only by $[Ca^{2+}]_i$, but also by a Ca^{2+}-sensitising effect as the force: Ca^{2+} ratio increases during sustained tension. The mechanisms contributing to this Ca^{2+} sensitisation are not clear. Two pathways have been implicated, a protein kinase C (PKC)-dependent pathway selectively activated by phorbol esters (Nishimura and van Breemen, 1989; Hori et al., 1993) and a PKC-independent pathway activated by

receptor agonists (Hori et al., 1993). However, some receptor agonists may generate enough diacylglycerol (DAG) to activate PKC to a level similar to that produced by phorbol esters and in turn also activate the PKC-dependent pathway. An altered balance between MLCK and MLC phosphatase activity has also been suggested to sensitise the myofilaments to Ca^{2+} (Gong et al., 1992).

1.2.1 Mechanisms for elevating smooth muscle $[Ca^{2+}]_i$

In smooth muscle, control of $[Ca^{2+}]_i$ is accomplished by transport systems that transport Ca^{2+} between four pools: extracellular space (ECS), cytoplasm, sarcoplasmic reticulum (SR) and mitochondria. Within these pools, $[Ca^{2+}]_i$ is regulated by Ca^{2+} influx/release, Ca^{2+} buffering, and Ca^{2+} efflux/removal processes.

The sources of activator Ca^{2+} are both extracellular and intracellular. Ca^{2+} enters the cell from the ECS by Ca^{2+} permeable channels in the cell membrane. There are at least three Ca^{2+} entry channels; (1) voltage-operated channels (VOCs), (2) receptor-operated channels (ROCs) (Bolton, 1979; Cauvin et al., 1984) and store-operated channels (SOCs) (Wayman et al., 1996a; Wayman et al., 1996b).

On the basis of their physiological and pharmacological properties, six VOCs have been identified, although the dihydropyridine-sensitive or L-type channel is the best characterised and predominates in most vascular smooth muscle cells (VSMCs) (Clapp and Gurney, 1991a; Gurney and Clapp, 1994). These channels are activated by membrane depolarisation, although vasoactive agents may also influence the probability of channel opening in response to a given depolarisation (Gurney, 1994).

In electrically quiescent smooth muscle such as the aorta, the opening of VOCs occurs in response to membrane depolarisation, brought about by agonist-induced activation of ROCs (Cauvin et al., 1984; van Breemen and Saida, 1989; Gurney, 1994).

ROCs constitute an alternative route of Ca^{2+} entry from the ECS to elevate $[Ca^{2+}]_i$ upon receptor activation. Evidence for the existence of these channels came from experiments where it was noted that agonists could induce contractions in fully depolarised smooth muscle and also that several agonists stimulated $^{45}Ca^{2+}$ influx into SMCs, an effect which was not blocked by dihydropyridine calcium antagonists (Bolton, 1979; van Breemen and Saida, 1989), which selectively block L-type VOCs. Thus, ROCs are opened by agonists such as noradrenaline (NA), binding to specific membrane receptors (Byrne and Large, 1988). Some agonists, such as adenosine 5-trisphosphate (ATP) acting on P2X receptors, directly open a channel (Benham and Tsien, 1987), whereas others may open ROCs indirectly, via a second messenger (van Breemen and Saida, 1989; Lepretre et al., 1994). ROCs are proposed to contribute more than VOCs to Ca^{2+} influx in large conduit arteries, because Ca^{2+} antagonists produce limited dilation of these arteries compared with smaller arteries (Cauvin et al., 1984).

In many cell types receptor-mediated Ca^{2+} release from internal stores is followed by Ca^{2+} influx across the plasma membrane, thought to result partly from the depletion of the intracellular store. At present, very little is known about the signal mechanism relating store depletion to activation of membrane Ca^{2+} influx, first proposed by

Putney (1986) in his capacitative model for receptor-regulated Ca^{2+} entry and subsequently observed by others in a variety of cell types (Takemura and Putney, 1989; Putney and Bird, 1993; Trafford et al., 1997), including smooth muscle (Wayman et al., 1996a; Gibson et al., 1998). In this capacitative model, depletion of the SR signals the direct entry of Ca^{2+} across the cell membrane (via SOCs) to refill the store. When the SR is full, entry is prevented.

The first demonstration that depletion of the SR leads to the activation of a Ca^{2+} channel current arose following experiments in rat peritoneal mast cells (Hoth and Penner, 1992). This highly selective Ca^{2+} current was designated the calcium release activated calcium current (I_{CRAC}) to distinguish it from other Ca^{2+} currents. More recently, a non-selective cation current has been suggested to underlie capacitative Ca^{2+} entry in rabbit aorta and mouse anococcygeus smooth muscle (Bolotina et al., 1995; Wayman et al., 1996a). Clues concerning the identity of the signal for capacitative Ca^{2+} entry have suggested that it is activated by an unidentified diffusible messenger, produced or released when the SR is depleted (Parekh et al., 1993). The negatively charged messenger was coined Ca^{2+} influx factor (CIF) and is thought to be a small, non-protein, phosphate-containing factor, the activity of which is lost when phosphate is removed (Randriamampita and Tsien, 1993). Phosphatase, the activity of which is controlled by the Ca^{2+} store content, appears to modulate the ability of the signal from the empty store to evoke influx, such that inhibition of phosphatase enhances Ca^{2+} influx (Randriamampita and Tsien, 1995). In addition to CIF, evidence has been presented for at a GTP-dependent step in capacitative Ca^{2+}

entry (Fasolato et al., 1993) and GTP may well be the diffusible messenger suggested by Parekh et al. (1993).

The SR is the intracellular source of Ca^{2+} to initiate contraction following agonist stimulation. Ca^{2+} may be released through either of two separate receptor/channels situated on the SR membrane. These are referred to as the inositol 1,4,5-trisphosphate (IP_3)-receptor channel and the ryanodine-receptor channel (Berridge, 1993; Ehrlich et al., 1994). IP_3 is formed in the cytosol in response to cell-surface receptor stimulation, upon which phospholipase C (PLC) is activated. PLC then catalyses the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2), releasing DAG and IP_3 (Berridge and Irvine, 1989; Berridge, 1993; Putney, 1993). Subsequently the interaction of IP_3 with the IP_3 receptor allows the release of Ca^{2+} into the cytosol, through a directly coupled Ca^{2+} selective channel (Somlyo et al., 1985; Ehrilch and Watras, 1988; Chadwick et al., 1990; Putney, 1993). Ca^{2+} may also be released through the ryanodine-receptor channel by Ca^{2+} that has entered the cell and binds to a site on the channel (Rousseau et al., 1987; Berridge, 1993; Putney, 1993; Sitsapesan and Williams, 1994), contributing to a further rise in $[Ca^{2+}]_i$. This receptor/channel can also be activated by caffeine, which facilitates the activation of the channel by Ca^{2+}, called the Ca^{2+}-induced Ca^{2+} release (CICR) mechanism (Endo, 1975; Leijten and van Breemen, 1984; Iino, 1989). Furthermore, ryanodine, which gives the receptor its name, induces a slow release of Ca^{2+} (Hwang and van Breemen, 1987; Sakai et al., 1988). It locks the channel in a reduced-conductance, high open probability state (Rousseau et al., 1987). At concentrations in excess of 10 μM, ryanodine can also cause inhibition of the channel (Meissner, 1986).

1.2.2 Mechanisms for removing cytoplasmic Ca^{2+}

A fall in $[Ca^{2+}]_i$ below a threshold level inactivates MLCK, permitting the dephosphorylation of MLC_{20} by MLC phosphatase, thus, deactivating actomyosin ATPase and causing relaxation (Somlyo and Somlyo, 1994). The removal of Ca^{2+} from the cytosol is achieved by a combination of Ca^{2+} regulating pathways, which sequester Ca^{2+} into intracellular stores or extrude it across the plasma membrane. These are: a Ca^{2+} pump in the SR, a Ca^{2+} pump in the plasma membrane, a Na^+/ Ca^{2+} exchanger in the plasma membrane, and a Ca^{2+} uniporter in the mitochondrial inner membrane. However, it is believed that the major route of Ca^{2+} removal in smooth muscle is the active uptake of Ca^{2+} into the SR (Kargacin and Fay, 1991; Ganitkevich and Isenberg, 1992) via a specific Ca^{2+}-adenosinetriphosphatase (ATPase). This ATPase is a very abundant protein, representing up to 90% of the total protein of the SR (Fleischer and Inui, 1989). It pumps Ca^{2+} in an ATP-dependent reaction, with 2 Ca^{2+} accumulated per molecule of ATP hydrolysed (Fleischer and Inui, 1989). The activity of the ATPase is regulated by phospholamban (Raeymaekers et al., 1988; Cornwell et al., 1991; Lincoln and Cornwell, 1993), a small inhibitory protein richly distributed throughout the membrane of the SR (Ferguson et al., 1988), which interacts with the Ca^{2+}-ATPase to inhibit Ca^{2+} transport activity. When phosphorylated phospholamban dissociates from the ATPase, leading to increased ATP hydrolysis and thus enhanced Ca^{2+} sequestration.

Two mechanisms mediate Ca^{2+} extrusion across the plasma membrane in many types of cells: an ATP-driven Ca^{2+} pump (Kamashima and McCarron, 1998) and Na^+/Ca^{2+}

exchange (Ashida and Blaustein, 1987). Ejection of Ca^{2+} across the plasma membrane is an active process mediated by the ATP-dependent Ca^{2+} extrusion pump (Suematsu et al., 1984; Wuytack et al., 1984), present in smooth muscle preparations (Wuytack et al., 1984; Popescu et al., 1985a; Popescu et al., 1985b; Vrolix et al., 1988; Magliola and Jones, 1990). The functional significance of this ATPase in Ca^{2+} removal was demonstrated in cerebral resistance artery cells (Kamashima and McCarron, 1998). It is a high affinity regulator of $[Ca^{2+}]_i$ (Popescu et al., 1985b) and can thus be considered as a fine tuner of $[Ca^{2+}]_i$. It co-operates with another membrane system involved in the regulation of $[Ca^{2+}]_i$, the specific Na^+/Ca^{2+} exchange mechanism.

The Na^+/Ca^{2+} exchanger is an ion exchange system that uses the inwardly directed transmembrane Na^+ gradient to extrude Ca^{2+} from the cell with a stoichiometry of 3 Na^+ to 1 Ca^{2+}. The Na^+/Ca^{2+} exchange mechanism can transport Ca^{2+} in either direction across the plasma membrane, depending on the prevailing electrochemical gradient for Na^+ (Leblanc and Hume, 1990). Although arguments have been advanced supporting the physiological significance of this Na^+/Ca^{2+} exchange in the plasma membrane (Bolton, 1979; Ashida and Blaustein, 1987; Bassani et al., 1992), its importance is not universally accepted (Baro and Eisner, 1995; Kamishima and McCarron, 1998). The co-distribution of the Na^+/K^+ pump and Na^+/Ca^{2+} exchanger in the plasma membrane of *Bufo marinus* stomach SMCs (Moore et al., 1993) suggested a strong functional linkage between the activities of these two ion-regulating systems. The Na^+/K^+ pump influences the activity of the Na^+/Ca^{2+} exchanger by maintaining a Na^+ gradient to drive exchanger activity. Arrest of the

Na^+/K^+ pump results in intracellular Na^+ accumulation, which suspends the activity of the Na^+/Ca^{2+} exchanger, thus reducing Ca^{2+} extrusion from the cell.

It was more recently shown that mitochondria could contribute to the lowering of $[Ca^{2+}]_i$ in smooth muscle, by accumulating Ca^{2+} via the Ca^{2+} uniporter in the inner mitochondrial membrane (Drummond and Fay, 1996). This mechanism has now been shown to contribute to the removal of Ca^{2+} from the cytosol of rabbit aorta smooth muscle (Gurney et al., 2000). Ca^{2+} accumulation via the uniporter is driven by the large negative potential across the inner membrane (Gunter et al., 1994; Drummond and Fay, 1996) while Ca^{2+} export from mitochondria occurs through both Na^+-dependent and -independent efflux mechanisms (Gunter et al., 1994). The high rate of uptake enables mitochondria to play a role in buffering Ca^{2+}, along with the other Ca^{2+} removal mechanisms. A very high degree of association between mitochondria and SR has been observed by several investigators (Hoth et al., 1997; Rizzuto et al., 1998; Drummond and Tuft, 1999; McCarron and Muir, 1999; Gurney et al., 2000). During measurements of capacitative Ca^{2+} signalling, Ca^{2+} influx was impaired by agents that prevent mitochondrial Ca^{2+} accumulation by dissipating the mitochondrial membrane potential (Hoth et al., 1997). This led to the proposal that mitochondrial Ca^{2+} buffering maintains a low subplasmalemmal $[Ca^{2+}]$ ($[Ca^{2+}]_s$), enabling greater store-operated Ca^{2+} influx and SR refilling (Hoth et al., 1997). Additional studies have revealed a close functional exchange of SR Ca^{2+} release and mitochondrial Ca^{2+} uptake in a variety of cells (Rizzuto et al., 1998; Drummond and Tuft, 1999; McCarron and Muir, 1999; Gurney et al., 2000). The close apposition of mitochondria and SR (Nixon et al., 1994; Rizzuto et al., 1998) results in the exposure

of mitochondria to high local [Ca^{2+}] near the SR Ca^{2+} release sites. Thus, Ca^{2+} uptake by the mitochondria may regulate local [Ca^{2+}] in the vicinity of the SR release channels and limit the concentration of Ca^{2+} exposed to the contractile proteins.

1.3 Superficial buffer barrier

Smooth muscle SR discharges Ca^{2+} during the initial phase of contraction and sequesters Ca^{2+} during relaxation. In addition to these functions it has been proposed that the peripheral SR also acts as a superficial buffer barrier (SBB) to direct Ca^{2+} entry from the ECS into the cytoplasm, before being extruded to raise $[Ca^{2+}]_i$ in the vicinity of the contractile proteins and activate contraction (van Breemen, 1977). This was derived from the observation that aortic smooth muscle contraction, in response to elevated extracellular K^+ concentration, depends to a greater extent on the rate of Ca^{2+} influx than on the amount of Ca^{2+} entering the cell. Thus, when the rate of Ca^{2+} influx was relatively slow i.e. at relatively low concentrations of extracellular K^+, the SR was able to remove a larger proportion of the entering Ca^{2+}, as evidenced by lower force development than produced by the same amount of Ca^{2+} entering at high extracellular K^+ concentrations. This was explained by the presence of a high capacity Ca^{2+} buffering system near the site of Ca^{2+} entry, which competes with myofilament activation sites for Ca^{2+} as it enters through the plasma membrane (van Breemen et al., 1995). Further support for a close functional relationship between the plasma membrane and SR was provided by several observations. K^+ depolarisation-induced contraction was delayed when the SR was depleted prior to stimulation of Ca^{2+} influx (Casteels and Droogmans, 1981). Conversely, increasing the SR content to near physiological levels diminished the buffering capacity of the

SR and so the Ca^{2+} influx threshold for initiation of contraction was reduced (van Breemen et al., 1988). Similar observations regarding SR Ca^{2+} buffering were reported in bovine coronary arterial SMCs, using fura-2 to directly measure $[Ca^{2+}]_i$ (Sturek et al., 1992). When the buffering capacity of the SR was increased by partially depleting the SR, the subsequent increase in $[Ca^{2+}]_i$ was slowed. This was because Ca^{2+} entering the cell was sequestered by the SR, as evidenced by the subsequent caffeine-induced $[Ca^{2+}]_i$ transient. Equally, inhibition of Ca^{2+} buffering resulted in a greater and more rapid increase in $[Ca^{2+}]_i$ (Sturek et al., 1992).

A critical component of this superficial buffering is the existence of an outwardly directed Ca^{2+} gradient beneath the plasma membrane surface (van Breemen and Saida, 1989), which appears to depend on the close proximity of the SR and plasma membrane. Experimental evidence for this Ca^{2+} gradient beneath the plasma membrane has been provided by several reports of large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels in the membrane, which were activated without contractile activity or increases in $[Ca^{2+}]_i$ being apparent (Benham and Bolton, 1986; Benham et al., 1986; Stehno-Bittel and Sturek, 1992). This can be explained by the spontaneous opening of ryanodine channels, which temporarily raises the $[Ca^{2+}]_i$ near the plasma membrane. Such localised transient elevations in smooth muscle $[Ca^{2+}]_i$ have been recorded and labelled as Ca^{2+} sparks (Nelson et al., 1995). Clear evidence for an SR-modulated Ca^{2+} gradient below the plasma membrane has also been shown by others simultaneously measuring force development and fura-2 fluorescence (Chen et al., 1992). Depletion of the SR prior to Ca^{2+} entry dissociated the $[Ca^{2+}]_i$ from

contraction, indicating that the $[Ca^{2+}]_i$ increased in the subplasmalemmal region before stimulating contraction.

If the SR is to function as a maintained buffer barrier, a selective discharge of Ca^{2+} towards the ECS (Leijten and van Breemen, 1986; Stehno-Bittel and Sturek, 1992) is required to prevent saturation and thus cease its function. The Na^+/Ca^{2+} exchanger and the Na^+/K^+ pump are largely co-distributed with the SR in smooth muscle (Moore et al., 1993) and this may have important functional implications for Ca^{2+} regulation. Because of the close anatomical proximity of the Na^+/Ca^{2+} exchanger in the plasma membrane, located within 50-100 nm from the SR (Devine et al., 1972), it may have preferential access to Ca^{2+} as it is released from the SR. $[Ca^{2+}]$ may vary significantly in this restricted 'fuzzy space', between the SR and plasma membrane (Lederer et al., 1990), from the average $[Ca^{2+}]_i$. Therefore, the Na^+/Ca^{2+} exchanger, exposed to a high $[Ca^{2+}]$ as it is released from the SR, could remove Ca^{2+} from the cell, despite its apparent low affinity for Ca^{2+}.

1.4 Sarcoplasmic reticulum structure

The SR clearly plays an important role in the regulation of VSM contraction. Based on electron microscopy and staining evidence, the ultrastructure of smooth muscle SR has been suggested to consist of an electron-dense, smooth-surfaced continuous network of membrane tubules (Devine et al., 1972; Bond et al., 1984), of which a considerable fraction in aorta is located centrally (Devine et al., 1972; Nixon et al., 1994). The peripheral SR is in close proximity to the plasma membrane (Bond et al., 1984), separated by an 8-10 nm gap crossed by 'bridging structures' (Devine et al.,

1972). However, the major store of Ca^{2+} in the aorta has been shown to be the central SR, which forms a continuous system connected to the peripheral SR (Nixon et al., 1994). Perhaps the central SR is mainly concerned with regulating $[Ca^{2+}]_i$ in the vicinity of the contractile proteins whereas the peripheral SR is more concerned with regulating the content of the SR (both central and peripheral), through close interactions with the plasma membrane and Ca^{2+} in the subplasmalemmal space. The $[Ca^{2+}]$ in smooth muscle SR is estimated to be ~ 5 mM (Leijten and van Breemen, 1984). However, the Ca^{2+} storage capacity of the SR in aorta is enhanced by the Ca^{2+}-binding protein calreticulin (Nixon et al., 1994).

1.5 Actions of nitric oxide and nitrovasodilators on Ca^{2+} regulation processes

The precise mechanisms responsible for the vasodilation produced by NO remain uncertain. It has been proposed to influence the regulation of $[Ca^{2+}]_i$ (Kobayashi et al., 1985; Karaki et al., 1988; McDaniel et al., 1992; Bolotina et al., 1994), although the decrease in $[Ca^{2+}]_i$ is much smaller than that in muscle contraction (Karaki et al., 1988; Yanagisawa et al., 1989; Gibson et al., 1994; Bolz et al., 1999). NO thus appears to have dual effects on $[Ca^{2+}]_i$ and Ca^{2+} desensitisation of the contractile proteins. MLC phosphatase has been demonstrated to be subject to activation by cGMP, which is increased by NO in VSM (Wu et al., 1996; Lee et al., 1997) and may account for the NO-induced Ca^{2+} desensitisation of contractile force in VSM.

Most of the mechanisms suggested to explain NO action centre on the lowering of $[Ca^{2+}]_i$ or the prevention of $[Ca^{2+}]_i$ from rising in response to a contractile stimulus (Kobayashi et al., 1985; Kai et al., 1987; Rashatwar et al., 1987; Karaki et al., 1988;

Cornwell and Lincoln, 1989; McDaniel et al., 1992; Bolotina et al., 1994; Andriantsitohaina et al., 1995). There is however conflicting data to support a variety of mechanisms that result in a decrease in $[Ca^{2+}]_i$.

1.5.1 K^+ channel activation

NO and its donors can activate K^+ channels in a variety of preparations (Thornbury et al., 1991; Robertson et al., 1993; Archer et al., 1994; Yuan et al., 1996; Khan et al., 1998; Lang and Watson, 1998; Mistry and Garland, 1998), including rabbit aorta (Bolotina et al., 1994). The resulting hyperpolarisation of the membrane, which subsequently inhibits opening of VOCs, thereby preventing evoked increases in $[Ca^{2+}]_i$, is apparently one of the mechanisms by which NO may induce relaxation. NO directly activated charybdotoxin (CTX)-sensitive BK_{Ca} channels in excised membrane patches of the rabbit aorta independently of cGMP (Bolotina et al., 1994). In contrast, Archer et al. (1994) reported that NO activation of BK_{Ca} channels in pulmonary arterial SMCs is mediated by cGMP. NO and sodium nitroprusside (SNP) have also been shown to activate a K^+ current in isolated SMCs and excised membrane patches of rat pulmonary artery and relax isolated pulmonary arteries (Archer et al., 1994; Yuan et al., 1996). Both K^+ channel activation and relaxation were blocked by 4-aminopyridine (4-AP) indicating that NO and SNP activated a delayed rectifier (K_v) channel (Archer et al., 1994; Yuan et al., 1996). However, incomplete reversal of NO-induced relaxation by 4-AP (Yuan et al., 1996) implied that the activation of K_v channels was not the primary mechanism responsible for NO-induced relaxation.

1.5.2 Reduced Ca^{2+} influx

The effect of NO on Ca^{2+} influx has been studied by monitoring $^{45}Ca^{2+}$ uptake into aortic smooth muscle segments. EDRF and nitrovasodilators were shown to inhibit NA-stimulated Ca^{2+} influx (Collins et al., 1986; Karaki et al., 1988; Lewis et al., 1988; Magliola and Jones, 1990) and K^+-stimulated Ca^{2+} influx (Karaki et al., 1988; Magliola and Jones, 1990) thereby reducing the intracellular $[Ca^{2+}]_i$ available for contraction. In accordance with these observations electrophysiological studies demonstrated that SNP reversibly decreased Ca^{2+} influx through dihydropyridine-sensitive VOCs of pulmonary arterial SMCs (Clapp and Gurney, 1991a). However, SNP can fully relax the pulmonary artery when pre-contracted with NA although voltage-operated Ca^{2+} influx only contributes ~ 20 % to the contraction (Clapp and Gurney, 1991a). Therefore inhibition of VOCs is unlikely to be the principal mechanism by which SNP relaxes VSM. This view is supported by Karaki et al. (1988) and Magliola and Jones (1990), who found that NA-stimulated Ca^{2+} influx was more potently inhibited by SNP than K^+-stimulated Ca^{2+} influx. Additional mechanisms are therefore necessary to fully explain the relaxant actions of NO and nitrovasodilators.

1.5.3 Enhanced Ca^{2+} extrusion

Glyceryl trinitrate (GTN) reduced $[Ca^{2+}]_i$ in both the presence and absence of extracellular Ca^{2+} in cultured VSMCs (Kobayashi et al., 1985). In addition, the finding that GTN did not affect the transient increase in $[Ca^{2+}]_i$ induced by the initial application of caffeine but markedly reduced subsequent increases in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} indicated that it may accelerate the extrusion of Ca^{2+}

through the plasma membrane (Kobayashi et al., 1985). These observations are consistent with the finding that GTN stimulated the ATP-dependent plasma membrane Ca^{2+}-ATPase isolated from coronary artery SMCs (Popescu et al., 1985a). Evidence in favour of stimulation by cGMP-dependent protein kinase (PKG) of the plasma membrane Ca^{2+}-ATPase of smooth muscle has been presented (Vrolix et al., 1988). This has been suggested to occur indirectly by increasing PI phosphate via the phosphorylation of an associated PI kinase (Vrolix et al., 1988). SNP enhanced $^{45}Ca^{2+}$ efflux from aortic smooth muscle presumably be enhancing the activity of a plasma membrane Ca^{2+}-ATPase (Magliola and Jones, 1990). Aortae contracted by NA were more sensitive to inhibition by SNP than high K^+ contracted tissues (Magliola and Jones, 1990). The differential sensitivity to SNP may reflect the inability of Ca^{2+} extrusion mechanisms to overcome the greater Ca^{2+} influx associated with a high K^+ contraction.

1.5.4 Inhibition of a Ca^{2+} store-depletion activated current

A non-selective cation current activated in response to intracellular Ca^{2+} store depletion (Wayman et al., 1996a) was inhibited by SNP in SMCs of the mouse anococcygeus (Wayman et al., 1996b) at concentrations similar to those which relax the whole muscle (Gibson et al., 1994). This action of SNP was apparent regardless of the agent used to deplete the store, and appeared to be mediated by cGMP, being mimicked by the membrane permeant analogue of cGMP, 8-bromo cyclic guanosine monophosphate (8-Br-cGMP), and blocked by 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), a selective inhibitor of cGC (Garthwaite et al., 1995). It was therefore proposed that this channel may be a potential target for the relaxant

effects of nitrates in non-VSM. Studies in aortic SMCs also suggested that NO through a cGMP pathway inhibited a Ca^{2+} permeable non-selective cation current activated by intracellular Ca^{2+} store depletion (Minowa et al., 1997). It is not known whether NO and nitrovasodilators interact directly with the ion channel or act to refill the depleted store so as to remove the stimulus for channel opening. However, SNP was unable to inhibit the non-selective cation current under conditions where the store could not be refilled i.e. in zero intracellular and extracellular Ca^{2+} (Wayman et al., 1996b).

1.5.5 Modulation of sarcoplasmic reticulum Ca^{2+} mobilisation

Two proposals concerning the mechanism of action of NO have gained considerable attention, which indicate that the major site of action of NO and nitrovasodilators may be intracellular rather than at the level of the membrane. One implicates that nitrovasodilators may modulate SR Ca^{2+} store function by inhibiting Ca^{2+} release from both caffeine- and IP_3-sensitive Ca^{2+} stores. This has been proposed to be achieved by blocking the ryanodine-receptor Ca^{2+} release channel (Meszaros et al., 1996; Aghdasi et al., 1997; Kannan et al., 1997) or by inhibiting PLC and the production of IP_3 (Rapoport et al., 1983; Cheung and MacKay, 1985; Lang and Lewis, 1989; Hirata et al., 1990) or its action. Despite the general acceptance that NO inhibits Ca^{2+} release from IP_3-sensitive stores in various cell systems, leading to a decrease in $[Ca^{2+}]_i$, studies examining the effects of NO on Ca^{2+} release through ryanodine receptors have been inconsistent (Publicover et al., 1993; Willmott et al., 1995; Willmott et al., 1996a).

The work of other investigators offers an alternative approach towards the understanding of the mechanism of action of NO. This hypothesis concentrates on enhanced Ca^{2+} sequestration via stimulation of the SR Ca^{2+}-ATPase (Lincoln, 1983; Twort and van Breemen, 1988; van Breemen et al., 1988; Cornwell et al., 1991; Andraintsitohaina et al., 1995).

SNP and 8-Br-cGMP were suggested to decrease contractile activity in the rat aorta by interfering with cellular activation of Ca^{2+} rather than Ca^{2+} influx because 8-Br-cGMP completely abolished tension development to NA in Ca^{2+}-free solution and in the presence of Ca^{2+} antagonists (Lincoln, 1983). Similar evidence for an effect of SNP on Ca^{2+} sequestration in rat aorta was later provided by Karaki et al. (1988). This is consistent with studies suggesting that cGMP activates the SR Ca^{2+}-ATPase via phosphorylation of the Ca^{2+}-ATPase regulatory protein, phospholamban (Cornwell et al., 1991) and that cGMP increased the removal of ~ 20 % of total Ca^{2+} in aortic cells by sequestering Ca^{2+} into the SR (Twort and van Breemen, 1988; van Breemen et al., 1988). It is also supported by electrophysiological evidence in isolated SMCs (Komori and Bolton, 1989; Clapp and Gurney, 1991a) and by the effects of ATPase inhibitors on the relaxant response to NO donors from a variety of vascular preparations (Luo et al., 1993; Andriantsitohaina et al., 1995; Raymond and Wendt, 1996; Khan et al., 1998).

1.6 Role of cGMP-dependent protein kinase in vascular smooth muscle function

The cascade of molecular events linking the rise in cGMP to the vasodilation caused by nitrovasodilators remains unclear but is thought to involve PKG (Murad et al.,

1985; Popescu et al., 1985b; Francis et al., 1988; Cornwell and Lincoln, 1989; Baltensperger et al., 1990; Lincoln and Cornwell, 1993). The occurrence of high levels of PKG in smooth muscle (Casnellie et al., 1980; Lincoln and Johnson, 1984; Felbel et al., 1988) suggests that the enzyme may occupy a pivotal role, for example, phosphorylation of membrane proteins (Casnellie et al., 1980), in the regulation of smooth muscle tone. Studies using cyclic nucleotide analogues and agents that increase cGMP levels support a role for PKG in relaxation of smooth muscle (Fiscus et al., 1984; Fiscus et al., 1985; Francis et al., 1988; Nishimura and van Breemen, 1989). In the former study, the cholinomimetic methacholine, and SNP, increased PKG activity in canine tracheal smooth muscle coincident with an increase in cGMP levels (Fiscus et al., 1984). Further evidence for the importance of PKG in smooth muscle was obtained in isolated and cultured cells in which activated PKG was directly associated with reduced $[Ca^{2+}]_i$ (Felbel et al., 1988; Cornwell and Lincoln, 1989). Agents that increase cGMP levels, decreased $[Ca^{2+}]_i$ in freshly isolated SMCs from bovine trachea (Felbel et al., 1988). To substantiate the involvement of PKG, the enzyme was introduced into the SMCs and was found to reduce, in a concentration-dependent manner, agonist-stimulated increases in $[Ca^{2+}]_i$. Furthermore, rat aortic SMCs were shown to lose their responsiveness to cGMP simultaneously with a reduction in the cellular content of PKG following cell culture (Cornwell and Lincoln, 1989). Conversely, introduction of PKG to the kinase-deficient cells subsequently restored cGMP responsiveness (Cornwell and Lincoln, 1989). These observations imply that PKG activation is necessary to mediate the $[Ca^{2+}]_i$ lowering and relaxant effects of cGMP. This was also found for adenosine 3':5'-cyclic monophosphate (cAMP) (Francis et al., 1988; Lincoln et al., 1990;

Lincoln and Cornwell, 1991; Lincoln and Cornwell, 1993), which can activate PKG in addition to cAMP-dependent protein kinase (PKA) (Silver et al., 1982; Lincoln and Fisher-Simpson, 1983). However, recent findings suggest that NO/cGMP-dependent modulation of vascular tone in the pulmonary circulation, while mediated by cGMP, may not be principally dependent on PKG activation (Fouty et al., 1998). Although inhibitors of cGC increased pulmonary vasoconstriction in the rat normotensive lung, inhibition of PKG activation did not affect pulmonary vascular tone. NO has also been proposed to have actions on rabbit aorta and rat mesenteric SMCs that are independent of cGMP (Bolotina et al., 1994; Mistry & Garland, 1998). Thus while cGMP and PKG are widely thought of as the central mediators of NO action, we cannot yet be certain that vasorelaxation does not employ alternative signalling pathways.

1.7 Aims of the study

NO relaxes VSM in part by reducing $[Ca^{2+}]_i$. The myriad of pathways that regulate $[Ca^{2+}]_i$ and VSM tone means that there are many potential targets for vasorelaxants to produce vasodilation. Therefore, not surprisingly, several cellular mechanism(s) by which NO can reduce $[Ca^{2+}]_i$ have been proposed. Each of these potential mechanisms has some support, but in spite of extensive research, the mechanisms predominant in NO-induced VSM relaxation remain elusive.

The objective of this study was to determine the mechanisms by which NO inhibits agonist-induced increases in $[Ca^{2+}]$ brought about by Ca^{2+} release from the SR. The project focused on the modulation of SR Ca^{2+} release and sequestration by

exogenous NO donors. Since the rabbit aorta is a widely used model for studying the regulation of vascular tone, SMCs freshly isolated from rabbit aorta were used as a model system. The study began by investigating the effects of NO donors on SR Ca^{2+} mobilisation by caffeine, using the whole-cell configuration of the patch-clamp technique to monitor Ca^{2+}-activated membrane currents as an estimate of $[Ca^{2+}]$ directly below the plasma membrane. Subsequent studies investigated the modulation of the bulk $[Ca^{2+}]_i$, using the fluorescent, Ca^{2+}-sensitive dye, fura-2, to measure the average in intact SMCs. The role of the cGMP signalling pathway in the observed effects of NO donors was investigated using agents that modulate cGMP synthesis or metabolism.

Chapter 2

Influence of SNP on K^+ currents and Ca^{2+}-dependent membrane currents in rabbit aortic smooth muscle cells

2.1 Introduction

One of the primary mechanisms that has been proposed to mediate the lowering of $[Ca^{2+}]_i$ in VSM by nitrovasodilators is an enhancement of Ca^{2+} sequestration by the SR, due to stimulation of the SR Ca^{2+}-ATPase. This was first proposed by Lincoln (1983) who suggested that SNP and the membrane permeant analogue of cGMP, 8-Br-cGMP, may relax the rat aorta by interfering with intracellular activation of Ca^{2+}. A similar mechanism was later supported by Karaki et al. (1988), who simultaneously measured $[Ca^{2+}]_i$ and contraction and showed that SNP reduced $[Ca^{2+}]_i$ along with contraction. However, if Ca^{2+} stores were depleted in Ca^{2+}-free medium and then allowed to refill by reintroducing Ca^{2+}, addition of SNP during this 'Ca^{2+} loading' period, augmented subsequent agonist-induced transient contractions. Enhanced Ca^{2+} accumulation by the SR was further supported by electrophysiological evidence from rat portal vein (Komori and Bolton, 1989), which showed that addition of cyclic nucleotides into cells, or extracellular application of their membrane permeable analogues, enhanced outward current responses to caffeine, an agonist known to release stored Ca^{2+} (Iino, 1989). This is consistent with studies examining the effects of cGMP on the phosphorylation of the Ca^{2+}-ATPase regulatory protein, phospholamban (Cornwell et al., 1991). Results suggested that one possible mechanism by which cGMP reduces $[Ca^{2+}]_i$ is the activation of the SR Ca^{2+}-ATPase via phosphorylation of phospholamban. In the same year, using the patch-clamp technique, Clapp and Gurney (1991a) found SNP to induce spontaneous transient outward currents (STOCs) in isolated VSMCs and a similar effect was observed with intracellular cGMP (Komori and Bolton, 1989). These spontaneous currents are thought to reflect the spontaneous release of Ca^{2+} from Ca^{2+}-overloaded

stores and activation of BK_{Ca} channels (Benham and Bolton, 1986). Confocal microscopy of SMCs of cerebral arteries confirmed that STOCs were mediated by spontaneous local increases in $[Ca^{2+}]_i$ directly below the plasma membrane (Ca^{2+} sparks), which arose from the opening of SR ryanodine receptors (Nelson et al., 1995). Directly supporting this idea, Perez et al. (1999) and ZhuGe et al. (1999) demonstrated that virtually all detectable Ca^{2+} sparks were associated with activation of STOCs through BK_{Ca} channels, in SMCs from cerebral arteries and *Bufo marinus* stomach. Recently, cyclic nucleotides were found to increase the frequency of STOC discharge and Ca^{2+} sparks by two- to three-fold (Porter et al., 1997; Porter et al., 1998). Such a result could be explained if cyclic nucleotides promote Ca^{2+} uptake into the SR thereby overloading the stores. Tension recordings from a variety of vascular preparations have also indicated that augmented sequestration of Ca^{2+} by the SR Ca^{2+}-ATPase may be necessary for elicitation of relaxation by cGMP-dependent nitrovasodilators (Luo et al., 1993; Andriantsitohaina et al., 1995; Khan et al., 1998). In each case, SR Ca^{2+}-ATPase inhibitors were found to non-competitively inhibit maximal nitrovasodilator relaxations.

Although NO has been suggested to interfere with SR Ca^{2+} release, leading to a decrease in $[Ca^{2+}]_i$, recent studies on the effects of NO and NO donors on Ca^{2+} release through ryanodine receptors have been inconsistent. In isolated SR from skeletal muscle, NO generated either by the exogenous NO donors S-nitroso-N-acetylpenicillamine (SNAP), SNP or 3-morpholinosydnonimine (SIN-1), or enzymatically from L-arginine, was shown to reduce the rate of Ca^{2+} release and the open probability of single ryanodine receptor channels (Meszaros et al., 1996).

Although SNAP prevented activation of the SR Ca^{2+} release channel from skeletal muscle, it increased activity at high concentrations (Aghdasi et al., 1997). In porcine tracheal SMCs, SNAP was shown to reduce the intracellular Ca^{2+} response to acetylcholine and caffeine by inhibiting SR Ca^{2+} release through both IP_3 and ryanodine receptors (Kannan et al., 1997). However, studies in rat aortic smooth muscle found that SNP did not influence intracellular Ca^{2+} release elicited by caffeine, but did inhibit IP_3-induced Ca^{2+} release (IICR) (Ji et al., 1998). A similar result was reported in pulmonary artery (Yuan et al., 1997). In a variety of other preparations, such as interstitial cells in the mammalian gut (Publicover et al., 1993), rat pancreatic β cells (Willmott et al., 1995) and sea urchin eggs (Willmott et al., 1996a), treatment with NO and NO donors was demonstrated to selectively mobilise stored Ca^{2+} through ryanodine receptors. In addition, cyclic ADP ribose (cADPR), the synthesis of which is stimulated by NO and cGMP, has been shown to stimulate Ca^{2+} release via a ryanodine-sensitive pathway in sea urchin eggs and PC12 cells (Willmott et al., 1996a; Clementi and Meldolesi, 1997). Fluorescence imaging studies have provided evidence that cADPR releases Ca^{2+} from the SR of VSM, but through a mechanism that is independent of activation of either the IP_3 or the ryanodine receptor (Kannan et al., 1996).

The main focus of the work described in this chapter was to examine the effects of SNP on $[Ca^{2+}]_i$ in rabbit aortic SMCs following Ca^{2+} mobilisation from the SR, using the current flowing through Ca^{2+}-dependent membrane ion channels to monitor $[Ca^{2+}]_i$ immediately under the cell membrane. The activation of Ca^{2+}-dependent membrane currents has previously been used to monitor such changes in $[Ca^{2+}]_i$.

These may be macroscopic currents activated by caffeine or IP_3, or STOCs observed in the absence of store modifying agents (Benham and Bolton, 1986; Komori and Bolton, 1989; Stehno-Bittel and Sturek, 1992). Caffeine is widely used as a tool for studying Ca^{2+} release from the SR in smooth muscle (Deth and Casteels, 1977; Iino, 1989). External application of caffeine releases stored Ca^{2+} by facilitating a CICR mechanism (Iino, 1989) to activate ryanodine receptors at resting $[Ca^{2+}]_i$ (Iino, 1989; Sitsapesan and Williams, 1990). Application of caffeine at low concentrations has been shown to initially increase STOC discharge frequency prior to a period of inhibition, presumably due to the rapid release of stored Ca^{2+}, which activated BK_{Ca} channels. A large, more long-lived, but still transient Ca^{2+}-dependent current was activated at higher caffeine concentrations (10-20 mM), indicating the massive release of Ca^{2+} from the stores and activation of Ca^{2+}-dependent channels (Benham and Bolton, 1986; Byrne and Large, 1987; Byrne and Large, 1988; Bolton and Lim, 1989; Komori and Bolton, 1989).

In the present study, caffeine was used to release stored Ca^{2+} and activate Ca^{2+}-dependent membrane currents. Caffeine was applied at 20 mM, because this concentration is known to release the entire pool of exchangeable SR Ca^{2+} (Gurney and Allam, 1995; Ji et al., 1998). Once released, the rise in $[Ca^{2+}]_i$ opens BK_{Ca} channels (Komori and Bolton, 1989) or Ca^{2+}-activated chloride (Cl_{Ca}) channels (Byrne and Large, 1988; Gurney et al., 2000) depending on the membrane potential. BK_{Ca} channels are calcium and voltage-dependent. In the absence of Ca^{2+} they rarely open at negative membrane potentials. When Ca^{2+} is elevated the open probability at all potentials is enhanced and they may open at relatively negative potentials.

Provided the membrane potential is above -80 mV, the equilibrium potential for K^+, the current produced is outward. Cl_{Ca} channels are voltage-independent and will therefore, open at any potential. With equimolar Cl^- on each side of the membrane, the equilibrium potential for chloride is close to 0 mV. Thus, outward currents would be produced at positive potentials, and inward currents at negative potentials. The outward BK_{Ca} current can be selectively recorded at 0 mV, while the inward Cl_{Ca} current can be selectively recorded by clamping cells at negative potentials. Both types of channel activate rapidly and produce currents that are transient in nature, indicative of a short-lived rise in $[Ca^{2+}]_i$.

Whilst Ca^{2+}-activated channels provide a useful means of studying the actions of NO on Ca^{2+} stores and $[Ca^{2+}]_s$, the results could however, be complicated by effects of NO on the channels themselves. There is substantial evidence that NO and the NO donors, SNP, SIN-1, GTN and S-nitroso-L-cysteine can activate K^+ channels in a variety of preparations (Thornbury et al., 1991; Robertson et al., 1993; Archer et al., 1994; Yuan et al., 1996; Khan et al., 1998; Lang and Watson, 1998; Mistry and Garland, 1998), including rabbit aorta (Bolotina et al., 1994). Thus, in some blood vessels, nitrovasodilators appear to act in a way comparable with the K^+-channel openers, increasing K^+ efflux, which leads to membrane hyperpolarisation and muscle relaxation. Since this could interfere with our ability to resolve the actions of SNP on $[Ca^{2+}]_i$, it was necessary to investigate whether or not SNP has effects on K^+ channel activity that occur independently of changes in $[Ca^{2+}]_i$. Only if it could be established that the effects of SNP did not include channel modulation, could caffeine-induced membrane currents be used to investigate its effects on $[Ca^{2+}]_i$. This

chapter therefore, examined the effect of SNP on K^+ currents and caffeine-induced membrane currents in rabbit aortic SMCs.

2.2 Materials and Methods

2.2.1 Tissue preparation

Male New Zealand rabbits (2-2.5 kg) were anaesthetised by lethal i.v. injection of sodium pentobarbitone (30 mg kg^{-1}; Rhone Merieux, Harlow, Essex) into the ear vein. The chest was then opened and a segment of descending thoracic aorta was carefully removed and placed directly in standard extracellular solution containing the following (mM): NaCl 122, KCl 5, N-[2-Hydroxyethyl]piperazine-N'-[2-ethane-sulfonic acid] (HEPES) 10, NaH_2PO_4 0.5, KH_2PO_4 0.5, $MgCl_2$ 1, $CaCl_2$ 1.8, glucose 11 (pH = 7.3 with NaOH). It was then cleaned of adhering fat and connective tissue, cut open longitudinally, and thin strips (approximately 2 mm wide by 10 mm long) cut transversely from the vessel.

2.2.2 Vascular smooth muscle cell isolation

The SMCs in a blood vessel such as the aorta are surrounded by extracellular connective tissue proteins such as elastin and collagen. Enzymes that break down the connective tissue are used to loosen the SMCs, so that they can be liberated by gentle trituration of the tissue. Two different methods of cell isolation were used. However, there were no differences in the properties of the cells isolated by the two methods. Routine cell preparation with both of these dissociation procedures (Method 1: Clapp and Gurney, 1991b; Method 2: Cotton et al., 1997) yielded a high proportion of relaxed, Ca^{2+} tolerant SMCs, which remained viable for up to 24 hrs. Following

isolation, cells were left to adhere to the bottom of the experimental chamber for 10-15 min.

2.2.2.1 Method 1-papain

Single VSMCs were enzymatically isolated using overnight (>16 hrs) incubation in a low concentration of papain (Fluka Chemicals Ltd., Dorset, U.K., ~13 units mg^{-1} protein). 5 or 6 vessel strips were transferred into a 7 ml air-tight glass bottle and stored in a 4 °C refrigerator overnight in a low $CaCl_2$ dissociation medium (DM) containing approximately 0.3 mg ml^{-1} papain and 5 % bovine serum albumin (BSA; fraction V, essentially fatty acid and globulin free, Sigma Chemical Co., Poole, Dorset, U.K.). The ionic composition of the DM was as follows (mM): KCl 5, NaCl 110, NaH_2PO_4 0.5, $CaCl_2$ 0.16, $NaHCO_3$ 10, KH_2PO_4 0.5, HEPES 10, $MgCl_2$ 2, glucose 10, phenol red 0.03, taurine 10, ethylenediaminetetraacetic acid (EDTA) 0.5. Ca^{2+}-free media facilitates the breakdown of connective tissue, but often results in Ca^{2+} overloading of the cells when the Ca^{2+} concentration is returned to normal at the end of the dissociation procedure. Therefore, to prevent this from happening, the Ca^{2+} concentration was kept low, but Ca^{2+} was not completely omitted. The DM was aerated for 15 min before use and pH adjusted to 7.0 with NaOH, the optimum for papain activity (Gurney, 1996). Free sulphydryl groups on papain are essential for activity (Gurney, 1996). For this reason EDTA (Sigma) was added to the DM to mop up heavy metals, which reduce enzyme activity by binding to the sulphydryl groups. In addition, to maintain sulphydryl groups in a reduced state, the solution was gassed with air rather than O_2. BSA and taurine are thought to have protective effects on cells during enzymatic dissociation (Isenberg and Klockner, 1982).

Papain is a protease with a broad substrate specificity, being able to hydrolyse a wide range of proteins. The proteolytic activity of papain is however, slowed during exposure to temperatures around 5 °C (Gurney, 1996). Overnight incubation at this temperature therefore allowed the enzyme to diffuse evenly throughout the tissue while causing little proteolysis. Thus, when activated, enzyme activity would be evenly distributed throughout the tissue, as opposed to digesting the tissue from the outside in, potentially causing overdigestion of surface cells. The following morning the reducing agent, dithiothreitol (DTT; 10 µl of a 100 mM stock solution, Sigma), was added to the DM containing the strips of muscle to maximise papain activity. It was then incubated in a static bath at 37 °C for 10 min. Enzymatic digestion of the connective tissue proceeds quickly on warming the tissue to 37 °C. At the end of the incubation period the muscle strips were transferred to 2 ml fresh, enzyme-free DM and cells were isolated by gentle trituration of the tissue with a wide bore (~2-3 mm) fire-polished Pasteur pipette to yield relaxed single VSMCs, which were stored in a refrigerator in DM until their use. The use of papain with DTT preserves receptor function in dissociated SMCs (Rusko et al., 1990).

2.2.2.2 Method 2-collagenase

5 or 6 vessel strips were transferred into a 7 ml air-tight glass bottle in the same low $CaCl_2$ DM as used for method 1, but containing the following agents: 3 mg ml^{-1} collagenase (Type 1a, Sigma), 0.2 mg ml^{-1} protease (Type XXIV, Sigma), 2 mg ml^{-1} BSA (fraction V, essentially fatty acid and globulin free, Sigma) and 2 mg ml^{-1} trypsin inhibitor (Sigma). The enzyme solution containing the vessel strips was incubated in a static bath at 37 °C for approximately 30-40 min to loosen the SMCs,

after which the tissue was transferred to fresh, enzyme-free DM and returned to the 37 °C water bath for approximately 30 min. Under these conditions the strips of blood vessel are digested from the outside in and cells from the outer regions of the tissue may become overdigested by the time the enzyme has reached deeper cells. This is the reason that the enzyme was removed at the end of the first incubation period and the strips of blood vessel were re-incubated for a further period in enzyme-free solution. During the second period of incubation the cells were gently triturated or shaken several times every 5-10 min. Cells were then isolated by further trituration of the tissue with a wide bore (~2-3 mm) fire-polished Pasteur pipette and stored in a refrigerator in DM until their use.

2.2.3 Electrophysiology

Voltage-clamp, first developed by Cole (1949) and Hodgkin et al. (1952), is a technique which allows the direct measurement of ion flow across the cell membrane as electric current, whilst the membrane potential is held under experimental control. With the use of the now well-established whole-cell configuration of the patch-clamp technique (Hamill et al., 1981), and the development of improved cell isolation procedures, it is possible to study the electrophysiological properties of single VSMCs that were previously unable to survive microelectrode penetration. The patch-clamp technique has refined the voltage-clamp method to permit recording of ionic currents flowing through an individual ion channel. The whole-cell configuration, used in the present study, has become the most widely employed method for recording currents from an entire cell population of channels, i.e. macroscopic currents.

2.2.3.1 Patch-clamp configurations

Whole-cell recording is one of four configurations utilised by the patch-clamp technique as outlined by Hamill et al. (1981), which are modifications of the extracellular patch-clamp technique first introduced by Neher and Sakmann (1976). Fig. 2.1 outlines each of the four recording configurations and how to achieve them. The whole-cell configuration of the patch-clamp technique is achieved by pressing a small heat-polished glass micropipette, filled with a suitable electrolyte solution, against the cell membrane surface to form a high electrical resistance seal. The resulting seals are described as 'giga-seals' as they have electrical resistances in excess of 10 GΩ. Under such conditions the electrode and cell membrane will be less than 1 nm apart, which ensures that the majority of the currents originating in the membrane patch flow into the pipette, and from there into the recording circuitry. Giga-seals are mechanically very stable, allowing the membrane patch at the tip of the electrode to be ruptured without disrupting the seal. Following seal formation in this study, suction was applied via a 1 ml syringe attached to the side-arm of the pipette holder. This deliberately ruptured the membrane patch trapped below the pipette tip, allowing direct access to the cell interior. A low resistance pathway was then formed between the electrode and the cell interior allowing measurement of current flow across the whole cell membrane and modulation of the intracellular environment.

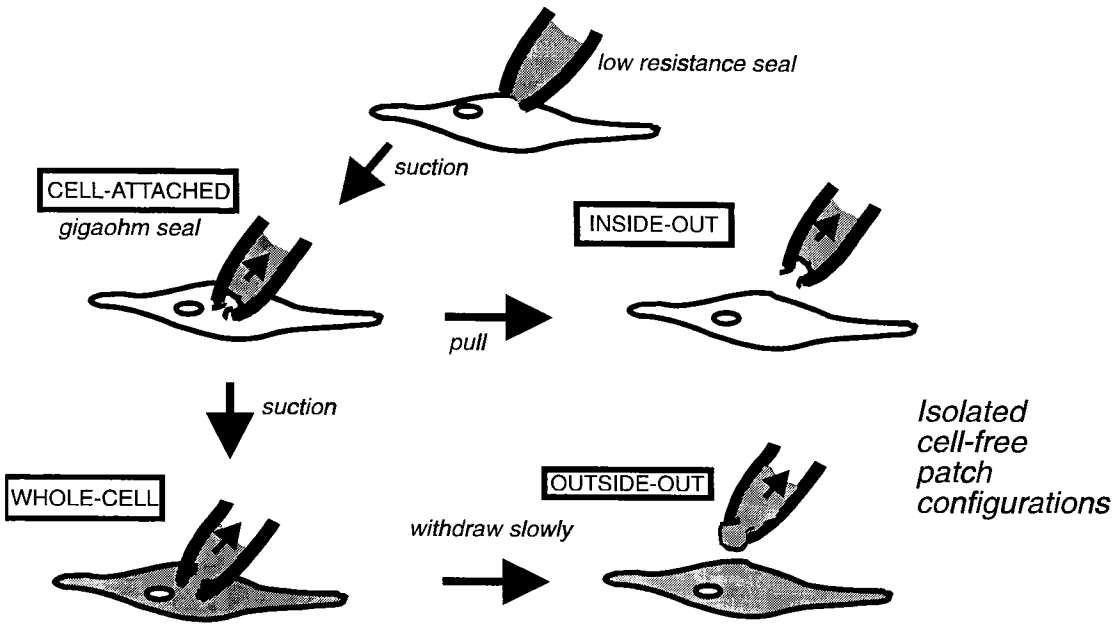

Fig. 2.1. *Schematic representation of the procedures for obtaining patch-clamp configurations. The four recording configurations are: 'cell-attached', 'whole-cell', 'inside-out', and 'outside-out'. A pipette is advanced toward the cell surface to form a giga-seal and the cell-attached mode. Abrupt withdrawl of the pipette from the cell produces the inside-out recording configuration, which exposes the intracellular surface of the membrane to the extracellular solution. If instead of withdrawing the pipette the patch is deliberately ruptured, the whole-cell configuration is formed, providing electrical continuity and diffusional exchange of pipette and cytoplasmic constituents. If the pipette is now slowly withdrawn from the cell, an outside-out patch is formed, exposing the extracellular surface to the bath solution. Modified from Hamill et al. (1981).*

2.2.3.2 Voltage-clamp

A diagramatic representation of the basic voltage-clamp circuit in the patch-clamp headstage used in electrophysiological experiments is illustrated in Fig. 2.2a. The headstage is the key component in current recording. It contains the feedback amplifier which controls membrane voltage whilst measuring current flow. The feedback amplifier compares the pipette potential (V_p) with a reference voltage (V_r), selected by the experimenter, and using the principle of negative feedback, continually adjusts its output to maintain a zero potential difference between the two inputs ($V_p = V_r$). When a discrepancy arises between the two inputs, the amplifier supplies current to the cell. In doing so, it compensates for ionic currents that would change the membrane potential. Therefore, the current that the amplifier supplies is equal and opposite to that carried by ions flowing across the cell membrane. Current flow (I) across the cell membrane should ideally be measured from the voltage difference across the feedback resistor (R_f) using Ohm's law:

$$V_{out} - V_p = I.R_f \qquad (1)$$

where V_{out} is the voltage output of the amplifier and R_f is the feedback resistance. However, in practice, it is the difference between V_{out} and V_r (which is equal to V_p) that is measured. To measure the voltage drop across R_f would mean physically adding components into the circuit at R_f, which would introduce extra capacitance. This would slow the voltage-clamp circuit as well as increasing noise. The feedback resistor used in the patch-clamp amplifier is associated with a stray capacitance (C_f) across its terminal, as shown in Fig. 2.2a. Therefore, the time taken to change V_p would be limited by the time taken to charge the capacitor across R_f. As a result, when V_r is changed, a capacitance current flows at the leading edge of the voltage

step, with a time constant determined by the product of C_f and R_f. This can be cancelled by using the capacitance compensation circuitry in the amplifier. From equation (1) it follows that the measured current is inversely proportional to R_f. Thus, larger feedback resistors cause a greater voltage drop for a given current, increasing the gain, and hence resolution of recording. In addition, thermal noise in the resistor, which contributes to noise in the current recording is lower with high values of R_f, which are therefore used for single channel recording. However, use of a high value R_f can cause saturation of the amplifier when large currents flow across the cell membrane, because the amplifier output cannot exceed its power supply, which is usually ± 12 V. Large whole-cell currents are therefore measured with a lower feedback resistor than single channel currents. The value of R_f in the headstage used to record K^+ currents from the rabbit aorta was 500 MΩ.

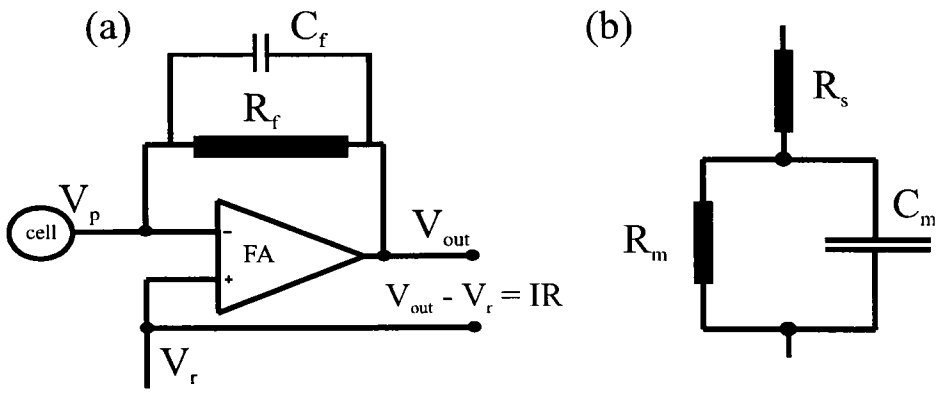

Fig. 2.2. *Circuit diagrams representing (a) the resistive feedback circuit in the patch-clamp headstage and (b) a simplified electrical circuit of the cell membrane. R_m is the membrane resistance, R_s is the series resistance and C_m is the membrane capacitance. Taken from (a) Ogden, (1987) and (b) Halliwell, et al. (1987).*

The basis of voltage-clamp may be understood by considering the model of a cell membrane illustrated in Fig. 2.2b. Since the cell membrane acts as an insulating barrier separating two conducting solutions (the cytoplasm and extracellular solution), it acts as a capacitor (C_m). The channels that allow ionic current to flow through the membrane are represented by the resistor, R_m. In practice, there is also a resistance in series with the cell membrane, represented by R_s, which corresponds to access resistance (pipette and other resistances). The pipette tip is small, and for this reason there can be considerable resistance to current flow from pipette to cytoplasm. When a change in membrane potential is produced, a transient capacitance current flows at the onset of the voltage step as the membrane charges. However, when the voltage is steady, the capacitance current has declined to zero to leave the voltage-clamped ionic current (I_i) flowing through the membrane resistance, or open ion channels. The total current (I_m) flowing across the membrane at any one time (t) will be the sum of I_i and the capacitance current (I_C) as follows:

$$I_m = I_i + I_c \quad \textbf{(2)}$$

$$\text{or } I_m = I_i + C_m \, dV/dt \quad \textbf{(3)}$$

where V is the membrane potential. So when the voltage change is complete ($dV/dt = 0$) $I_m = I_i$.

Both R_s and C_m may be calculated from the capacitance current. The area under the capacitive transient is given by:

$$C_m = Q / dV \quad \textbf{(4)}$$

Where Q is the charge (coulombs) on the membrane capacitor and dV is the change in the membrane potential. In my experiments, Q was estimated by integrating the

current response to a 10 mV hyperpolarisation step in a potential region where no ion channels were open (i.e. –80 to –90 mV), following leak subtraction of the current traces, with Clampfit (V6 Axon Instruments) software. C_m was then obtained by dividing Q by the voltage step and was calculated to be 19 ± 1 pF (n = 29). The decay of the capacitive transient followed a single exponential time course, the average time constant (τ) of which was 0.11 ± 0.01 ms (n = 29). This relates to C_m and R_s according to:

$$\tau = R_s.C_m \quad \textbf{(5)}$$

From this equation R_s was calculated for each cell and found to be 5.1 ± 0.4 MΩ (n = 29) before compensation.

The main error in whole-cell recording results from the resistance in series with the membrane, which gives rise to a voltage error between the measured potential (V_m) and the true membrane potential. This is due to a drop in voltage across R_s as current flows into the cell from the feedback amplifier. The size of the error equals $I_m.R_s$, so that the problem becomes more serious when large membrane currents are recorded. The maximum voltage error in this study was 8.2 mV calculated from a peak current of 3.9 nA, recorded with R_s = 2.1 MΩ. Minimising R_s is important for reducing errors in the measurement of membrane voltage and current. Up to 90 % of the R_s was usually compensated for electronically, by manually adjusting the compensation controls on the patch-clamp amplifier.

2.2.3.3 Formation of a giga-seal

Several factors promote giga-seal formation, including cleanliness of the pipette tip, a clean cell surface and clean bath and pipette solutions (Hamill et al., 1981). The solutions were routinely filtered through a 0.2 μm pore membrane prior to their use. On entry of the pipette into the bath solution, a junction potential between the pipette and bath solution was formed at the pipette tip. This potential arose owing to the different ionic compositions of bath and pipette solutions, and the different concentrations and mobility of the ionic species in the two solutions. Before proceeding, this potential was electronically cancelled by adjusting the offset on the patch-clamp amplifier.

During the course of giga-seal formation, a rapidly repeating 10 mV pulse was applied to the pipette, enabling the pipette resistance to be measured and seal formation to be monitored as a loss in current response to the voltage step. While observing the current trace on the oscilloscope, the pipette was moved towards the cell. Contact with the cell surface was indicated by an increase in the pipette resistance, seen on the oscilloscope as a slight reduction of current amplitude. When this occurred gentle suction was applied from a syringe connected to a side arm on the pipette holder. The current response then became negligible, indicating seal formation. This sometimes occurred immediately or could take some time to develop. In some cases giga-seals developed spontaneously without suction.

After achieving a giga-seal, small but fast capacity transients were seen due to pipette and other stray capacitance. These were cancelled using fast capacity

compensation controls, which injected current via a capacitor into the headstage, which was opposite in direction to the transient. Rupture of the membrane was then achieved by applying further suction. This was visualised as the sudden appearance of large capacitance transients at the leading and trailing edges of the voltage pulse, due to charging of the cell membrane.

2.2.4 Electrophysiological recording instrumentation

A schematic representation of the recording setup is shown in Fig. 2.3. The headstage (CV 201A; Axon Instruments, Foster City, CA, USA) was mounted on a three-dimensional hydraulic micromanipulator (Narishige MO103; Narishige Scientific Co., Tokyo, Japan), through which mechanical contact between the recording electrode and cell was controlled. The pipette was filled with an electrolyte solution designed to mimic the normal intracellular environment (composition on page 46), which connected to the headstage circuitry via a chloridized silver wire protruding from the pipette holder. To stabilise the junction potential formed between the pipette solution and the silver wire, the wire was dipped in chlorine bleach for ~10 min to form a non-polarisable AgCl coating. This was repeated every few weeks as the coating gradually deteriorated. The bathing solution within the perfusion chamber (composition on page 30) was connected to ground via a Ag-AgCl pellet placed at the outlet of the perfusing solution and connected directly to the ground input on the headstage. A glass-bottom perfusion chamber was rigidly attached to the stage of an inverted microscope (Nikon Diaphot; Nikon Corporation, Tokyo, Japan), mounted on a vibration isolation table (Technical Manufacturing Corp., Peabody, Massachussetts) to dampen mechanical vibration. Measurement of

currents can be hampered by interference from unwanted signals. Electrical interference was therefore removed by enclosing the apparatus, mounted on the vibration isolation table, in a grounded Faraday cage, leaving one side open to allow access to the equipment. Interference could only enter through the opening, or via some non-earthed conductor in the cage. Within the cage, all conducting components, including the microscope, manipulator and baseplate were grounded to a common earth point on the front of the oscilloscope. In addition the internal a.c. power source of the microscope was disconnected, so that it would not serve as a source of 50 Hz noise within the cage. The light for the microscope was instead powered by a d.c. power supply outside the cage. Although the headstage must be close to the cells, all other equipment for recording or displaying the signals was placed outside the cage.

The output of the headstage was connected to an Axopatch 200-A patch-clamp amplifier operating in the voltage-clamp mode (Axon Instruments). Current signals were filtered at 2 kHz by the patch-clamp amplifier and digitised, using a DigiData 1200 interface (Axon Instruments) for computer display, collection and analysis. Current and voltage signals were monitored with a digital storage oscilloscope (Hitachi VC 6023; Hitachi Denshi Ltd., Tokyo, Japan) and personal computer (OPUS PC VI). Data was also stored either on digital audio tape (DAT122, HHb; DTR-1200), video tape (Samsung SV 30IK; Samsung Electronics, Tokyo, Japan) following digitisation by an Instrutech CRC VR-100B recorder (Instrutech Corp., Great Neck, N.Y., U.S.A.) or on chart records (BBC Goerz Metrawatt SE 120;

Recorderlab Services, Surrey). Data stored on tape was transferred to a PC for illustration and 'off-line' analysis.

Calibration of the experimental setup was checked prior to use. A resistor of known value was connected between the input of the headstage, and ground. A known voltage was applied across the resistor and the current measured was as expected from Ohm's law.

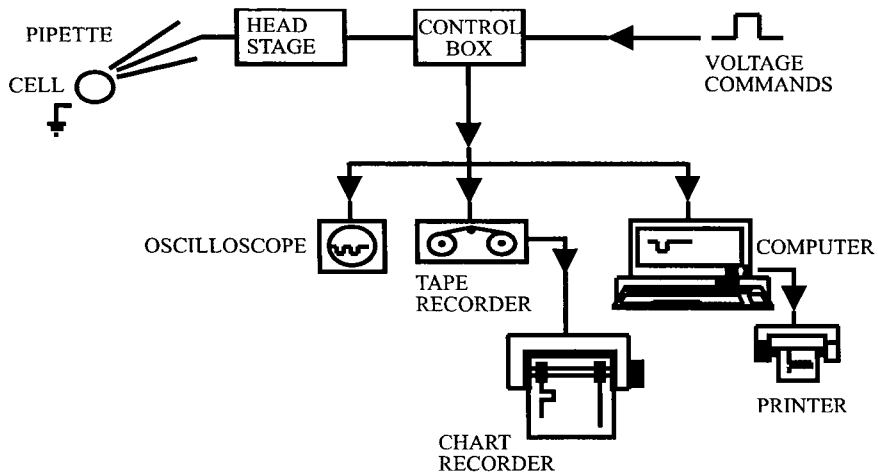

Fig. 2.3. *A schematic diagram of the patch-clamp recording system. The patch pipette carries current signals from the cell to the headstage. The output from the headstage is amplified and then displayed by the oscilloscope and computer. Taken from Gurney (1990 a).*

2.2.5 Preparation of the recording electrode

The recording electrode or pipette was made in three stages: (1) a glass capillary was pulled to form two pipettes (2) the pipette tip was heat polished and (3) the shank was coated with beeswax.

Pipettes were pulled in two stages (Fig. 2.4) from thin-walled, filamented, borosilicate, glass capillaries (1.5 mm O.D. x 1.17 mm I.D.; Clark Electromedical Instruments, Berks, U.K.) using a vertical micropipette puller (Narishige PB-7; Narishige Scientific Co.). During the initial pull the centre of the capillary was heated and extended by gravity over a length of 8 mm. After cooling and re-centring the narrowed region in the heating coil, the glass was then pulled at a lower temperature until it separated into two equally sized pipettes. This procedure produced a steep taper, which reduces electrode resistance. The shape of the pipette tip was largely dependent on the heat received during the first pull, with the heat during the second pull determining the diameter of the pipette tip orifice. Pipettes had resistances of 2-4 MΩ when filled with pipette solution (composition on page 46).

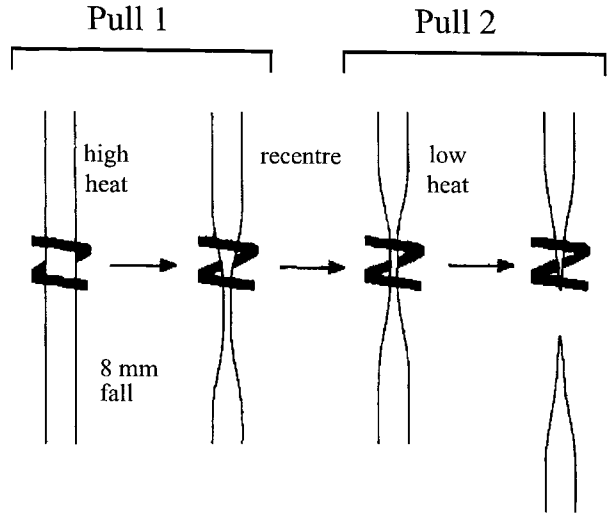

Fig. 2.4. Two stage pull of patch pipettes. Taken from Ogden & Stanfield (1987).

The tip of each electrode was heat polished using a heated filament under high magnification (400 x) as shown in Fig. 2.5. This facilitated the formation of a giga-seal between the pipette and cell membrane. The point of the filament was coated in glass to prevent the metal from becoming deposited on the pipette tip during heating. Before heating, the tip of the pipette was usually observed as a clear hole, which became smaller and appeared more rounded and darker after heating.

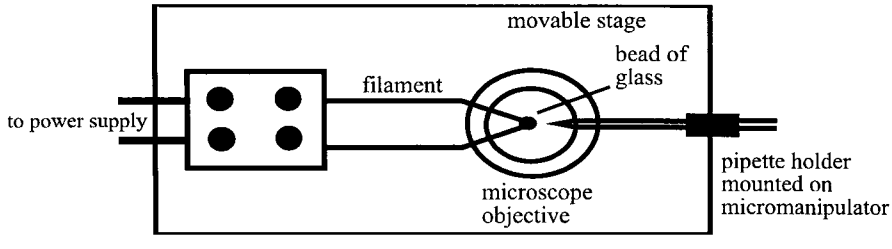

Fig. 2.5. *The heated platinum wire filament was arranged in a V-shape above a 40 x objective on an inverted microscope so that it could be brought to within a few microns of the pipette tip under direct observation. Taken from Gurney, (1990a).*

The pipette wall behaves as a capacitor and, as such, is a source of noise. Since capacitance is inversely proportional to the thickness of the glass wall, it can be reduced by coating the pipette with a hydrophobic substance. Since most of the pipette capacitance resides at the tip, it is important to ensure that the coating extends as close to the tip as possible without blocking it. This also reduces the area of glass contributing to capacitance, by preventing creep of the bath solution up the outer wall of the pipette. Beeswax was applied to the pipettes using a small glass hook, taking care not to block the tip. Three additional measures were taken to reduce electrical noise; minimal volume of solution in the pipette (just enough to make contact with the wire), minimal depth of immersion of the pipette in the bathing solution, and the pipette holder was kept clean and dry.

2.2.6 Pipette and bath solutions

The ionic composition of the pipette (intracellular) and bath (extracellular) solutions were designed to mimic the physiological intracellular and extracellular environment of the cell. Patch pipettes were back-filled with ~ 5 μl intracellular solution of the following composition (mM): KCl 112, HEPES 20, ethylene glycol-bis(β-aminoethyl ester) N, N, N', N',-tetraacetic acid (EGTA) 0.1, ATP-Mg 3, GTP-Na_2 0.3, with pH adjusted to 7.2 with KOH. An internal filament facilitated filling and air bubbles remaining in the shank were removed by gently tapping the stem of the pipette. Since the tips become dirty, pipettes were only used once. A low concentration of EGTA (Sigma) was present in the pipette solution to provide minimal Ca^{2+} buffering. HEPES (Sigma) was included to ensure buffering at physiological pH. Magnesium-adenosine 5'-triphosphate (ATP-Mg, Sigma) and

sodium-guanine 5'-triphosphate (GTP-Na_2, Sigma) were included in the pipette solution, as many cellular processes, including the modulation of Ca^{2+}-pumps and ion channels, require ATP and GTP. Pipette solution osmolarity was regularly checked using an automatic osmometer (Osmette S 4002; Schuco Int., Lond., Ltd.) and found to be ~ 270 mOsm. The composition of the external bath solution used was (mM): NaCl 122, KCl 5, HEPES 10, NaH_2PO_4 0.5, KH_2PO_4 0.5, $MgCl_2$ 1, $CaCl_2$ 1.8, glucose 11. The pH was adjusted to 7.3 with NaOH.

2.2.7 Drug application

Cells were continuously superfused with either drug-free extracellular solution or drug-containing solutions at a rate of approximately 2 ml min^{-1}. Switching between solutions was achieved manually with total replacement of the bathing solution achieved within 30 s. Caffeine was either applied directly to the chamber via a gilson pipette or ejected from a nearby pipette at a pressure of ~ 2-4 psi. These pipettes were pulled in a similar way to the recording electrodes, but had a slightly larger tip orifice and were not heat polished or coated with wax. The tip of the micropipette was placed 100-250 μm from the cell. The duration of caffeine application was usually approximately 2-5 s. Caffeine was ejected against the flow of the superfusing solution, thereby allowing rapid and precise control of delivery. The concentration of caffeine noted in the text refers to the final bath concentration if applied in the bath or to that in the micropipette. It is likely, however, that some dilution of pressure-ejected caffeine occurred, due to the small volume of solution ejected and the distance of the pipettes from the cells.

The following drugs and chemicals were used: tetraethylammonium chloride (TEA, Fluka), charybdotoxin (CTX, Sigma), caffeine (Sigma) and sodium nitroprusside (SNP, Sigma). Drug solutions were freshly prepared each day. Caffeine, TEA and CTX were dissolved directly in the bath solution. SNP was stored in a dark flask at 4 °C as a 20 mM stock solution and used on the same day as it was prepared.

2.2.8 Data analysis and statistics

The pClamp data acquisition program, Clampex, consists of a set of user definable parameters configured to generate voltage protocols. It was used to investigate voltage-activated K^+ currents. During each stimulus protocol, the data generated were displayed on the computer screen and stored on the hard disc drive. The cell membrane potential was clamped at –80 mV and voltage steps of 100 ms duration applied at 5 s intervals, starting with a step to –90 mV and incrementing the step by 10 mV each time, to a maximum test potential of +60 mV.

Clampfit V6 (Axon Instruments) and Origin V4.1 (Microcal Software Inc.) software were used to perform off-line analysis of data generated by Clampex. Two components of outward K^+ current, as described by Halliday et al. (1995), were measured. The peak current (I_t) was measured within the first 20 ms of the depolarising pulse. The sustained current (I_{sus}) was measured as the average of the digitised points comprising the last 20 ms of the current during the depolarising pulse. Current amplitudes were plotted as a function of the test potential at which they were obtained, thereby giving the current-voltage relationship for each cell.

The currents induced by caffeine were collected, stored and analysed using Axotape V2 and Origin software. By convention, outward currents are depicted by upward, and inward currents by downward deflections. A 5 min period of dialysis was allowed before commencing any experimental protocol. Statistical comparisons were made using a Student's t-test or one-way analysis of variance (ANOVA) with $P < 0.05$ taken as indicating a significant difference. Experiments were routinely conducted at room temperature (22-25 °C) in a 1 ml perfusion chamber, as isolated cells remain relaxed for a longer period at room temperature than at higher temperatures. Data are expressed as mean ± standard error of the mean (S.E.M.) of *n* cells unless otherwise stated.

2.3. Results

2.3.1. Outward membrane currents in isolated rabbit aorta smooth muscle cells

After establishing the whole-cell configuration of the patch-clamp technique, recordings of macroscopic currents were made from cells superfused with standard extracellular solution. Examples of currents activated by stepping to a range of test potentials, from –90 mV to +60 mV, from a holding potential of –80 mV are shown in Fig. 2.6. Since the pipettes were filled with solution containing a low concentration of EGTA, there was minimal cytosolic Ca^{2+} buffering and as a result cells demonstrated a noisy outward current at positive potentials, upon which were sometimes superimposed STOCs. This suggests the contribution of channels with a large single channel conductance e.g. BK_{Ca} (Beech and Bolton, 1989; Halliday et al., 1995).

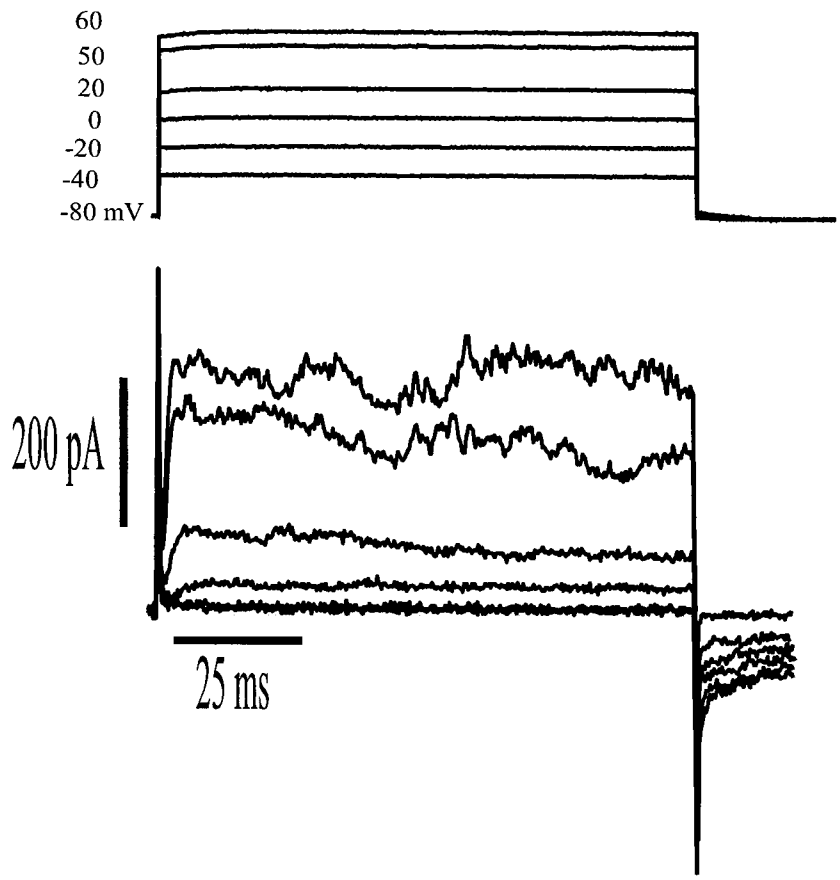

Fig. 2.6. *Whole-cell outward currents elicited by stepping to various test potentials for 100 ms from a holding potential of –80 mV, with an intrerpulse interval of 5 s. The threshold for activation of sustained currents was ~ -30 mV. The dotted line indicates the zero current level (I = 0). Records of the step changes in membrane potential used to elicit the currents are displayed above the current traces.*

Depolarisation elicited outward currents at or above -30 mV. At least two components of time- and voltage-dependent outward current could be distinguished during depolarising steps (Halliday et al., 1995): the rapidly activating transient component (I_t), which inactivated within 15 ms, and the sustained current component (I_{sus}). The magnitude of the transient current varied considerably from cell to cell and in some was too small to be easily distinguished (e.g. Fig. 2.6). The noisier, sustained component failed to inactivate by the end of the 100 ms pulse. Results similar to those of Fig. 2.6 were obtained in a total of 78 experiments. The mean current-voltage relationships for I_t and I_{sus}; plotted separately for each component, are shown in Fig. 2.7. With progressive depolarisation, the amplitude of both components of outward current increased in a similar way, showing that they were both voltage-sensitive as previously shown by Halliday et al. (1995). The mean current amplitudes measured in the present study were, however, larger. For example, the current activated at +60 mV was 1.0 ± 0.2 nA in this study (Fig. 2.7) compared with ~ 800 pA in Halliday et al. (1995).

2.3.2. Effects of K^+ channel blockers on outward current

2.3.2.1. Tetraethylammonium chloride

TEA is known to block most classes of K^+ channel with varying affinities. However, where potency of block from inside and outside the cell has been compared, significant differences in sensitivity to TEA were apparent between different channel types. When applied externally to VSM at low millimolar concentrations, TEA has been shown to act as a relatively selective inhibitor of BK_{Ca} currents (Haeusler and

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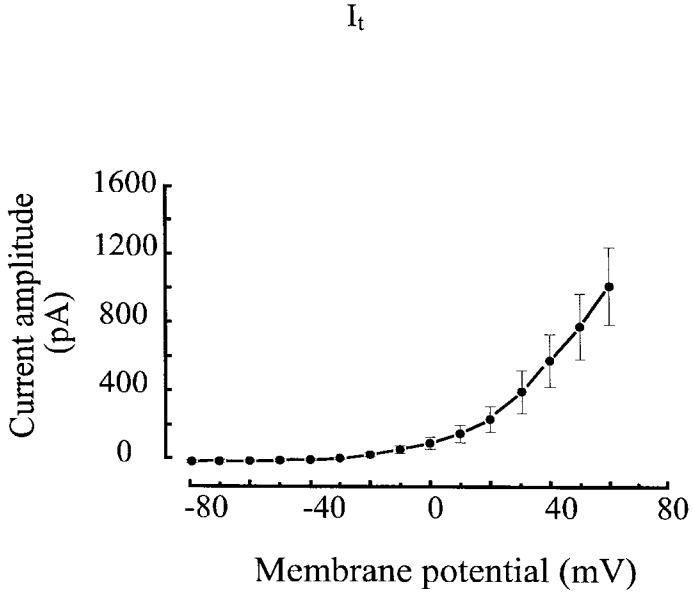

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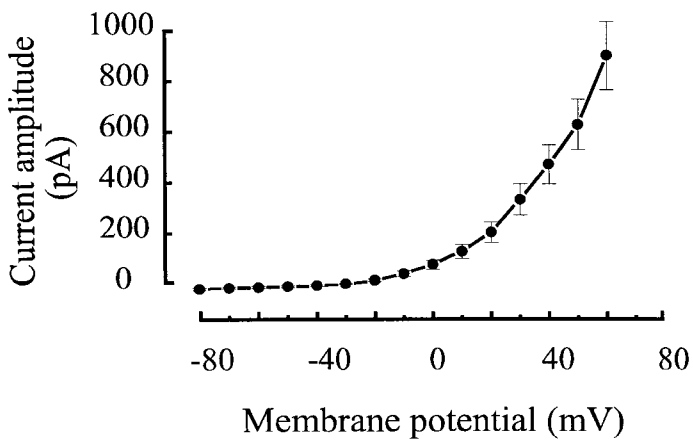

Fig. 2.7. *Current-voltage relationships for the transient current, I_t (a) and sustained current, I_{sus} (b). In each case the current amplitude is plotted as a function of the membrane potential. Data are expressed as mean ± S.E.M. (n = 78).*

Thorens, 1980; Vergara et al., 1984; Benham et al., 1985; Inoue et al., 1985; Huang et al., 1990; Nelson and Quayle, 1995; Carl and Sanders, 1996). At these concentrations it does however, block certain subtypes of delayed rectifier channels, such as $K_v1.1$, $K_v1.3$, $K_v2.1$ and $K_v3.1$ (Vogalis et al., 1993; Grissmer et al., 1994; Patel et al., 1997). TEA is known to suppress I_{sus} in rabbit aortic smooth muscle (Halliday et al., 1995). Its effect was previously found to be maximal at 1 mM, with no further block observed on increasing the TEA concentration to 10 mM. This was proposed to reflect inhibition by TEA of BK_{Ca} channels.

To determine the percentage of outward current carried through BK_{Ca} channels in this study, the effect of 10 mM, extracellularly (bath)-applied TEA was examined on outward currents activated at a test potential of 0 mV, as illustrated in Fig. 2.8a. From the raw traces it appears that TEA may block I_t. Overall, however, I_t was only slightly reduced (5 ± 1 %) in the presence of TEA (Fig. 2.8b), and this reduction was not significant ($P > 0.05$). Thus, approximately 60 % of I_{sus} at 0 mV appeared to be carried through BK_{Ca} channels. As shown in Fig. 2.8, the application of 10 mM TEA significantly ($P < 0.05$) attenuated I_{sus} relative to control by 60 ± 5 % ($n = 15$). Inhibition of I_{sus} was observed in all cells and was associated with a concomitant reduction in current noise (Fig. 2.8a). Blockade of I_{sus} by TEA could be completely reversed by washing the drug out for 5 min.

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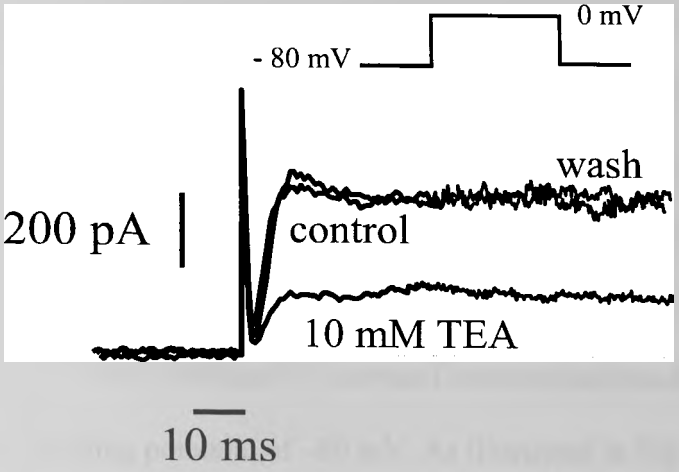

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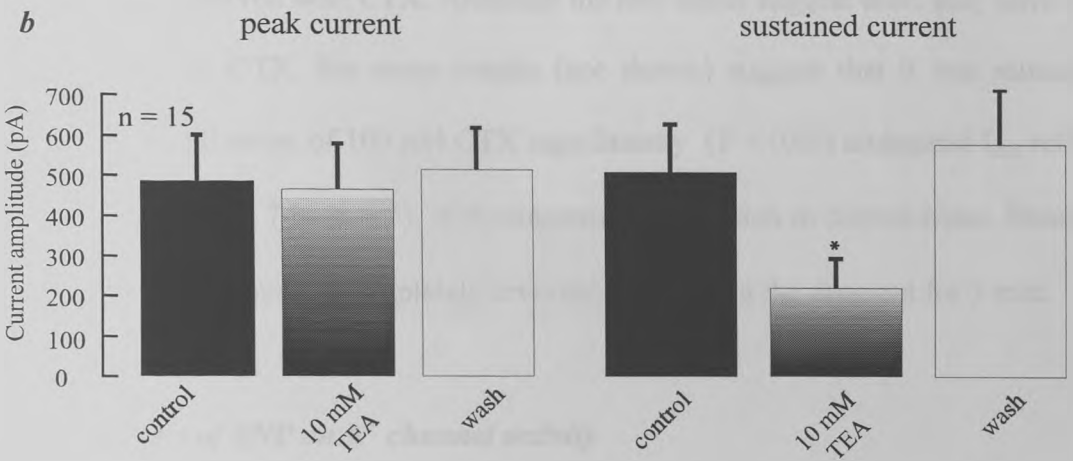

Fig. 2.8. *a Inhibition of K^+ currents by 10 mM TEA. Representative current traces from an individual cell illustrate the TEA-induced inhibition of outward K^+ current. At 10 mM, TEA caused a substantial block of I_{sus}. TEA was applied to the cell for 5 min prior to recording. After washing the drug out the current returned to the control level. Currents were recorded by stepping to 0 mV for 100 ms from a holding potential of –80 mV, with an interpulse interval of 5 s. The dotted line indicates the zero current level (I = 0). b Mean results from 15 cells showing significant ($P < 0.05$) inhibition of I_{sus} by TEA and full recovery upon washing. Data are expressed as mean ± S.E.M. (n = 15).*

2.3.2.2. Charybdotoxin

CTX is a more potent and selective blocker of BK_{Ca} channels than TEA, although it can also block monomeric $K_v1.2$, $K_v1.3$ and K_v3 channels (Oliva et al., 1991; Goldstein and Miller, 1992; Grissmer et al., 1994). To further test the contribution of BK_{Ca} channels to the sustained outward current observed in rabbit aortic SMCs, the effects of CTX were also tested. As with TEA, the effect of extracellularly-applied CTX (100 nM) was examined on outward currents activated by depolarisation to 0 mV, from a holding potential of –80 mV. As illustrated in Fig. 2.9a, similar effects to TEA were observed with CTX. Although the raw traces suggest there may have been block of I_t by CTX, the mean results (not shown) suggest that it was minimally affected. Application of 100 nM CTX significantly ($P < 0.05$) attenuated I_{sus} relative to control by 40 ± 7 % (n = 5), with concomitant reduction in current noise. Blockade of I_{sus} by CTX could be completely reversed by washing the drug out for 5 min.

2.3.3. Effect of SNP on K^+ channel activity

The effects of SNP on K^+ currents were investigated in a similar way to the K^+ channel blockers. K^+ currents were evoked by 100 ms depolarising steps applied from a holding potential of –80 mV, before and during the application of SNP at either 1 μM or 10 μM and again following washout of the drug from the recording chamber. Typical responses to SNP are shown in Fig. 2.10. K^+ currents were inconsistently altered by SNP at both concentrations. Of all the cells studied, 5 cells showed an increase and 4 cells showed a decrease in K^+ current amplitude, in the

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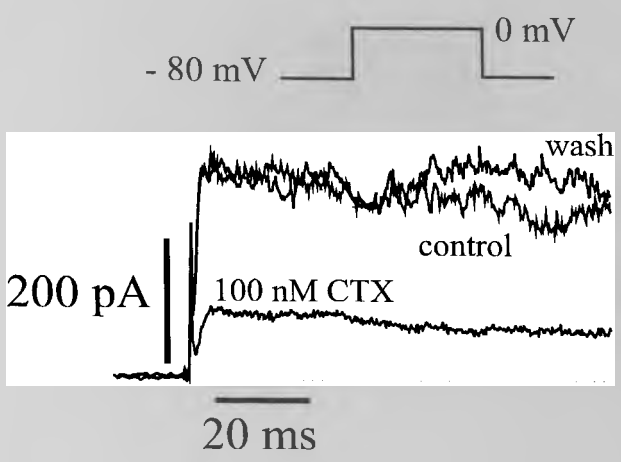

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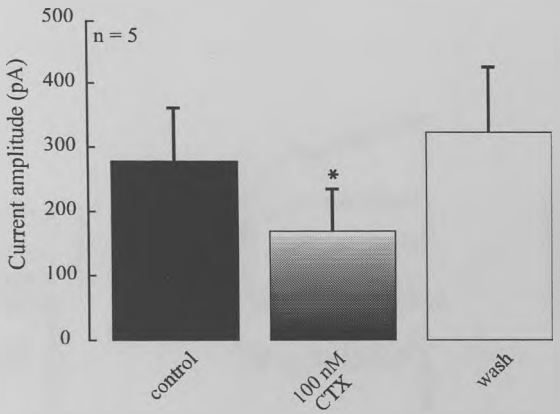

Fig. 2.9. *a Inhibition of K^+ currents by CTX. Representative current traces from an individual cell illustrate the CTX-induced inhibition of outward K^+ current. At 100 nM, CTX caused a substantial block of I_{sus}, and upon washing the drug out the current returned to its previous level. CTX was applied to the cell 5 min prior to recording. Currents were recorded by stepping to 0 mV for 100 ms from a holding potential of –80 mV, with an interpulse interval of 5 s. The dotted line indicates the zero current level (I = 0). b Mean results from 5 cells showing that CTX significantly attenuated I_{sus} and that responses to CTX were recovered on washing. * Significantly different from control, $P < 0.05$. Data are expressed as mean ± S.E.M. (n = 5).*

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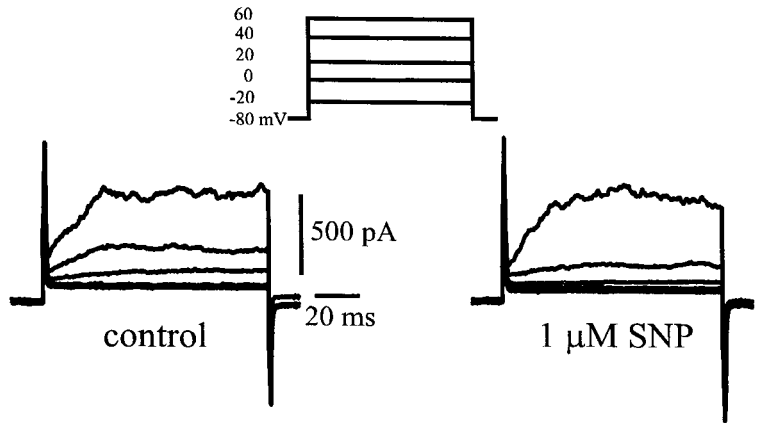

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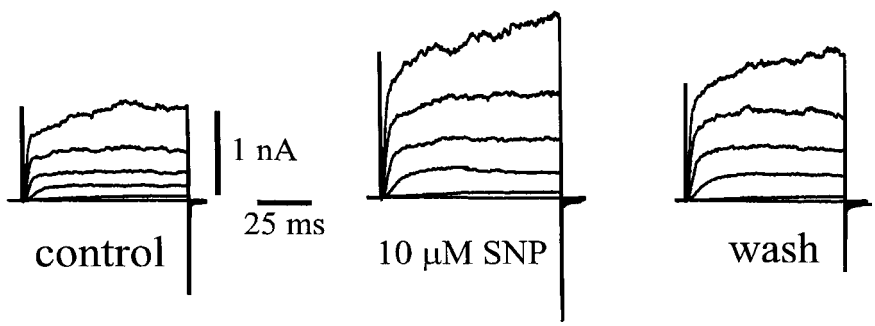


Fig. 2.10. *Effect of SNP on whole-cell K^+ currents. Current traces were obtained in control conditions, then from the same cell following SNP application at 1 μM (a) or 10 μM (b) and following washout of the drug. Currents were elicited by 100 ms depolarising steps to various test potentials from a holding potential of -80 mV, with an interpulse interval of 5 s. SNP was applied to the bath in the superfusate. Currents measured in the presence of 1 μM SNP were generally similar to control. In the example shown, 10 μM SNP reversibly enhanced current amplitude, but this was not consistently observed. The voltage protocol is displayed above the current traces.*

presence of 1 μM SNP. Similarly, at 10 μM, SNP increased the K^+ current amplitude in 10 cells, but reduced the current amplitude in 5 cells. Recovery after washout of SNP was achieved in only 2 cells following an increase in current amplitude. Thus, both increases and decreases in K^+ current amplitude were seen, with no clear overall effect apparent. When examining the mean effects on these cells, SNP at 1 μM or 10 μM did not significantly ($P > 0.05$) affect I_{sus} relative to control. Mean I_{sus} before treatment was measured as 220 ± 18 pA at 0 mV, whereas the mean current amplitude in the same cells in the presence of 1 μM SNP was 190 ± 11 pA (n = 9). Similarly, mean I_{sus} before treatment with 10 μM SNP was measured as 250 ± 45 pA at 0 mV, compared with a mean current amplitude in the presence of SNP of 258 ± 53 pA and 264 ± 32 pA (n = 15) following washout of the drug. Mean I_t was also measured in the presence and absence of SNP (1 μM and 10 μM) and as with I_{sus}, SNP did not significantly ($P > 0.05$) affect the mean peak current amplitude at either concentration. Mean I_t before treatment was 117 ± 47 pA at 0 mV, compared with a mean current amplitude in the presence of 1 μM SNP of 128 ± 64 pA (n = 9). Similarly, mean I_t before treatment with 10 μM SNP was 239 ± 45 pA at 0 mV, compared with a mean current amplitude in the presence of SNP of 238 ± 54 pA and 245 ± 67 pA following washout of the drug (n = 15). Cells were exposed to SNP (1 μM or 10 μM) for up to 15 min.

The effect of SNP on K^+ channel activity was studied over a range of potentials. The mean current-voltage relationships for I_t and I_{sus}, before and during SNP (1 μM and 10 μM) application, are shown for the voltage range –80 mV to +60 mV in Fig. 2.11.

The current amplitudes recorded during the depolarising pulse (I) were normalised by expressing them as a fraction of the current amplitude recorded at +60 mV under control conditions (I_{60}) and plotted as a function of the membrane potential. From the mean results, it is clear that overall, SNP had no significant effect on K^+ channel activity at either 1 μM (n = 9) or 10 μM (n = 15) at any membrane potential studied. This was true for both I_t and I_{sus}. To be certain that cells were exposed to SNP for long enough to be certain of detecting any potential effect, prolonged exposure times (> 30 min) were often used. Recovery was difficult to obtain under these conditions as cells were often lost before wash was complete. For this reason, current amplitudes following washout of 1 μM SNP are not included in Fig. 2.11a.

2.3.4. Caffeine-induced mobilisation of Ca^{2+} from the SR

Application of caffeine to SMCs stimulated the rapid activation of a transient current. Fig. 2.12 shows typical examples of the currents induced in cells held at 0 mV and -60 mV. Under the experimental conditions employed, caffeine induced an outward current at 0 mV following Ca^{2+} release from the SR. At -60 mV, caffeine induced an inward current. Current rose to its peak level in 19 ± 1 s (n = 10) and 22 ± 1 s (n = 5), in cells held at 0 mV and –60 mV, respectively. Recovery to basal current levels followed a single exponential time course, the mean time constant of which was 38 ± 3 s (n = 10; 0 mV) and 46 ± 4 s (n = 5; -60 mV). The rise to peak current and return to baseline did not differ significantly between –60 mV and 0 mV. To allow for replenishing of the stores, intervals of 5 min were imposed between consecutive

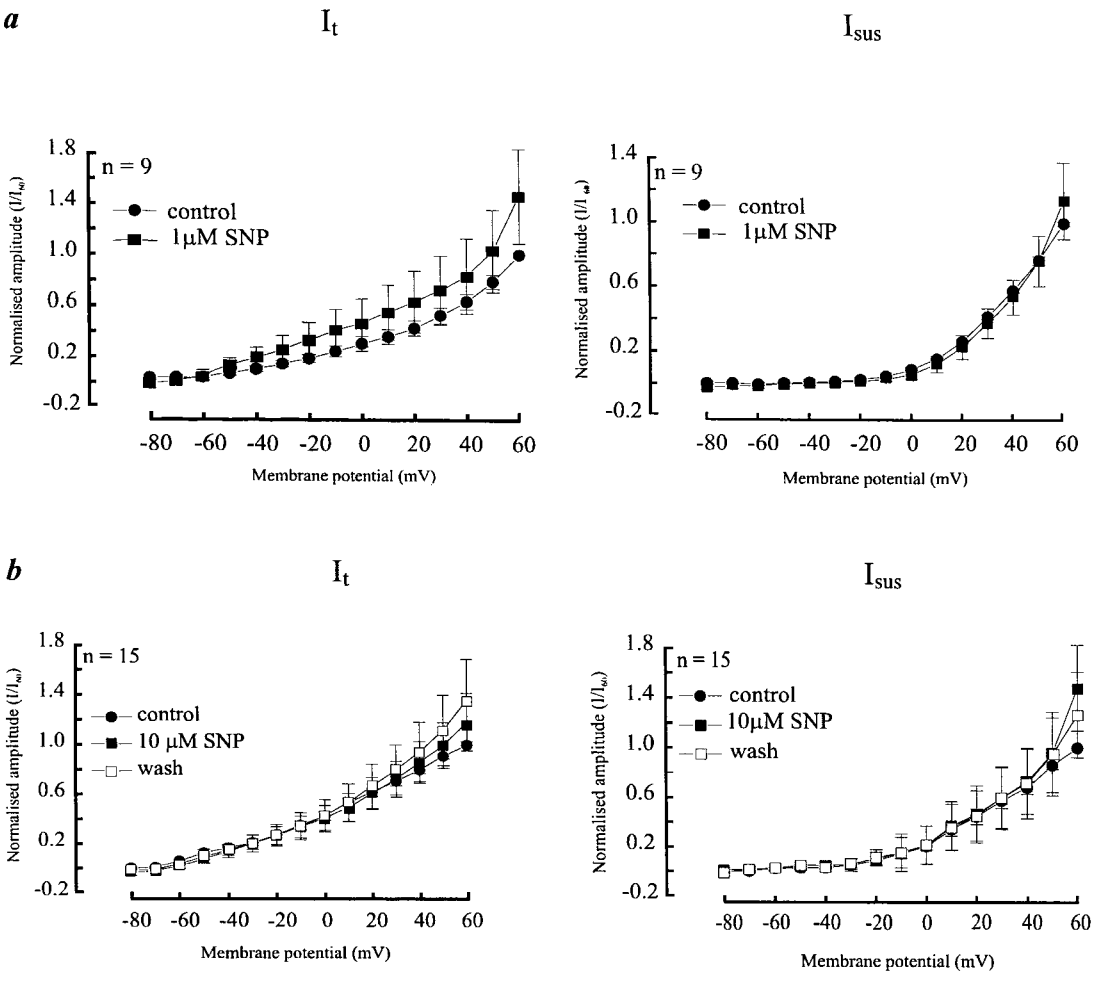
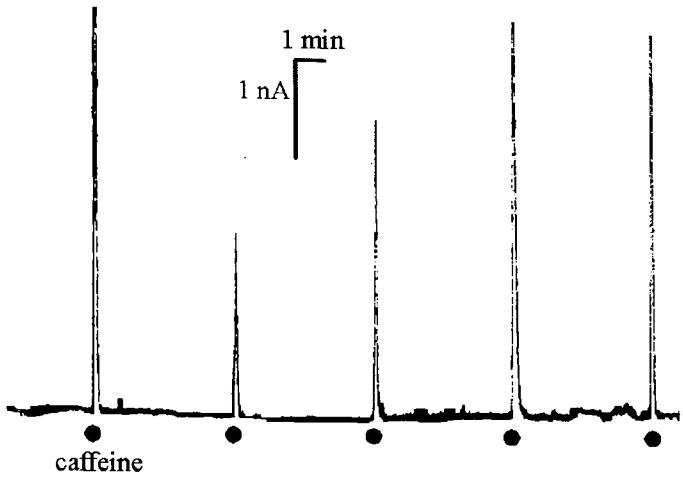


Fig. 2.11. *Influence of SNP on I_t and I_{sus}. The current-voltage plots are shown for I_t (left hand panel) and I_{sus} (right hand panel) in the absence and presence of 1 μM SNP (a) and 10 μM SNP (b). SNP had no effect apparent over the entire range of step potentials applied. Data are expressed as the mean ± S.E.M. (n = 9 and 15).*

a



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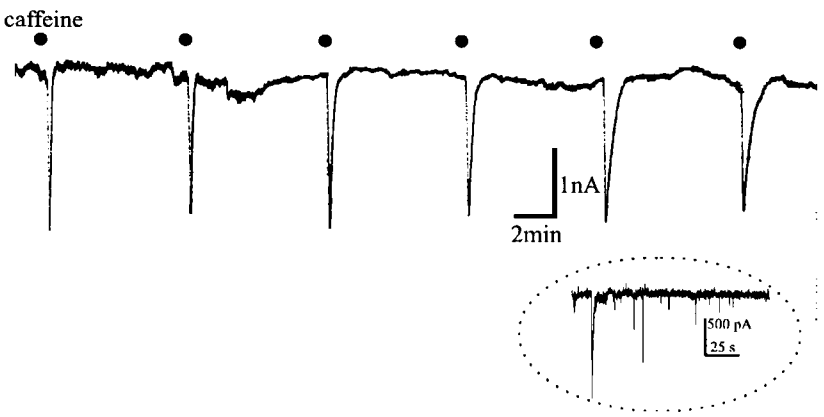
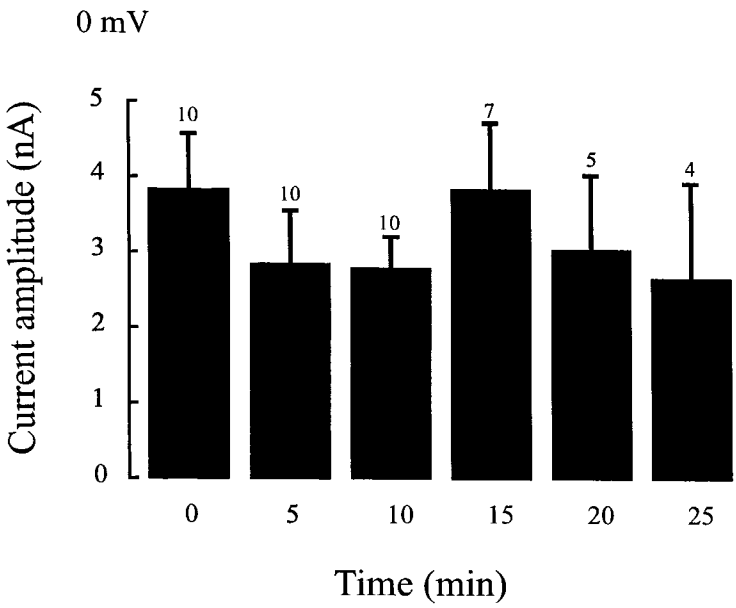


Fig. 2.12. *Representative whole-cell membrane current responses to caffeine in single rabbit aortic SMCs held under voltage-clamp at 0 mV (a) and -60 mV (b). Caffeine was applied at the times indicated (•). Outward currents in response to caffeine application indicate the transient increase in* $[Ca^{2+}]_i$ *in relation to* BK_{Ca} *channels, and inward currents indicate the transient increase in* $[Ca^{2+}]_i$ *in relation to* Cl_{Ca} *channels. Inset shows an example of spontaneous transient inward currents (STICs). Application of caffeine evoked reversible and reproducible current responses.*

caffeine applications. Repeated applications of caffeine over a period of 30 min elicited responses that were similar in amplitude and shape compared to the initial responses. Cells could not be held in voltage-clamp for longer than 30 min. A few experiments were carried out to determine the minimum time interval allowed between consecutive caffeine applications to refill the stores. Repeated caffeine application at intervals shorter than 5 min progressively suppressed responses to caffeine. Only those cells exhibiting repeated caffeine responses of similar magnitude were selected. There was no significant ($P > 0.05$) change in the mean, peak current amplitude with time following repeated caffeine application (Fig. 2.13.). Since it was impossible for some cells to be held long enough to obtain a large number of repeated responses and the current amplitudes varied widely from cell to cell, the differences in current responses were analysed by normalising current amplitudes against the first response in each cell, which was set at 100 %. The reproducibility measured in this way is illustrated in Fig. 2.14 where the mean caffeine responses (plotted as % 1^{st} response) from cells held at 0 mV or -60 mV are plotted as a function of time. The large error bars clearly illustrate that there was variability among caffeine responses in individual cells, but overall there was no significant change with time. In a few cells STOCs or STICs could be seen at 0 mV or -60 mV, respectively, as illustrated in Fig. 2.12b. Generally STOC or STIC activity was abolished in the presence of caffeine.

a



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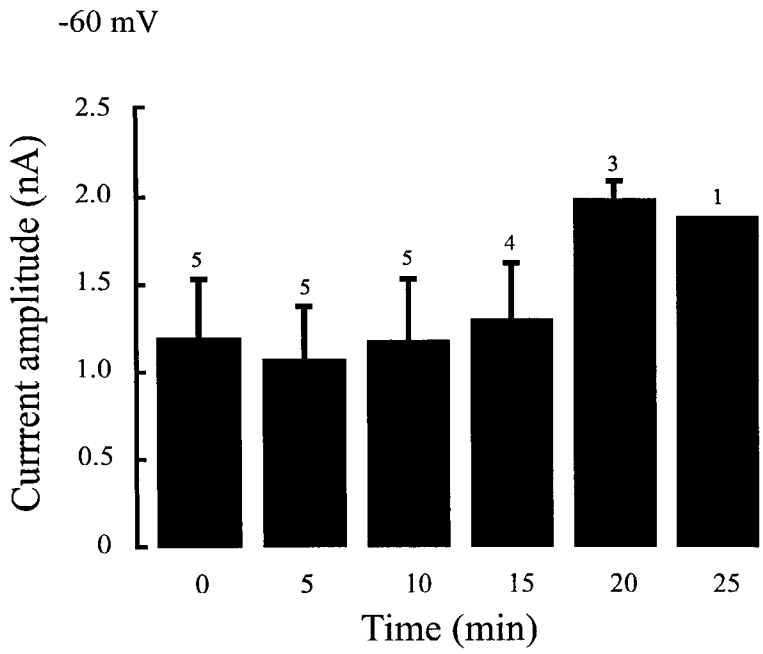


Fig. 2.13. *Mean current amplitude of consecutive responses to caffeine applications repeated at 5 min intervals, in cells held under voltage-clamp at 0 mV (a) and -60 mV (b). Current amplitudes are expressed as mean ± S.E.M. Values above S.E.M. bars indicate the total number of cells tested.*

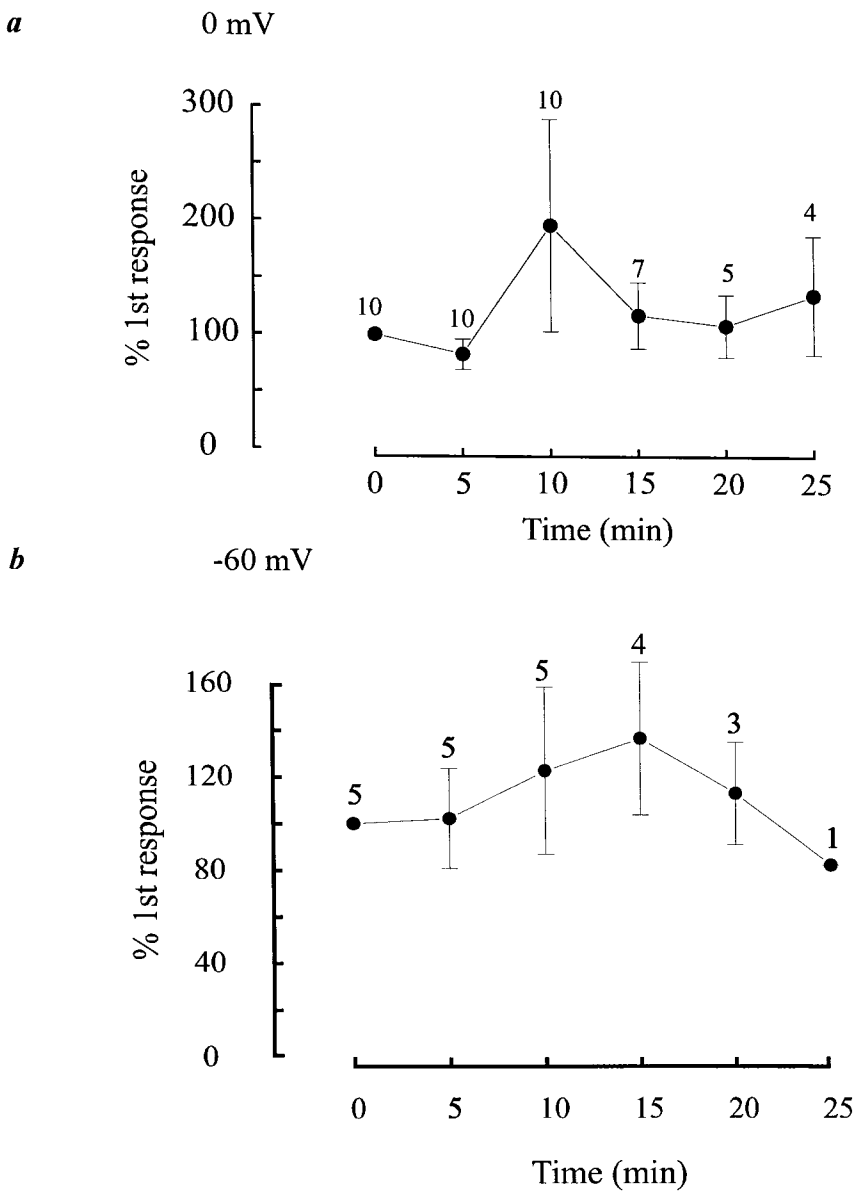


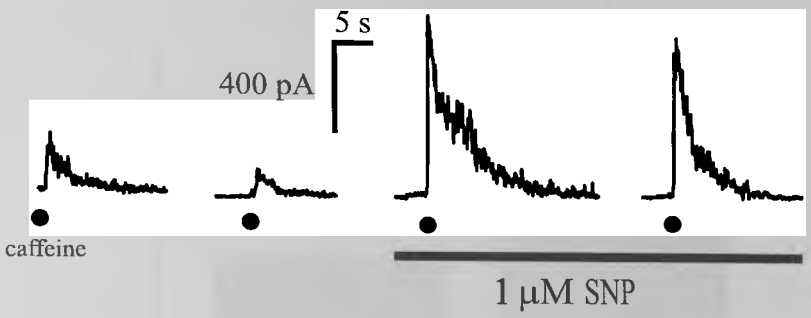
Fig. 2.14. *Mean responses to caffeine as a function of the time following the first application. Each response is plotted as a percent of the first response in cells held at 0 mV (a) and –60 mV (b). The response was measured as the peak amplitude of the transient current activated by caffeine. There were no significant differences between responses to consecutive caffeine applications. Points and bars represent the mean ± S.E.M. of 3-10 cells. Values above S.E.M. bars indicate the total number of cells tested.*

2.3.5. Effect of SNP on caffeine-induced outward currents

To investigate the effect of SNP on $[Ca^{2+}]_i$ mobilisation from the SR by caffeine, caffeine-induced currents were recorded in the presence and absence of 1 μM or 10 μM SNP at 0 mV. In the example shown in Fig. 2.15a, caffeine responses were increased in the presence of 1 μM SNP by 233 %. This was not seen when the SNP concentration was increased to 10 μM. In another cell, illustrated in Fig. 2.15b, 10 μM SNP reduced the response to caffeine by 95 %, but the effect did not develop immediately. Generally, the response to the first application of caffeine in the presence of 10 μM SNP was only slightly reduced, but a second caffeine application, 5 min later, showed a larger percent decrease in outward current amplitude.

Fig. 2.16 illustrates the mean effect of SNP at 1 μM and 10 μM on the outward current response to caffeine at 0 mV. In cells held at 0 mV the current was significantly ($P < 0.05$) enhanced in the presence of 1 μM SNP by 77 ± 7 % ($n = 5$). The mean current amplitude recorded in the presence of 1μM SNP was 1.1 ± 0.5 nA, which was 1.7-fold larger than the mean current amplitude recorded in control conditions (622 ± 282 pA). Recovery was observed in 1 cell where washout of SNP was achieved. All other cells were unable to be held long enough to wash the drug off. Although the overriding effect of 1 μM SNP was enhancement of the response to caffeine, the response decreased in 2 of the 7 cells examined. Under the same experimental conditions, when the SNP concentration was raised to 10 μM, membrane current responses to caffeine were significantly ($P < 0.05$) reduced by 40

a



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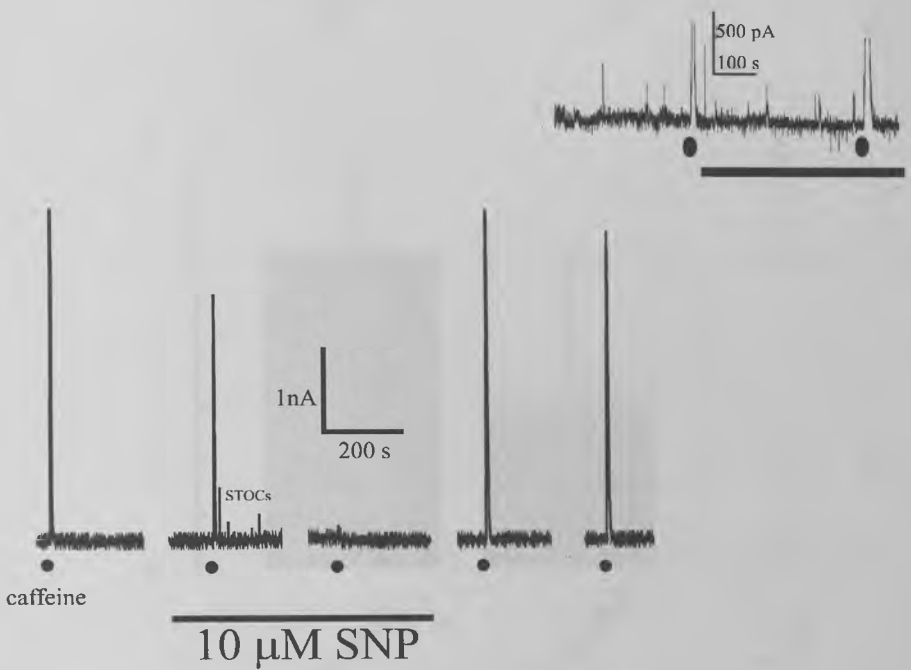
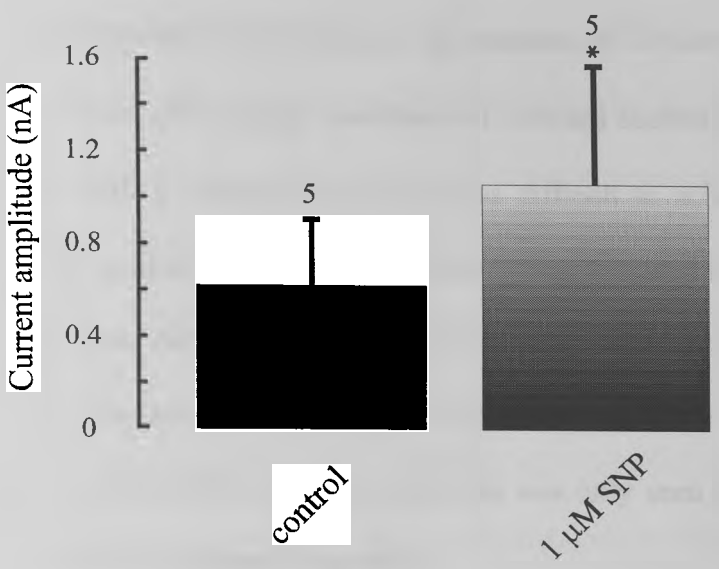


Fig. 2.15. *Effect of SNP on caffeine-induced outward currents in cells held at 0 mV. At 1 μM (a), SNP increased, whereas at 10 μM (b), SNP decreased, the caffeine-induced outward currents. SNP was applied for the duration indicated by the horizontal bars and for 5 min before testing caffeine. Gaps in the records represent 5 min recovery periods between caffeine applications. Inset shows STOCs in the absence and presence of 10 μM SNP. Caffeine was applied at the times indicated (•).*

a



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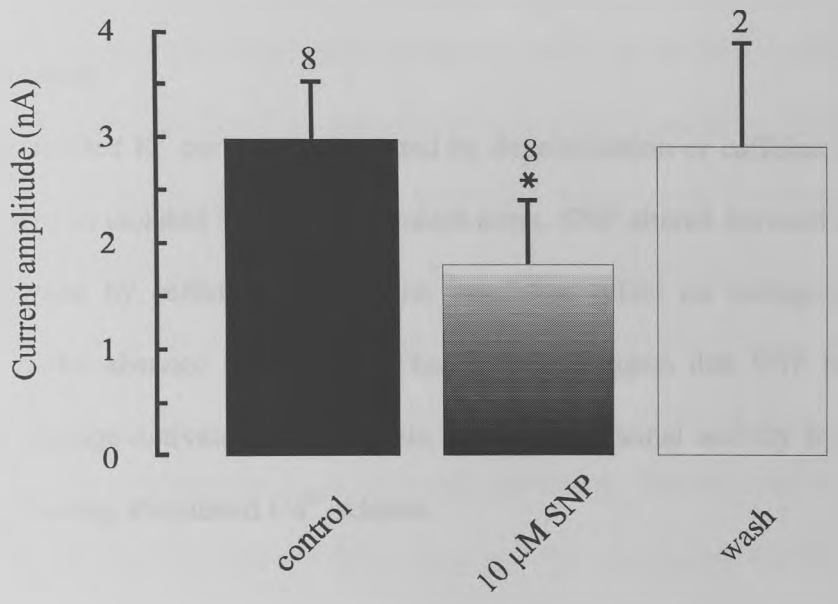


Fig. 2.16. *Peak amplitude of caffeine-induced outward current at 0 mV were significantly increased by 1 μM SNP and significantly decreased by 10 μM SNP. * Significantly different from control, $P < 0.05$. Current amplitudes are expressed as mean ± S.E.M. (n =2-8). Values above S.E.M. bars indicate the total number of cells tested.*

± 8 % (n = 8). The mean current amplitude recorded in control conditions was 3.0 ± 0.5 nA compared with 1.8 ± 0.6 nA in the presence of 10 μM SNP. In 4 out of 8 cells tested, 10 μM SNP largely abolished all outward current responses to caffeine at 0 mV. As with 1 μM SNP, recovery was difficult to achieve due to the long recording time needed. However, full recovery was achieved in 2 cells on return to drug-free solution. An increase in STOC discharge frequency and amplitude was seen in 2 cells exposed to 10 μM SNP (Fig 2.15 (inset)). Since STOCs were present in these cells prior to SNP perfusion and this was only seen in a small number of cells, it was difficult to quantify the effect.

2.4. Discussion

Voltage-activated K^+ currents, stimulated by depolarisation or caffeine, were shown to be present in isolated SMCs of the rabbit aorta. SNP altered outward currents that were activated by caffeine, but had no consistent effect on voltage-activated K^+ currents in the absence of caffeine. These results suggest that SNP has no direct effect on voltage-activated K^+ channels, but alters channel activity by modulating $[Ca^{2+}]_i$ following stimulated Ca^{2+} release.

2.4.1. BK_{Ca} contributes to voltage-activated K^+ current in aortic smooth muscle cells

The classical K^+ channel blocking drugs TEA and CTX (Nelson and Quayle, 1995; Giminez-Gallego et al., 1988) were used to demonstrate that BK_{Ca} channels contribute to the outward K^+ current activated by depolarisation in rabbit aortic SMCs. The voltage-dependence and high noise during the depolarising pulse are

characteristic of BK_{Ca} currents, which have been previously identified in a number of VSMCs, including the rabbit aorta (Benham et al., 1986; Sadoshima et al., 1988; Beech and Bolton, 1989; Rusko et al., 1992; Smirnov and Aaronson, 1992; Halliday et al., 1995; Cotton et al., 1997). Moreover, in my experiments the majority of I_{sus} was consistently suppressed by TEA and CTX, with a concomitant reduction in current noise at concentrations expected for the BK_{Ca} channel (Benham and Bolton, 1986; Halliday et al., 1995). A low concentration of EGTA was used in the pipette solution to minimise cytosolic Ca^{2+} buffering and promote BK_{Ca} channel activity, because I was particularly interested in investigating the potential modulation of this channel by SNP. Approximately 60 % of the outward current at 0 mV was blocked by TEA. Previously published information on rabbit aortic SMCs (Halliday et al., 1995) showed that 40 % of I_{sus} was sensitive to TEA when using a higher (1 mM) pipette EGTA concentration. Thus the lower Ca^{2+} buffering in the present study did result in greater BK_{Ca} channel activity. At the concentration used in this study TEA does, however, additionally block subtypes of K_v channels (Vogalis et al., 1993; Grissmer et al., 1994; Patel et al., 1997). Therefore, some of the TEA-sensitive current may be attributable to K_v channel activation. Studies with CTX, a more potent and selective inhibitor of BK_{Ca} channels, than TEA, showed 40 % inhibition of I_{sus}. The discrepancy between the TEA- and CTX-sensitive current may be explained by effects on channels other than BK_{Ca}. In addition to inhibiting BK_{Ca} channels, both drugs have previously been shown to block $K_v1.2$, $K_v1.3$ and $K_v2.1$ channels (Grissmer et al., 1994), which are present in VSM (Patel et al., 1997; Clapp and Tinker, 1998). So 40 % may be an overestimate of the contribution of BK_{Ca} channels to the voltage-activated current. Consistent with the results of Halliday et

al. (1995), a residual outward current, consisting of transient and sustained components, remained following treatment with TEA or CTX. These currents were previously characterised by Halliday et al. (1995) as 'A'-type and delayed rectifier K^+ currents, respectively.

2.4.2. SNP does not directly activate voltage-gated K^+ current in aortic smooth muscle cells

Although it was established that BK_{Ca} channels contributed to the depolarisation-activated K^+ current, SNP consistently failed to modulate the voltage-activated K^+ current at all potentials studied. Neither I_t nor I_{sus} were affected at 1 or 10 μM SNP. It is feasible that BK_{Ca} channels were already maximally activated during depolarisation due to low Ca^{2+} buffering. On the other hand, 1 μM SNP potentiated the K^+ currents activated by caffeine at 0 mV. Evidence from the literature also argues against this. BK_{Ca} channels have been shown to be modulated by NO in VSMCs studied with the perforated-patch technique where there would be less interference with cytosolic Ca^{2+} buffering than in the present study (Mistry and Garland, 1998). The present results therefore contradict previous studies that implicated both a direct and cGMP-dependent activation of K^+ channels in NO-mediated vascular relaxation, including rabbit aorta (Hamaguchi et al., 1992; Bolotina et al., 1994; Archer et al., 1994; Yuan et al., 1996; Lang and Watson, 1998; Mistry and Garland, 1998).

Evidence was presented for direct BK_{Ca} channel activation by NO in excised membrane patches of rabbit aortic SMCs (Bolotina et al., 1994). However, since it

has been shown that a functional Ca^{2+} store can be retained in excised patches (Xiong et al., 1992), it is feasible that apparent K^+ channel activation in excised patches could have occurred as a consequence of Ca^{2+} released from a local store. Consistent with this suggestion, highly localised increases in $[Ca^{2+}]_i$ (Ca^{2+} sparks) resulting from SR Ca^{2+} release, can activate BK_{Ca} channels in VSMCs (Nelson et al., 1995). Bolotina et al. (1994) also provided evidence for the involvement of BK_{Ca} channel activation in NO-induced relaxation of arterial rings, by both exogenous NO and endothelium-derived NO. The mechanism was suggested to be independent of cGMP, because relaxations to NO that persisted in the presence of the GC inhibitor, methylene blue (MB), were blocked by CTX. However, MB is not a selective inhibitor of GC. In particular, it can directly activate BK_{Ca} channels in human mesangial cells (Stockand and Sansom, 1996). Thus, the ability of CTX to block relaxation in the presence of MB is not convincing evidence in favour of cGMP-independent activation of BK_{Ca} channels by NO. Moreover, the proposed contribution of direct BK_{Ca} channel activation to the NO-dependent relaxation was small and it appeared to prevail only in diseased i.e. atherosclerotic arteries (Bolotina et al., 1994). Other studies found no definite effect of CTX on relaxations to SNP, GTN or 8-Br-cGMP on the rabbit aortic ring preparation, although it did inhibit relaxation in bovine tracheal smooth muscle and rat pulmonary artery (Hamaguchi et al., 1992; Archer et al., 1994). Some of the inhibitory effects of K^+ channel blockers on NO-induced relaxation may reflect physiological antagonism. For example, TEA and CTX are known to enhance agonist-induced depolarisation and contraction in VSM (Brayden and Nelson, 1992; Plane et al., 1998), presumably because BK_{Ca}

channels are activated by the rise in $[Ca^{2+}]_i$ during contraction. This would have a negative-feedback effect by hyperpolarising SMCs and limiting further contraction.

NO and SNP (5-10 μM) were also suggested to activate K_v channels in isolated SMCs and excised membrane patches of rat pulmonary artery (Archer et al., 1994; Yuan et al., 1996), because they activated a K^+ current that was blocked by 4-AP, which also significantly inhibited NO-induced relaxation of isolated pulmonary arteries (Archer et al., 1994; Yuan et al., 1996). However, 4-AP incompletely reversed the NO-induced relaxation (Yuan et al., 1996) suggesting that the activation of K_v channels was not the sole mechanism responsible for NO-induced relaxation.

My results are consistent with several studies that concluded that K^+ channel activation is not a major mechanism of NO-induced relaxation. Thus, Hamaguchi et al. (1992) and Bialecki and Stinson-Fisher (1995) showed that not only CTX, but the more selective BK_{Ca} channel blocker iberiotoxin, had little effect on the relaxant effects of NO donors on rabbit aorta, although they did reduce the response on other smooth muscles (Hamaguchi et al., 1992). In the present study SNP failed to activate BK_{Ca} or K_v channels even at 10 μM. This is a high concentration, more than needed to cause maximal relaxation (Karaki et al., 1988). Thus, K^+ channel activation is unlikely to contribute to NO-induced relaxation of rabbit aorta.

2.4.3. Influence of SNP on caffeine-induced Ca^{2+} transients

Caffeine activated outward current at 0 mV and inward current at –60 mV. At 0 mV the dominant outward current was carried by K^+ channels (Komori and Bolton, 1989). Although Cl^- channels would also be open at this potential, there would be no net Cl^- current, because this is close to the Cl^- equilibrium potential of -2 mV. At -60 mV the dominant current was carried by Cl_{Ca} channels (Byrne and Large, 1988; Gurney et al., 2000). Whether recording K^+ or Cl^- current, responses were reproducible during repeated caffeine application. In a few experiments STOCs or STICs were seen, which are believed to represent the simultaneous opening of several hundred or so BK_{Ca} or Cl_{Ca} channels, respectively, due to the spontaneous release of Ca^{2+} from the SR (Benham and Bolton, 1986; Wang et al., 1992). In each case STOCs or STICs were abolished following caffeine application, due to the depletion of Ca^{2+} from the SR.

Responses to caffeine at 0 mV were significantly enhanced in the presence of 1μM SNP. This did not reflect an effect on BK_{Ca} channels, because it was shown in this study that SNP had no effect on these K^+ currents in the same cells. The simplest explanation is that there was an increase in $[Ca^{2+}]_i$ in the vicinity of the BK_{Ca} channels. This in turn suggests that caffeine released a larger amount of Ca^{2+}. This could have resulted if SNP increased either Ca^{2+} uptake into and/or Ca^{2+} release from the store, although the former more obviously correlates with muscle relaxation. There is however, substantial evidence in support of both mechanisms. Experiments carried out in skinned cultured aortic SMCs demonstrated an increase in the rate of $^{45}Ca^{2+}$ uptake and the content of the SR by cGMP (Twort and van Breemen, 1988;

van Breemen et al., 1988). Karaki et al. (1988) similarly demonstrated in rat aortic smooth muscle that SNP lowered $[Ca^{2+}]_i$ but could also augment agonist-induced transient contractions. This is further supported by evidence from rat portal vein (Komori and Bolton, 1989) and rat aortic smooth muscle (Cornwell et al., 1991). In the former study, introduction of cGMP into the cell or application of 8-Br-cGMP increased STOC discharge and outward current responses to caffeine. SNP was also found to increase STOC discharge in pulmonary arterial SMCs (Clapp and Gurney, 1991a). This likely reflected an increase in the frequency of Ca^{2+} sparks as shown in myocytes isolated from rat coronary and cerebral arteries (Porter et al., 1998). In view of the evidence that STOCs result from overloaded Ca^{2+} stores (Benham and Bolton, 1986), the increased frequency of STOCs may reflect increased Ca^{2+} uptake and fuller stores (ZhuGe et al., 1999). This would explain the observed inhibition of STOCs and Ca^{2+} sparks in SMCs when the stores were depleted by caffeine (Bolton and Lim, 1989; Stehno-Bittel and Sturek, 1992; Ganitkevich and Isenberg, 1995) or by ryanodine, or the SR Ca^{2+}-ATPase inhibitor thapsigargin (TG) (Nelson et al., 1995). Others have noted that STOC discharge is increased when stores are loaded (Stehno-Bittel and Sturek, 1992; ZhuGe and Fay, 1997; ZhuGe et al., 1999) and STOC discharge was correlated with refilling of the stores (ZhuGe and Fay, 1997). SNP increased SR $[Ca^{2+}]$, STOC activity and the rate of refilling of the stores (ZhuGe and Fay, 1997), suggesting that SR $[Ca^{2+}]$ may act as a determinant of STOC generation in SMCs.

Other investigators suggested that PKG regulates Ca^{2+}-ATPase activity in aortic smooth muscle SR via phosphorylation of phospholamban (Cornwell et al., 1991), a

regulatory protein that inhibits the SR Ca^{2+}-ATPase activity. In this study, SNP was shown to increase the phosphorylation of phospholamban, which presumably leads to the reduction of $[Ca^{2+}]_i$ by stimulating SR Ca^{2+} uptake. In addition, confocal laser scanning microscopy provided evidence that PKG was localised to the same cellular region(s) as phospholamban, in areas corresponding to the SR (Cornwell et al., 1991).

The concentration of caffeine used in the present study releases the entire store (Gurney and Allam, 1995; Ji et al., 1998), therefore, the responses to caffeine reflected the maximum rise in $[Ca^{2+}]_i$ caused by releasing the complete store. Since SNP increased the caffeine response, it is therefore reasonable to propose that it increased the amount of stored Ca^{2+}. Moreover, tension studies in a variety of vascular preparations indicate that augmented sequestration of Ca^{2+} by the SR Ca^{2+}-ATPase is important for elicitation of relaxation by cGMP-dependent nitrovasodilators (Luo et al., 1993; Andriantsitohaina et al., 1995; Khan et al., 1998). In each case, SR Ca^{2+}-ATPase inhibitors were shown to produce a pronounced inhibition of nitrovasodilator relaxations.

The alternative possibility that the potentiation of caffeine-induced BK_{Ca} current by SNP may be due to increased Ca^{2+} release from the SR cannot be discounted. It is possible that the rate of Ca^{2+} release was enhanced while the rate of Ca^{2+} removal remained unchanged, such that a larger peak response was reached. Microfluorimetric and video fluorescence imaging techniques have demonstrated that the release of stored Ca^{2+} through SR ryanodine receptors can be stimulated by

NO and NO donors in a variety of preparations, including, interstitial cells in the mammalian gut (Publicover et al., 1993), rat pancreatic β cells (Willmott et al., 1995) and sea urchin eggs (Willmott et al., 1996a). There is also evidence that such an action may be mediated via a signal transduction pathway involving cADPR (Willmott et al., 1996a; Clementi and Meldolesi, 1997). This may involve activation of cGC with the resulting accumulation of cGMP and activation of PKG, which then phosphorylates ADP-ribosyl cyclase (regulatory enzyme), resulting in an increase in cADPR levels (Willmott et al., 1996a). cADPR may then bind to the ryanodine receptor leading to its opening and subsequent Ca^{2+} release. An increase in Ca^{2+} release would only be consistent with a vasodilatory effect of SNP if the increase was localised near the plasma membrane and was rapidly removed from the cell by the plasma membrane Ca^{2+}-ATPase or Na^{+}/Ca^{2+} exchange. There is evidence that this can happen. Confocal imaging studies by Nelson et al. (1995) have suggested that local, transient increases in $[Ca^{2+}]_i$, resulting from the activation of the ryanodine receptor in the SR, may lead to arterial dilation by activating BK_{Ca} channels and causing membrane hyperpolarisation. However, Publicover et al. (1993) showed that although SR Ca^{2+} release was stimulated by NO in interstitial cells, it did not occur in SMCs from the same colon preparation.

When the concentration of SNP was increased to 10 μM, transient responses to caffeine were substantially suppressed, and in some cells, completely abolished. Generally, the response to a second application of caffeine in the presence of SNP was suppressed more than the first, suggesting a slower onset of this effect. These results suggest that higher concentrations of SNP inhibit Ca^{2+} release from the store.

This is consistent with several previous observations. In isolated skeletal muscle SR, NO, generated either by the exogenous NO donors, SNAP, SNP and SIN-1, or enzymatically from L-arginine, was shown to reduce the rate of Ca^{2+} release and the open probability of single ryanodine receptors (Meszaros et al., 1996; Aghdasi et al., 1997). The ryanodine receptor of skeletal muscle (*ryr1*) shares common properties with that of the smooth muscle counterpart (*ryr3*) (Devine et al., 1972; Imagawa et al., 1987; Iino, 1989; Lesh et al., 1998). Moreover, SNAP was also shown, using video fluorescence imaging, to reduce the intracellular Ca^{2+} response to caffeine in porcine tracheal SMCs (Kannan et al., 1997). Therefore, at high concentrations, SNP may inhibit SR Ca^{2+} release in rabbit aortic SMCs by blocking the ryanodine receptor/channel or reducing the Ca^{2+} sensitivity of the channel, such that it is no longer activated at physiological $[Ca^{2+}]_i$. The inhibitory effect of SNP on the caffeine response was observed at a concentration comparable to the NO donors used in other studies (Meszaros et al., 1996; Aghdasi et al., 1997; Kannan et al., 1997).

SNP is known to almost completely relax aortic smooth muscle at 1 μM (Karaki et al., 1988) and at this concentration it appeared to enhance store filling, so that caffeine released a greater amount of Ca^{2+} from the store. Although NO and NO donors can inhibit SR Ca^{2+} release this effect appeared to become important in rabbit aortic SMCs only at relatively high concentrations. In fact in most studies showing inhibition of SR Ca^{2+} release by NO donors, the drugs were applied at high concentrations (10 μM-1 mM). The exception is Kannan et al. (1997) who found this effect in porcine trachea at relatively low concentrations (1 μM).

Although stimulated Ca^{2+} uptake could lead to overloading of the Ca^{2+} stores and increased STOC discharge, inhibition of Ca^{2+} release from the SR could have the same effect. Both mechanisms would tend to lower $[Ca^{2+}]_i$. It is likely therefore that at high concentrations of NO or NO donors, increased uptake and reduced release work together to keep $[Ca^{2+}]_i$ low. Inhibition of release may also occur at lower SNP concentrations in rabbit aortic SMCs, but was not apparent due to the predominant effect on Ca^{2+} uptake. It was only when the effect on release became stronger that the response to caffeine was reduced, despite the increased store filling caused by increased SR Ca^{2+} uptake.

In conclusion, SNP had no direct effect on BK_{Ca} channel activity, but concentration-dependently altered BK_{Ca} currents that were activated by caffeine, implying that they did this by altering $[Ca^{2+}]_i$ storage. It is proposed that at 1 μM, SNP stimulated Ca^{2+} accumulation by the SR, but at higher concentrations it inhibited Ca^{2+} release through ryanodine receptors. Both mechanisms would lead to reduced $[Ca^{2+}]_i$ and relaxation.

Chapter 3

Role of SR Ca^{2+}-ATPase in the regulation of $[Ca^{2+}]_i$ in rabbit aortic smooth muscle cells following stimulation with caffeine or noradrenaline

3.1 Introduction

The SR is likely to make the largest contribution to Ca^{2+} removal from the SMC after cell activation (Kargacin and Fay, 1991; Ganitkevich and Isenberg, 1992). From direct measurements of Ca^{2+} uptake, the SR of smooth muscle may be expected to lower intracellular Ca^{2+} at a rate ~ 45-75 % of the rate of Ca^{2+} removal from the cytoplasm seen in cells during the declining phase of intracellular Ca^{2+} transients (Kargacin and Kargacin, 1995). However, the relative contribution of the SR to the contraction-relaxation cycle depends on several factors that may vary in specific VSMCs, such as the relative size of the SR and the amount of Ca^{2+} stored.

The SR contains two structurally and functionally distinct classes of intracellular Ca^{2+} release channels, the ryanodine receptor/channel, and the IP_3 receptor/channel, which are essential for Ca^{2+} mobilisation. Ca^{2+} can be released from the SR through either of these two receptor-operated mechanisms/Ca^{2+} release channels. Ryanodine receptor/release channels are functionally distinct from IP_3, which are activated by agonists such as phenylephrine (PE), NA, or low concentrations of intracellularly applied IP_3 (Somlyo et al., 1985). The IP_3 channel is thought to be the major channel mediating pharmacomechanical coupling in VSM (Somlyo et al., 1985; Chadwick et al., 1990).

The contribution of CICR and IICR to SR Ca^{2+} release has been suggested to vary in different smooth muscle types. From measurements of contractile force and stimulated ^{45}Ca release, the caffeine- and IP_3-sensitive stores have been suggested to overlap completely in rabbit aorta (Leijten and van Breemen, 1984). Thus, both

CICR and IICR release the same pool of Ca^{2+} when stimulated, although Ca^{2+} leaves the SR through different channels. Thus, agents that modify Ca^{2+} accumulation by the SR Ca^{2+}-ATPase might be expected to alter the amount of Ca^{2+} released by IP_3-dependent agonists, such as NA, as well as caffeine, as well as influencing the rate of Ca^{2+} removal from the cytoplasm. In contrast to these results, at least two physically separated Ca^{2+} storage compartments, distinguishable by their location, and mechanism of Ca^{2+} release, IP_3 sensitivity and Ca^{2+} affinity, have been reported to in rat aortic and porcine aortic VSMCs (Kanaide et al., 1987; Missiaen et al., 1991).

A greater understanding of how Ca^{2+} is regulated by the SR Ca^{2+}-ATPase has been achieved with specific pharmacological probes. The naturally occurring sesquiterpene lactone, TG, and an indole tetramic acid metabolite of *Aspergillus* and *Penicillium*, cyclopiazonic acid (CPA), selectively inhibit the SR Ca^{2+}-ATPase (Seidler, 1989; Thastrup, 1990; Thastrup et al., 1990; Lytton et al., 1991; Uyama et al., 1992). It has been suggested that CPA competitively inhibits ATPase activity, as increasing ATP concentrations counteracted its effect (Seidler et al., 1989). In contrast TG interacts irreversibly and stoichiometrically with the SR Ca^{2+}-ATPase, thereby irreversibly inhibiting Ca^{2+} uptake (Lytton et al., 1991). These properties are in accordance with contractility studies that reported TG and CPA inhibit repletion of the PE-sensitive store in aorta and mesenteric artery (Low et al., 1991; Gurney and Allam, 1995). In the former study, inhibition of SR Ca^{2+} sequestration, presumably leading to elevated $[Ca^{2+}]_i$, was shown to elevate vascular tone in isolated strip preparations and cause cell shortening in single VSMCs. TG and CPA also modulated tension in other smooth muscle preparations (Shima and Blaustein, 1992;

Gibson et al., 1994; Kwan et al., 1994; Takemoto et al., 1998) and inhibited Ca^{2+} sequestration, causing subsequent failure of Ca^{2+} release in permeabilised guinea-pig ileal SMCs (Uyama et al., 1992), guinea-pig ileal and urinary bladder SMCs (Suzuki et al., 1992), and A7r5 VSMCs (Missiaen et al., 1992).

Many additional studies are consistent with the contention that SR Ca^{2+}-ATPase inhibitors enhance vascular tone by elevating $[Ca^{2+}]_i$. For example, using the fluorescent Ca^{2+} indicators indo-1 and fura-2, several laboratories found a variety of cells respond to TG and CPA with a pronounced rise in $[Ca^{2+}]_i$ (Thastrup et al., 1990; Mason et al., 1991; Xuan et al., 1992; Raymond and Wendt, 1996). In addition to inhibiting the SR Ca^{2+}-ATPase, however, TG and CPA have also been shown to effectively release stored Ca^{2+} (Law et al., 1990; Thastrup et al., 1990; Mason et al., 1991; Xuan et al., 1992; Kargacin and Kargacin, 1995). Although the mechanism by which they release Ca^{2+} is not well established, it was suggested to reflect unmasking of a passive leak of Ca^{2+} from the store upon inhibition of the SR Ca^{2+}-ATPase. In several studies, both inhibitors subsequently activated Ca^{2+} entry into the cell (Mason et al., 1991; Xuan et al., 1992; Takemoto et al., 1998). However, increasingly high concentrations of CPA, greater than those required for depletion of the stores, have also been shown to produce a secondary inhibition of Ca^{2+} entry in thymic lymphocytes (Mason et al., 1991). Neither drug has been shown to inhibit the sarcolemmal Ca^{2+}-ATPase or to stimulate IP_3 formation (Seidler et al., 1989; Takemura et al., 1989; Law et al., 1990; Thastrup et al., 1990; Lytton et al., 1991; Mason et al., 1991; Demaurex et al., 1992; Xuan et al., 1992).

The work described in this chapter investigated $[Ca^{2+}]_i$ transients activated by caffeine or NA, and the importance of the SR Ca^{2+}-ATPase for the removal of Ca^{2+} from the cytoplasm of rabbit aortic SMCs. Experiments employed the fluorescent Ca^{2+}-sensitive indicator, fura-2 (Grynkiewicz et al., 1985), to measure $[Ca^{2+}]_i$ in isolated rabbit aortic SMCs, as well as TG and CPA, to selectively inhibit SR Ca^{2+} uptake via the ATPase.

3.2 Materials and Methods

3.2.1 Fura-2 loading

Intracellular free Ca^{2+} concentrations were measured using the fluorescent Ca^{2+}-sensitive dye, fura-2, derived from the Ca^{2+} chelator 1,2-bis (2 aminophenoxy) ethane N, N, N', N'-tetraacetic acid (Tsien, 1980; Grynkiewicz et al., 1985). Cells were loaded by incubation with the membrane permeant acetoxymethyl (AM) ester of fura-2 (fura-2/AM; 5 μM, Molecular Probes, Eugene, Oregon, USA or Calbiochem-Novabiochem U.K. Ltd., Beeston, Nottingham) for 1hr at either room temperature (22-25 °C) or 4 °C in DM. In some experiments cells were also loaded for 1hr at 37 °C to determine the effect of temperature on loading. Loading appeared to be reduced at higher temperatures. After the loading procedure cells were kept on ice. Fura-2/AM being uncharged and hydrophobic (Thomas & Delaville, 1991) diffuses across the cell membrane in a non-invasive way. Once inside the cells, hydrolysis of the AM ester by intracellular hydrolases releases fura-2, enabling $[Ca^{2+}]_i$ to be measured in the intact cell. Once the extracellular fura-2 has been removed, fluorescence signals arise only from the cytoplasmic dye. There are some

disadvantages to this method of loading. For example fura-2/AM may be accumulated into subcellular compartments (Williams et al., 1985) and hydrolysis may be incomplete (Scanlon et al., 1987). These effects are often worsened by prolonged exposure to the ester. This gives rise to errors in the measurement of $[Ca^{2+}]_i$, since fluorescence is not limited to the Ca^{2+}-bound and Ca^{2+}-free fura-2 in the cytoplasm. The amount of dye in non-cytoplasmic compartments can be reduced by lowering the temperature at which loading is carried out (Thomas & Delaville, 1991). Since dye loading depends on diffusion across the cell membrane, the concentration inside the cell is not readily controlled, so the fura-2 concentration could become sufficiently high to buffer the $[Ca^{2+}]_i$. This would lower basal $[Ca^{2+}]_i$ and dampen $[Ca^{2+}]_i$ transients. However, since fura-2 has a high efficiency of fluorescence (Grynkiewicz et al., 1985), the levels required for loading are low and Ca^{2+} buffering can be minimised. Despite the potential problems, this method for intracellular loading of fluorescent Ca^{2+} indicators remains the most straightforward and minimally disruptive technique for measuring $[Ca^{2+}]_i$.

3.2.2 Relationship between fluorescence and $[Ca^{2+}]_i$

Fluorescent molecules absorb light at one wavelength and emit light at a longer wavelength. With fura-2, the intensity of the emitted light is a function of the amount of Ca^{2+} bound to the molecule. Fura-2 displays a characteristic excitation spectrum, which shifts on binding Ca^{2+} (Fig. 3.1). Changes in the ratio of the fluorescence measured at two wavelengths on either side of the isosbestic point, where the fluorescence intensity is independent of the $[Ca^{2+}]_i$, are directly related to changes in Ca^{2+} binding (Grynkiewicz et al., 1985). The excitation and emission maxima are

well separated with peak absorption (335 nm) in the near UV region and emission (510 nm) (not shown) in the green region of the spectrum (Grynkiewicz et al., 1985).

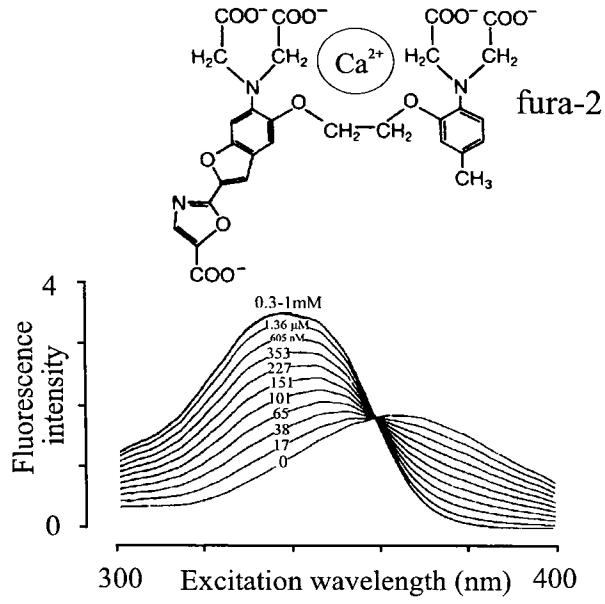


Fig. 3.1. *Structure of fura-2 and its excitation spectrum in varying $[Ca^{2+}]_i$. Emission was collected at 510 nm. Increasing the free $[Ca^{2+}]$ increases the fluorescence excited at 340 nm and decreases the fluorescence at 380 nm. Taken from Grynkiewicz et al. (1985).*

Fura-2 is well suited to measuring $[Ca^{2+}]_i$ in the physiological range. It can be seen from Fig. 3.1 that it is a tetracarboxylate indicator. The Ca^{2+} binding site is a 'cavity' formed in the chelator molecule by the disposition of the 4 carboxylate groups. The dye binds Ca^{2+} with high selectivity over other divalent and trivalent cations. This is mainly determined by the size of the cavity, which is just the correct size to promote Ca^{2+} binding, but is unable to constrict further to accommodate other divalent cations. Of particular importance are Mg^{2+} ions as they can potentially influence the

Ca^{2+} measurement and are present at mM concentrations in cells. The main effect of Mg^{2+} is to lower the affinity of fura-2 for Ca^{2+} (Grynkiewicz et al., 1985; Gurney, 1990b). Binding of Ca^{2+} withdraws the nitrogen lone of pair electrons from the aromatic rings resulting in altered fluorescence. Fluorescence is therefore related to the proportion of dye in the Ca^{2+}-bound and Ca^{2+}-free forms. Advantages of using fura-2 over other fluorescent indicators include its high fluorescence intensity, its relative insensitivity to pH and Mg^{2+} changes in the physiological range, sensitivity at low illumination intensities and lack of toxicity (Tsien, 1980; Grynkiewicz et al., 1985).

3.2.3 Determination of free cytoplasmic calcium concentration

Since the excitation spectrum of fura-2 shifts upon binding Ca^{2+}, the ratio (R) of fluorescence at two excitation wavelengths, λ_1 and λ_2, can be used to calculate $[Ca^{2+}]_i$. In an indicator solution containing Ca^{2+}, the fluorescence at each wavelength is dependent not only on the fura-2 concentration and $[Ca^{2+}]_i$, but also on the excitation intensity, extinction coefficient, path length, quantum efficiency and the instrumental efficiency. These constants contribute to a proportionality factor (S), which can be measured from the fluorescence intensities of calibration solutions containing known concentrations of Ca^{2+}-free and Ca^{2+}-saturated dye. For the free dye, S_{f1} represents the proportionality factor measured at wavelength λ_1, with S_{f2} measured at wavelength λ_2. S_{b1} and S_{b2} represent the proportionality factors for Ca^{2+} bound dye at λ_1 and λ_2. The fluorescence intensities (F_1 and F_2) at wavelengths λ_1 and λ_2 are then given by the following equations:

$$F_1 = S_{f1}c_f + S_{b1}c_b \quad \textbf{(1)}$$

$$F_2 = S_{f2}c_f + S_{b2}c_b \quad (2)$$

where c_f and c_b are the respective concentrations of the free and bound indicator. Fura-2 forms a 1:1 stoichiometric complex with Ca^{2+} (Grynkiewicz et al., 1985) so

$$c_b = c_f [Ca^{2+}] / K_d \quad (3)$$

where K_d is the dissociation constant of the indicator:Ca^{2+} complex. The measured fluorescence ratio ($R = F_1/F_2$) at the two exciting wavelengths (λ_1 and λ_2) is therefore given by:

$$R = (S_{f1} + S_{b1}[Ca^{2+}] / K_d) / (S_{f2} + S_{b2}[Ca^{2+}] / K_d) \quad (4)$$

which can be re-arranged to:

$$[Ca^{2+}] = K_d \{(R-(S_{f1} / S_{f2}) / (S_{b1} / S_{b2}) - R)\} (S_{f2} / S_{b2}) \quad (5)$$

This equation may be re-written as:

$$[Ca^{2+}] = K_d \{(R-R_{min}) / (R_{max} - R)\} (S_{f2} / S_{b2}) \quad (6)$$

where $R_{min} = S_{f1} / S_{f2}$ and $R_{max} = S_{b1} / S_{b2}$ are the limiting ratios of fluorescence in Ca^{2+}-free and Ca^{2+} saturated solutions, respectively. It is more often written as:

$$[Ca^{2+}] = K_d \{(R-R_{min}) / (R_{max} - R)\} \beta \quad (7)$$

where $\beta = S_{f2} / S_{b2}$ defines the ratio of fluorescence at λ_2 of the Ca^{2+}-free to Ca^{2+}-bound indicator.

Dual wavelength ratiometric measurements of $[Ca^{2+}]_i$ provide a more reliable indicator of $[Ca^{2+}]_i$ than single wavelength measurements, because they are independent of uncertainties in optical efficiency, instrumental sensitivity, and variations in dye concentration and distribution (Tsien, 1980; Grynkiewicz et al., 1985; Minta et al., 1989; Thomas & Delaville, 1991). Signals can be calibrated without knowledge of the exact concentration of the dye. This is particularly

important when making measurements from muscle cells, which change shape and thickness in response to changes in $[Ca^{2+}]_i$.

Fura-2 shows considerable sensitivity to Ca^{2+} at 340 nm and 380 nm, the peak wavelengths at which signals change inversely in response to a change in $[Ca^{2+}]_i$. Fluorescence increases at 340 nm on binding Ca^{2+}, while decreasing at 380 nm. A measure of $[Ca^{2+}]_i$ was therefore obtained from the ratio of fluorescence at these two wavelengths, i.e. $\lambda_1 = 340$ nm and $\lambda_2 = 380$ nm.

3.2.4 Instrumentation for measuring fluorescence

Dye-loaded cells were placed in a glass-bottom perfusion chamber mounted on an inverted microscope (Nikon Diaphot, Nikon Corporation) equipped for epifluorescence microscopy and photometry. The output of the light source (Nikon mercury-xenon 75 W arc lamp, Nikon Corporation) was directed through a motor-driven rotating filter wheel that had both 340 nm and 380 nm interference filters (band pass ~10 nm) to provide excitation at the 2 wavelengths. The intensity was modified by neutral density filters placed in the light path. A cell was illuminated every 160 ms with each wavelength, rapidly alternating excitation light being reflected onto the cell by a dichroic mirror placed at 45° below a 40 x oil-immersion fluorescence objective with a numerical aperture of 1.3 (Nikon Corporation). Emitted light at each of the two excitation wavelengths returned through the objective, and was transmitted by the dichroic mirror towards a photomultiplier tube (PMT; AFX-IIA; Nikon Corporation) mounted directly on a side-port of the microscope. A band pass (10 nm) emission filter allowed only light at 510 nm to strike the PMT. The

light was measured by photon counting and displayed and stored directly on a PC using PhoCal Cell Fluorescence Analyser V5.2b (University of Strathclyde), for later analysis. The cell could also be observed on a video monitor (Hitachi) via a CCD camera (CICR, Japan) mounted on the same microscope port as the PMT. At any one time, light was directed to either the PMT or video camera, controlled by an exposure meter (Nikon AFX-IIA; Nikon Corporation). The experimental setup for measuring fluorescence signals can be seen in Fig. 3.2.

To restrict the light reaching the PMT to only the fluorescence emitted by the fura-2 loaded cell, an adjustable rectangular diaphragm placed in front of the PMT and video camera was used to limit the field to a rectangular area of ~ 200 μm^2 in the centre of the field of view around the loaded cell. For each cell, background images (adjacent to the cell but containing no cells) were routinely subtracted from the measured fluorescence signals before calculating the ratio. A potential problem, particularly with single cell studies, is that the intense excitation light can cause a significant amount of photobleaching and phototoxicity (Becker and Fay, 1987), which can reduce the accuracy of measurement and its interpretation. A manually-controlled shutter in front of the excitation light was used to prevent fluorescence excitation. During experimental fluorescence recording, the excitation light was blocked between stimuli in order to minimise photobleaching and cell damage. Thus, records were obtained only during cell excitation. Background noise was minimised by working in a dark room with the only light, other than the excitation light, originating from the recording equipment. These were masked where possible with red acetate sheets, to minimise short wavelength sources. Data collection started

within approximately 1-2 hrs of loading with fura-2 as the cells were seen to loose responsiveness when loaded for extended periods.

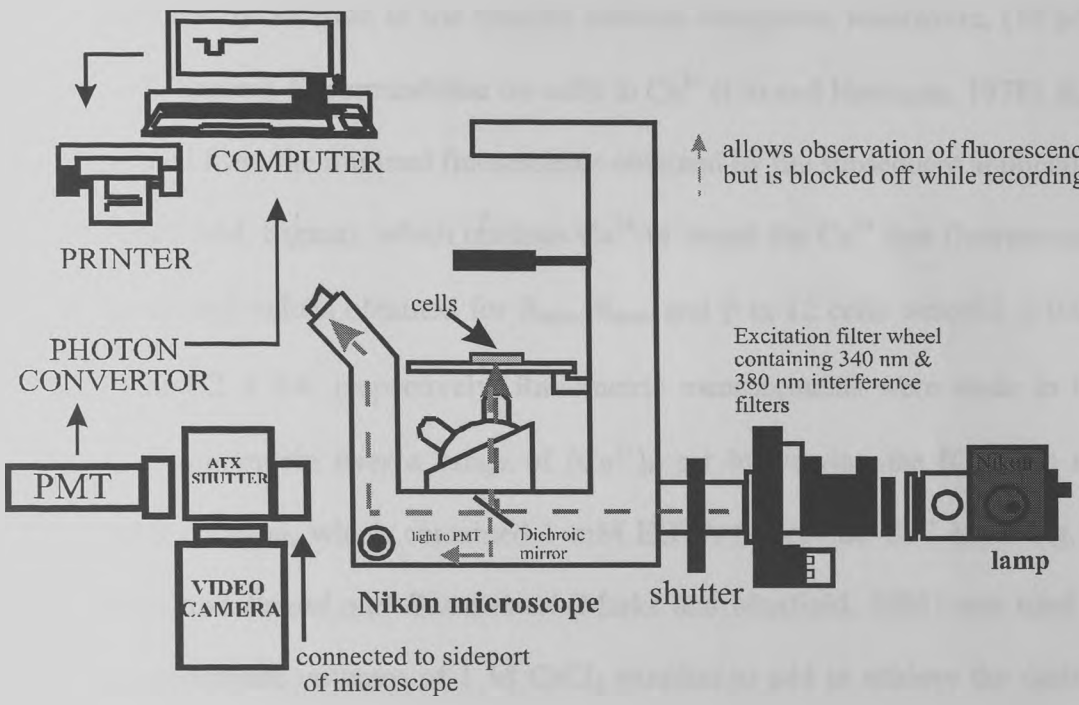


Fig. 3.2. *Measurement of* $[Ca^{2+}]_i$ *using fura-2 requires a dual excitation wavelength recording system. The location of the lamp and excitation filter wheel connected to the Nikon microscope can be seen. A dichroic mirror directed excitation light to cells and emitted light from cells to the PMT.*

The PhoCal software displayed the fluorescence signals at both 340 nm and 380 nm as they were collected, while simultaneously storing them on the PC's hard disc. The programme automatically calculated the ratio and displayed it on the computer screen, together with the raw wavelength data.

Ratiometric data were converted to $[Ca^{2+}]_i$ according to equation 7 using values of K_d, β, R_{min} and R_{max} that were determined experimentally from calibrations performed on dye-loaded cells as shown in Fig. 3.3. Maximal fluorescence (R_{max}) was determined by addition of the specific calcium ionophore, ionomycin, (10 μM, calcium salt, Sigma), to permeabilise the cells to Ca^{2+} (Liu and Hermann, 1978). R_{min} was calculated from the minimal fluorescence obtained by the subsequent application of EGTA (10 mM, Sigma), which chelates Ca^{2+} to reveal the Ca^{2+} free fluorescence. The experimental values obtained for R_{min}, R_{max} and β in 12 cells were 0.2 ± 0.02, 2.4 ± 0.1 and 2 ± 0.4, respectively. Ratiometric measurements were made in the presence of ionomycin over a range of $[Ca^{2+}]_i$, set by varying the $[Ca^{2+}]$ in the extracellular solution, which contained 1 mM EGTA to provide Ca^{2+} buffering. A program entitled *Bound and Determined* (Marks and Maxfield, 1991) was used to calculate appropriate volumes of 1 M $CaCl_2$ solution to add to achieve the desired $[Ca^{2+}]$ across a range from 25-1000 nM. Fig. 3.4 shows the relationship between the ratio of fluorescence at 340 and 380 nm ($R_{340/380}$) recorded from fura-2 loaded cells and the $[Ca^{2+}]_i$. The K_d was determined graphically according to equation 7 and calculated as 158 nM. PhoCal used the experimentally obtained values for R_{min}, R_{max}, β and K_d to calculate $[Ca^{2+}]_i$ values for each experiment. Subsequent display and analysis used Origin V4.1 (Microcal Software Inc.). Results are presented as $R_{340/380}$ and calibrated $[Ca^{2+}]_i$.

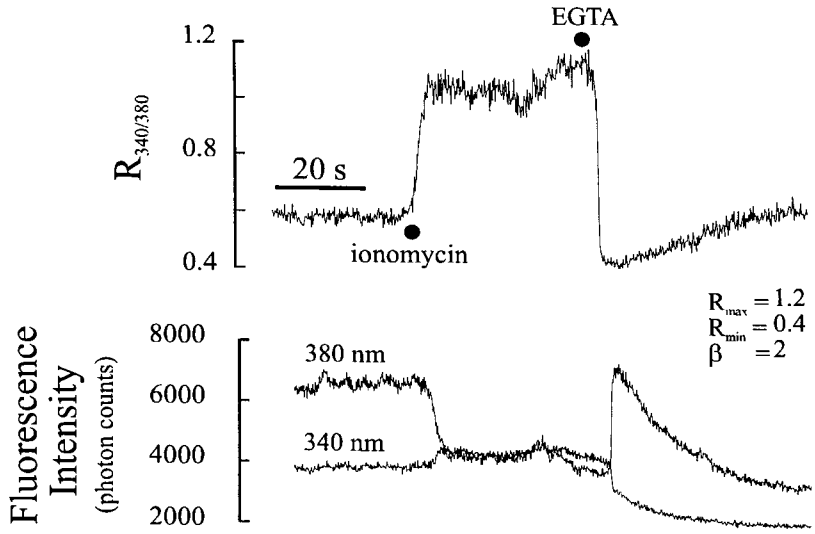


Fig. 3.3. *A representative example of a calibration performed on a fura-2 loaded rabbit aortic SMC. 10 μM ionomycin was added to determine the maximum level of* R_{340}/R_{380} *(*R_{max}*). Subsequently 10 mM EGTA was added to determine the minimum level of* R_{340}/R_{380} *(*R_{min}*).* R_{340}/R_{380} *was converted to* $[Ca^{2+}]_i$ *by the method of Grynkiewicz et al. (1985).* R_{max}*,* R_{min} *and* β *values are given.*

The correct K_d for the experimental conditions under study needs to be used for the accurate determination of the cytoplasmic $[Ca^{2+}]_{free}$ because the K_d of fura-2 is sensitive to the environment. Nevertheless, the majority of investigators still refer to the original *in vitro* K_d as determined by Grynkiewicz et al (1985), in which the experimental conditions were arbitrarily set. Although fura-2 was calibrated for use in rabbit aortic SMCs, the calibrated signals remain as a best approximation. This is because compartmentalisation or incomplete hydrolysis of intracellular fura-2 could give rise to an error (Williams et al., 1985; Scanlon et al., 1987).

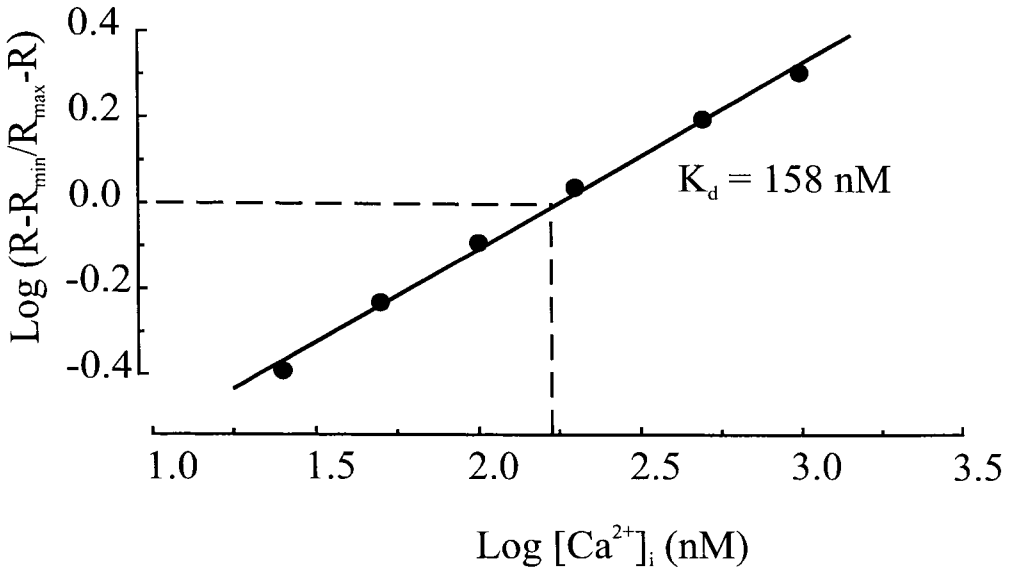


Fig. 3.4. *Calibration of the fura-2 $[Ca^{2+}]_i$ relationship. Solutions with known Ca^{2+} concentrations in the nanomolar range, buffered with 1 mM EGTA, were prepared. If $log[(R-R_{min})/(R_{max}-R)]$ is plotted as a function of $log[Ca^{2+}]_i$, the Ca^{2+} response of the indicator is linear with the x-intercept being equal to the log of the K_d. The K_d was determined as 158 nM at room temperature. It was determined over a course of several weeks using different calibration solutions prepared using the same $CaCl_2$ and EGTA stock solutions.*

3.2.5 The kinetics of $[Ca^{2+}]_i$ decay

To study the rate at which Ca^{2+} was removed from a cell after a stimulus, the decline in fura-2 $R_{340/380}$ was fitted with a first order exponential equation of the form

$$R_{340/380} = Ae^{-\tau/t} \quad \textbf{(8)}$$

where A = was the peak $R_{340/380}$ achieved during the Ca^{2+} transient, t = time and τ = the time constant.

3.2.6 Bath solutions

The ionic composition of the bath (extracellular) solution was designed to mimic the physiological extracellular environment of the cell. Its composition was (mM): NaCl 122, KCl 5, HEPES 10, NaH_2PO_4 0.5, KH_2PO_4 0.5, $MgCl_2$ 1, $CaCl_2$ 1.8, glucose 11. The pH was adjusted to 7.3 with NaOH.

3.2.7 Drug application

Cells were continuously superfused with either drug-free or drug-containing solutions at a rate of approximately 2 ml min^{-1}, with total replacement of the bathing solution achieved within 30 s. Switching between solutions was achieved manually. The delay between changing reservoirs and arrival of the drug in the bath was approximately 25 s. To initiate agonist responses, drugs were either applied directly to the chamber or ejected from a nearby pipette as in chapter 2. The concentration of each agonist noted in the text refers to the final bath concentration or that in the micropipette if applied by pressure ejection. It is likely, however, that some dilution of ejected drug occurred, due to the small volume of solution ejected and the distance of the pipette from the cell.

3.2.8 Data analysis and statistics

Statistical comparisons were made using a Student's t-test or ANOVA with $P < 0.05$ taken as indicating a significant difference. Experiments were routinely conducted at room temperature (22-25 °C) in a 1 ml perfusion chamber. Data are expressed as mean ± standard error of the mean (S.E.M.) of *n* cells unless otherwise stated.

3.2.9 Drugs and chemicals

The following drugs and chemicals were used: caffeine (Sigma), noradrenaline (arterenol, bitartrate salt, Sigma), thapsigargin (TG, Sigma or Calbiochem), cyclopiazonic acid (CPA, Calbiochem), fura-2/AM (Molecular Probes and Calbiochem) and ionomycin (calcium salt, Sigma). Drugs prepared on the day of each experiment were routinely dissolved in distilled water as stock solutions, and further dilutions were made in the same solution bathing the cells, with the exception of caffeine, which was dissolved directly in bath solution. Fura-2 (1 mM) and ionomycin (10 mM) were stored at –20 °C as stock solutions in dimethylsulphoxide (DMSO) and diluted in DM (fura-2) or bath solution (ionomycin) before adding to the cells. TG and CPA were dissolved as stock solutions in DMSO and further diluted in bath solution. All solutions were routinely filtered before use through a pore size of 0.2 μm (Anotop disposable filters, Whatman International Ltd., Kent).

3.3 Results

3.3.1 Caffeine-induced mobilisation of $[Ca^{2+}]_i$

Application of caffeine (20 mM) to individual cells in standard extracellular solution resulted in the rapid and transient elevation of $[Ca^{2+}]_i$, as assessed by the change in fura-2 fluorescence. Representative records of fura-2 fluorescence evoked by fast application of caffeine are shown in Fig. 3.5. The fluorescence intensities measured at 510 nm emission in response to excitation at 340 nm and 380 nm are shown, together with the ratio of fluorescence at the two wavelengths ($R_{340/380}$). The fluorescence intensity was increased at 340 nm and decreased at 380 nm in response

to caffeine, giving rise to an increase in $R_{340/380}$. The ratio reached a peak 18 s, after caffeine application started, and then rapidly declined to basal levels. Furthermore, a second application of caffeine, applied 5 min after the first, produced a transient increase in $R_{340/380}$ which did not differ significantly ($P > 0.05$) from that produced by the first application. A few experiments were carried out to determine the minimum time interval required between consecutive caffeine applications for complete refilling of the stores. Repeated caffeine application at intervals shorter than 3 and 4 min progressively suppressed the caffeine response. Thus, to allow replenishing of the stores, an interval of 5 min was imposed between caffeine applications in future experiments. However, only 1 in 4 cells responded to caffeine. The reason for this is not known. Results similar to that in Fig. 3.5 were obtained in a total of 12 experiments. The time to peak increase in $R_{340/380}$ upon addition of caffeine was 12 ± 0.7 s (n = 12). Fig. 3.6a indicates the mean $[Ca^{2+}]_i$ detected by fura-2 under basal conditions and at the peak response to caffeine, during repeated application of caffeine at 5 min intervals. Fig. 3.6b illustrates the mean increases in $[Ca^{2+}]_i$ produced by repeated caffeine applications. In each cell the increases were normalised against the first response (100 %). Overall, there was no significant change in either the basal $[Ca^{2+}]_i$ or in the response to caffeine with time of recording. The mean basal $[Ca^{2+}]_i$, and $[Ca^{2+}]_i$ at the peak of the response to caffeine from all cells tested were calculated as 70 ± 20 nM and 300 ± 40 nM, respectively (n = 85).

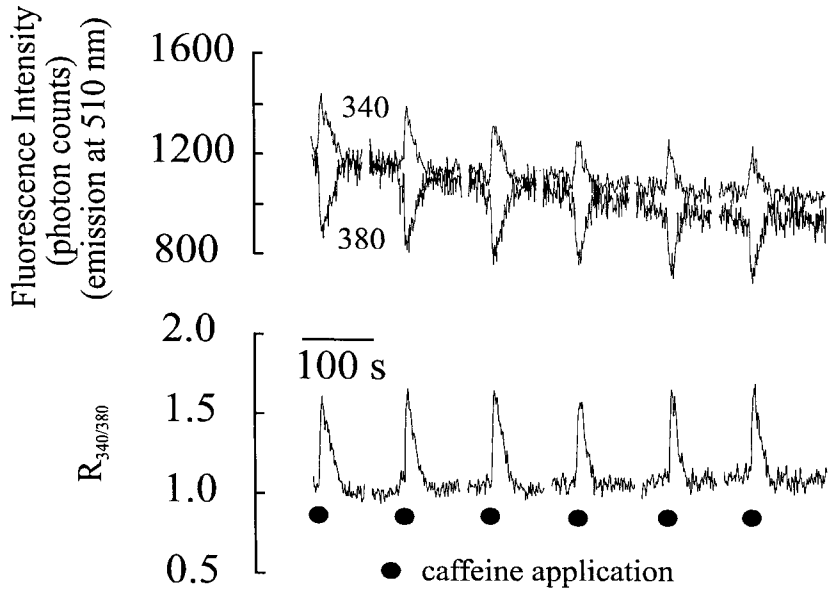
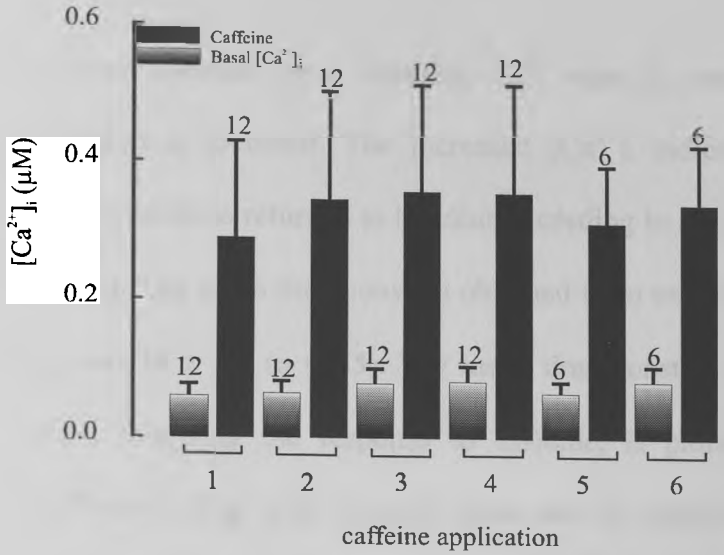


Fig. 3.5. *Caffeine-induced $[Ca^{2+}]_i$ transients detected by fura-2 in a single aortic SMC. Application of caffeine produced a transient increase in $[Ca^{2+}]_i$ as indicated by $R_{340/380}$. Following a 5 min wash, caffeine was applied for a second time and produced a response very similar to the first. 6 consecutive applications of caffeine produced reproducible and reversible responses. Fluorescence evoked by excitation at 340 nm and 380 nm is shown at the top. The ratio $R_{340/380}$ is shown below. Gaps in the records represent 5 min recovery periods between caffeine applications. Caffeine was applied at the times indicated (•).*

a



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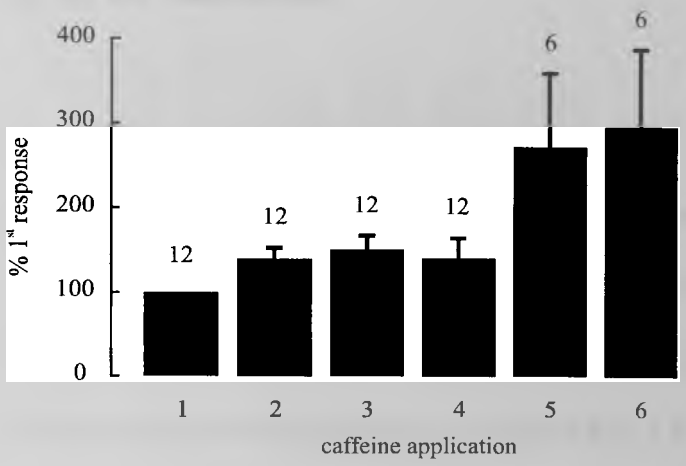


Fig. 3.6. *a Mean $[Ca^{2+}]_i$ measured before (basal) and at the peak of the response to caffeine in 12 cells during repeated applications at 5 min intervals. b Mean amplitudes of the caffeine responses measured as a percent of the first response in each cell. There were no significant differences between responses to consecutive caffeine applications. Data are expressed as mean ± S.E.M. (n = 6-12). Values above S.E.M. bars indicate the total number of cells tested.*

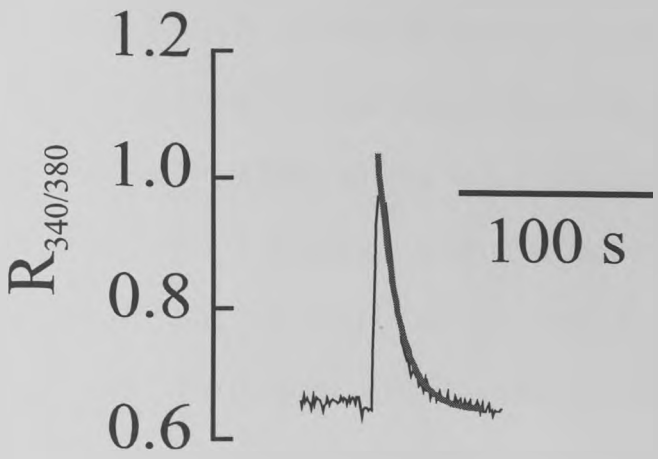
3.3.2 Determination of the rate of $[Ca^{2+}]_i$ decline following caffeine-induced Ca^{2+} release

Once $[Ca^{2+}]_i$ has been elevated by a stimulus, Ca^{2+} must be removed from the cytoplasm if relaxation is to occur. The increased $[Ca^{2+}]_i$ indicated by $R_{340/380}$, induced in response to caffeine returned to baseline according to a single exponential time course (Fig 3.7a). The mean time constant obtained from exponential fits to the decline of $R_{340/380}$ was 18 ± 3 s (n = 85). The mean time constant of the decay of $R_{340/380}$ in 12 cells, following the response to caffeine, is plotted for repeated applications of caffeine in Fig. 3.7b. Overall, there was no significant ($P > 0.05$) change in the rate of $[Ca^{2+}]_i$ decline following caffeine applications when caffeine was repeatedly applied at 5 min intervals.

3.3.3 NA-induced mobilisation of $[Ca^{2+}]_i$

Addition of the Ca^{2+}-mobilising agonist, NA (10 μM), which releases Ca^{2+} through IP_3-sensitive channels, also stimulated the elevation of $[Ca^{2+}]_i$ in single cells, again with considerable variability between individual cells. Representative records of $R_{340/380}$ during fast application of NA (10 μM) are shown in Fig. 3.8. $R_{340/380}$ reached a peak after application of NA and then rapidly declined to basal levels. Furthermore, a second application of NA applied 5 min after the first produced a transient increase in $R_{340/380}$ comparable to that produced by the first application. The time to peak increase in $R_{340/380}$, measured from when NA application was started, was 27 ± 2 s (n = 9). This was significantly ($P < 0.05$) longer than that measured with caffeine. Responses to repeated NA applications, similar to those of Fig. 3.8, were obtained in

a



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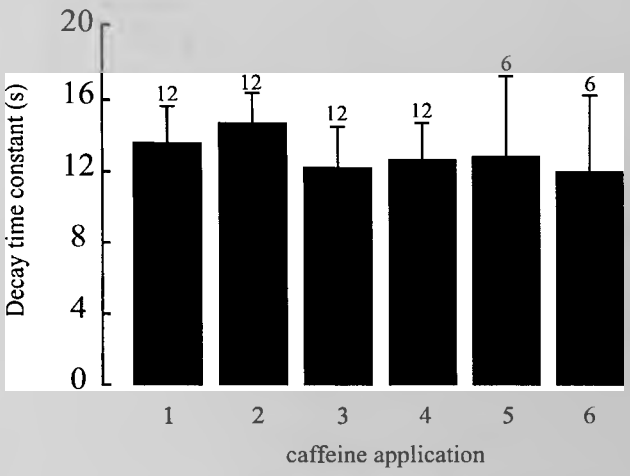


Fig. 3.7. *a Raw record of $[Ca^{2+}]_i$ response to caffeine showing a fitted curve superimposed upon the decline to the basal level. b Mean $[Ca^{2+}]_i$ decay time constants during repeated caffeine applications at 5 min intervals. There were no significant differences between consecutive $[Ca^{2+}]_i$ decay time constants. Data are expressed as mean ± S.E.M. (n = 6-12). Values above S.E.M. bars indicate the total number of cells tested.*

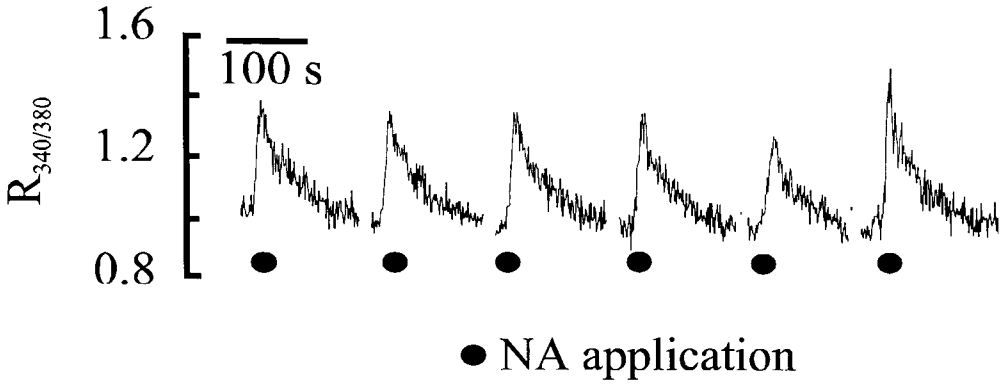


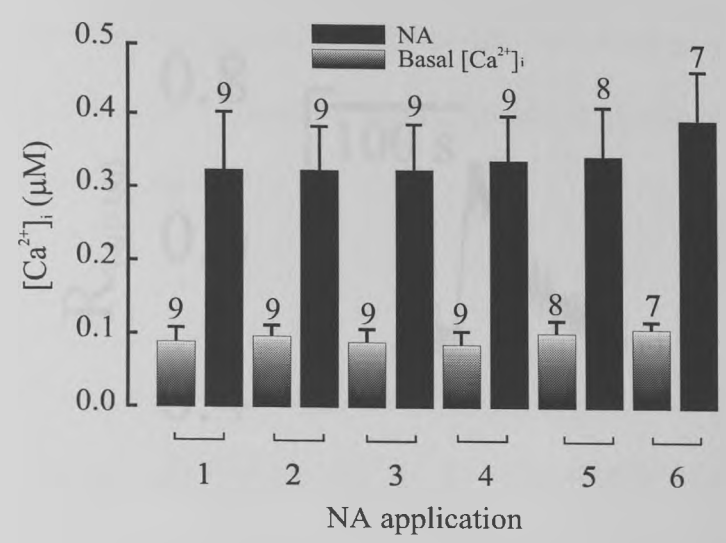
Fig. 3.8. *NA-induced $[Ca^{2+}]_i$ transients in an intact single aortic SMC detected by fura-2. Application of 10 μM NA produced a transient increase in $[Ca^{2+}]_i$ as indicated by $R_{340/380}$. Following a 5 min wash, NA was applied for a second time and produced a response similar to the first. 6 consecutive applications of NA produced reproducible and reversible responses. Gaps in the records represent 5 min recovery periods between NA applications. NA was applied at the times indicated (•).*

a total of 9 experiments. As with caffeine, only a proportion (50 %) of the cells responded to NA. Fig. 3.9a indicates the mean $[Ca^{2+}]_i$ determined by fura-2 under basal conditions and during the peak response to NA in 9 cells exposed to repeated application of NA at 5 min intervals. Fig. 3.9b illustrates the mean percent increases in $[Ca^{2+}]_i$ recorded during repeated NA applications. Responses were normalised against the first response (100 %) in each cell. There was no significant change in either the basal $[Ca^{2+}]_i$ or the response to NA with time of recording. The mean basal $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$ at the peak of the response to NA, calculated from all cells tested was 50 ± 10 nM and 240 ± 20 nM, respectively (n = 95).

3.3.4 Determination of the rate of $[Ca^{2+}]_i$ decline following NA-induced Ca^{2+} release

The increase in $[Ca^{2+}]_i$ induced in response to NA returned to baseline according to a single exponential time course (Fig. 3.10a). The mean time constant determined from exponential fits to the decline of $R_{340/380}$ was 44 ± 6 s (n = 95). This was significantly ($P < 0.05$) longer than that measured with caffeine. The mean time constants for 9 cells subjected to repeated applications of NA are plotted in Fig. 3.10b. There was no significant change in the rate of $[Ca^{2+}]_i$ decline following NA application when NA was repeatedly applied at 5 min intervals.

a



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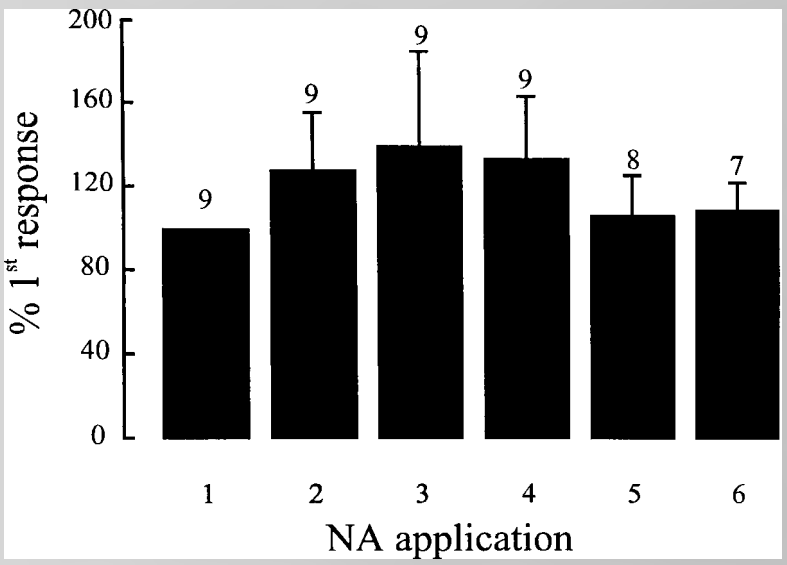
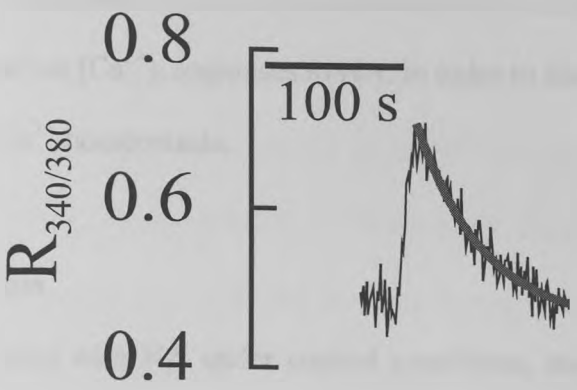


Fig. 3.9. *a Mean $[Ca^{2+}]_i$ measured before (basal) and at the peak of the response to 10 μM NA during repeated application at 5 min intervals. b Mean amplitude of the NA responses measured as a percent of the first response in each cell. There were no significant differences between responses to consecutive NA applications. Data are expressed as mean ± S.E.M. (n = 7-9). Values above S.E.M. bars indicate the total number of cells tested.*

a



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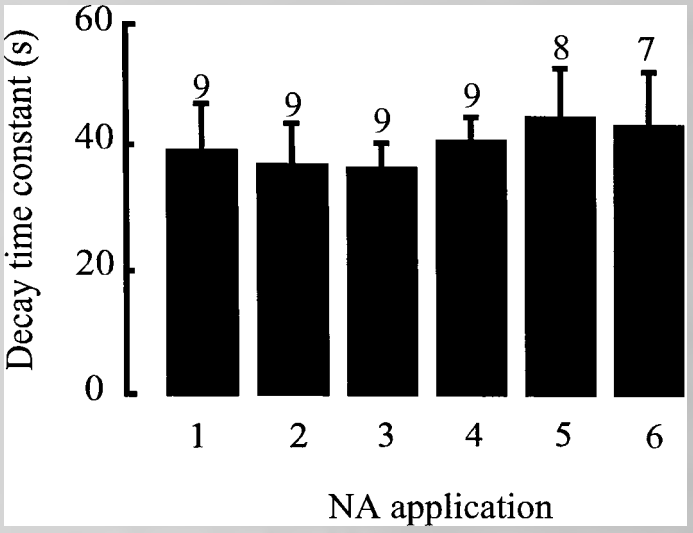


Fig. 3.10. *a $[Ca^{2+}]_i$ indicated by $R_{340/380}$ declined exponentially following the peak response to NA. The curve superimposed on the decay of the Ca^{2+} transient indicates the best fit to $R_{340/380}$ decline, from which a time constant of 38 s was derived. b Mean $[Ca^{2+}]_i$ decay time constant during repeated NA applications at 5 min intervals in 9 cells. There were no significant differences between consecutive $[Ca^{2+}]_i$ decay time constants. Data are expressed as mean ± S.E.M., with the values above S.E.M. bars indicating the number of cells tested.*

3.3.5 Effect of SR Ca^{2+}-ATPase inhibitors on $[Ca^{2+}]_i$ transients activated by NA

The effects of the inhibitors TG and CPA of the SR Ca^{2+}-ATPase were investigated on basal $[Ca^{2+}]_i$ and on $[Ca^{2+}]_i$ responses to NA, in order to elucidate the contribution of the ATPase to Ca^{2+} homeostasis.

3.3.5.1 Thapsigargin

Cells were stimulated with NA under control conditions, and when a reproducible response was obtained, TG was applied and the cells stimulated again. Basal and stimulated $[Ca^{2+}]_i$ were measured for repeated responses prior to and during TG exposure. Addition of TG (0.1 μM and 1 μM) increased basal $R_{340/380}$ by 150 ± 4 % (n = 8) and 180 ± 4 % (n = 11), respectively as shown in Fig. 3.11, this represented an increase in basal $[Ca^{2+}]_i$ from 40 ± 10 nM (n = 8) under control conditions to 100 ± 10 nM in the presence of 0.1 μM TG, and from 50 ± 10 nM (n = 11) under basal conditions to 140 ± 10 nM (n = 11) in the presence of 1 μM TG. The increase in basal $[Ca^{2+}]_i$ following TG addition was observed before NA was applied to stimulate SR Ca^{2+} release and was maintained throughout the remainder of the experiment. At the same time, 0.1 μM TG significantly ($P < 0.05$) reduced the peak $[Ca^{2+}]_i$ reached in the presence of NA by 65 ± 3 % (n = 8), from 600 ± 100 nM (n = 8) to 210 ± 100 nM (n = 8). The change in $[Ca^{2+}]_i$ induced by NA was significantly ($P < 0.05$) reduced from 560 ± 100 nM (n = 8) to 110 ± 10 nM (n = 8) in the presence of 0.1 μM TG. It can be seen from Fig. 3.11a that the effects of 0.1 μM TG on basal $[Ca^{2+}]_i$ and the peak $[Ca^{2+}]_i$ response were augmented in the continued presence of the drug, such that NA eventually failed to stimulate a rise in $[Ca^{2+}]_i$.

A different effect was seen following the addition of a higher concentration of TG (1 μM). Out of 11 cells tested, 5 cells showed an increase in the peak $[Ca^{2+}]_i$ reached in the presence of NA, following treatment with TG (1 μM). Although the remaining 6 cells tested showed a reduction, overall, the peak $[Ca^{2+}]_i$ reached during the response to NA was significantly increased by 31 ± 3 % (n = 11), from 160 ± 40 nM (n = 11) under control conditions to 210 ± 60 nM (n = 11) in the presence of 1 μM TG. This did not reflect a larger increase in $[Ca^{2+}]_i$ in response to NA, but resulted because basal $[Ca^{2+}]_i$ was already very high. The change in $[Ca^{2+}]_i$ induced by NA was actually reduced from 110 ± 30 nM (n = 11) under control conditions to 70 ± 30 nM (n = 11) in the presence of 1 μM TG, although these values are not significantly ($P > 0.05$) different. The effect of TG was essentially irreversible. Although there appears to be a little recovery of the peak response to NA in Fig. 3.11, basal $[Ca^{2+}]_i$ remained elevated.

3.3.5.2 Cyclopiazonic Acid

Figs. 3.12 and 3.13 illustrate the effect of another SR Ca^{2+}-ATPase inhibitor, CPA, on basal $[Ca^{2+}]_i$ and stimulated $[Ca^{2+}]_i$. Cells were stimulated with NA under control conditions, and when a reproducible response was obtained, CPA was applied and the cells stimulated again. Representative records of $R_{340/380}$ in the absence and presence of 30 μM CPA are shown in Fig. 3.12. CPA had a qualitatively similar effect to 0.1 μM TG. In general basal $[Ca^{2+}]_i$ was increased, whereas the peak $[Ca^{2+}]_i$ reached during the response to NA was decreased. In 2 of the cells studied, NA-stimulated Ca^{2+} transients were fully recovered following washout of 30 μM CPA (Fig. 3.12).

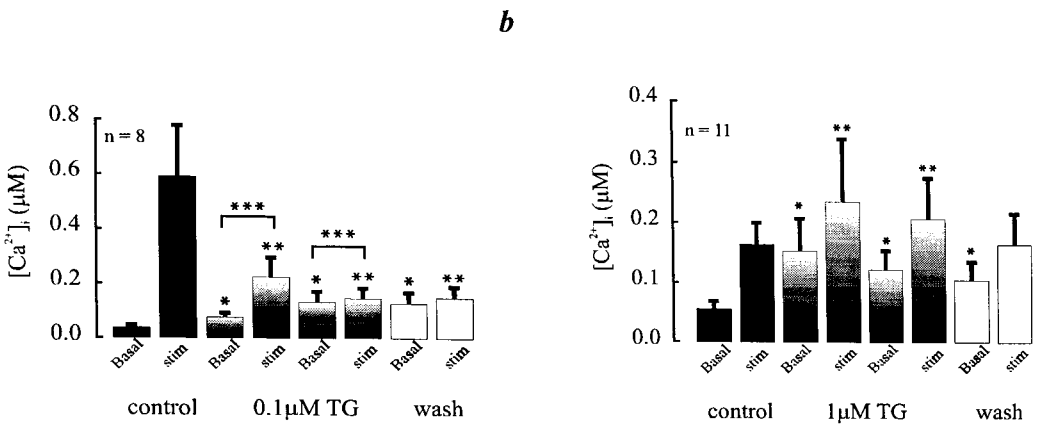


Fig. 3.11. *Mean $[Ca^{2+}]_i$ measured before (basal) and at the peak of the response to NA (stim). Plots show consecutive responses to NA applied at 5 min intervals. TG was applied immediately after the first NA application and washed after the third. Responses to TG were taken at the second application of NA. At 0.1 μM (a) and 1 μM (b), TG significantly increased basal $[Ca^{2+}]_i$. At 0.1 μM TG (a) the $[Ca^{2+}]_i$ reached at the peak of the response to NA was lower than in control conditions in contrast to 1 μM TG (b), which significantly increased the peak of the response to NA in comparison to control. Note that subsequent basal measurements of $[Ca^{2+}]_i$ were augmented and NA-stimulated measurements of $[Ca^{2+}]_i$ were reduced in the continued presence of 0.1 μM TG. * Significantly different from basal control, ** significantly different from stimulated control, *** significant difference between stimulated and basal in comparison to control, $P < 0.05$. Data are expressed as mean ± S.E.M. (n = 8 (a) and 11 (b)).*

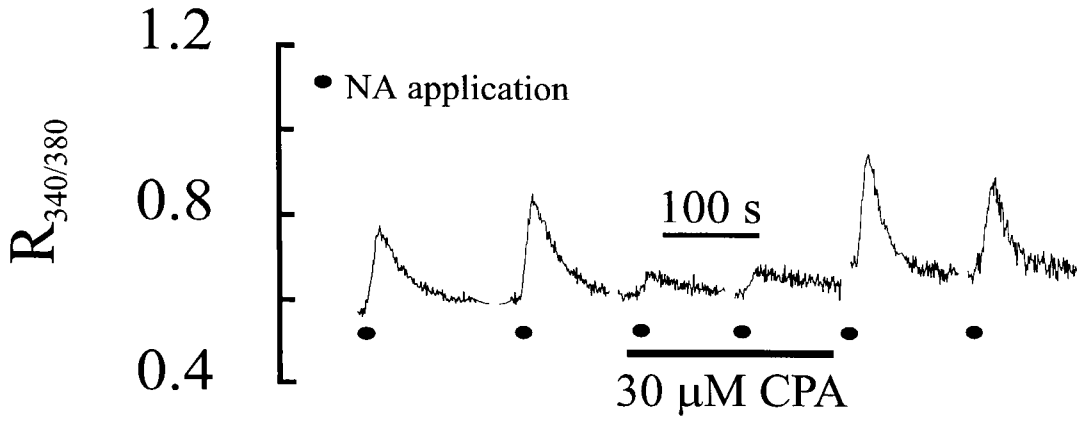


Fig. 3.12. *NA-induced $[Ca^{2+}]_i$ transients before, during and after the application of 30 μM CPA indicated by $R_{340/380}$. In this particular cell responses to NA were recovered on washing (seen in only 2 cells). The maximal effect of CPA was obtained at 30 μM. Gaps in the records represent 5 min recovery periods. NA was applied at the times indicated (•). CPA was applied to the cell for the duration indicated by the horizontal bar and for 5 min before testing NA.*

Basal $[Ca^{2+}]_i$ was increased in a concentration-dependent manner by CPA, (Fig. 3.13) by 44 ± 5 % (n = 15) at 0.1μM and by 95 ± 6 % (n = 13) at 30 μM, as assessed by the increase in $R_{340/380}$. This represented an increase in basal $[Ca^{2+}]_i$ from 39 ± 5 nM (n = 15) under control conditions to 56 ± 10 nM (n = 15) at 0.1 μM and from 41 ± 9 nM (n = 13) to 80 ± 5 nM (n = 13) at 30 μM. The concentration dependence of the effects of CPA on basal $[Ca^{2+}]_i$ is clear in Fig. 3.14, which shows the CPA-induced increase in $[Ca^{2+}]_i$. The increase in basal $[Ca^{2+}]_i$ was maintained throughout exposure to CPA. CPA treatment significantly ($P < 0.05$) reduced peak $[Ca^{2+}]_i$ responses to NA in all cells examined. Measured from the first response to NA in the presence of CPA, this inhibition ranged from 60 ± 10 % (n = 15) at 0.1 μM to 18 ± 4 % (n = 13) at 30 μM, equivalent to a reduction of the peak $[Ca^{2+}]_i$ from 390 ± 120 nM (n = 15) to 155 ± 30 nM (n = 15) at 0.1 μM and from 110 ± 20 nM (n = 13) to 90 ± 20 nM (n = 13) at 30 μM CPA. There was therefore less Ca^{2+} released from the SR by NA in the presence of CPA. The change in $[Ca^{2+}]_i$ induced by NA was significantly ($P < 0.05$) reduced from 351 ± 100 nM (n = 15) to 99 ± 1 nM (n = 15) in the presence of 0.1 μM CPA and from 69 ± 10 nM (n = 13) to 10 ± 1 nM (n = 13) in the presence of 30 μM CPA. With continued exposure to CPA, NA failed to stimulate Ca^{2+} release even at the lowest concentration of CPA (0.1 μM). Although in 2 cells full reversal of $[Ca^{2+}]_i$ was obtained after removing CPA, it was not easily washed out and in most cells recovery was not observed.

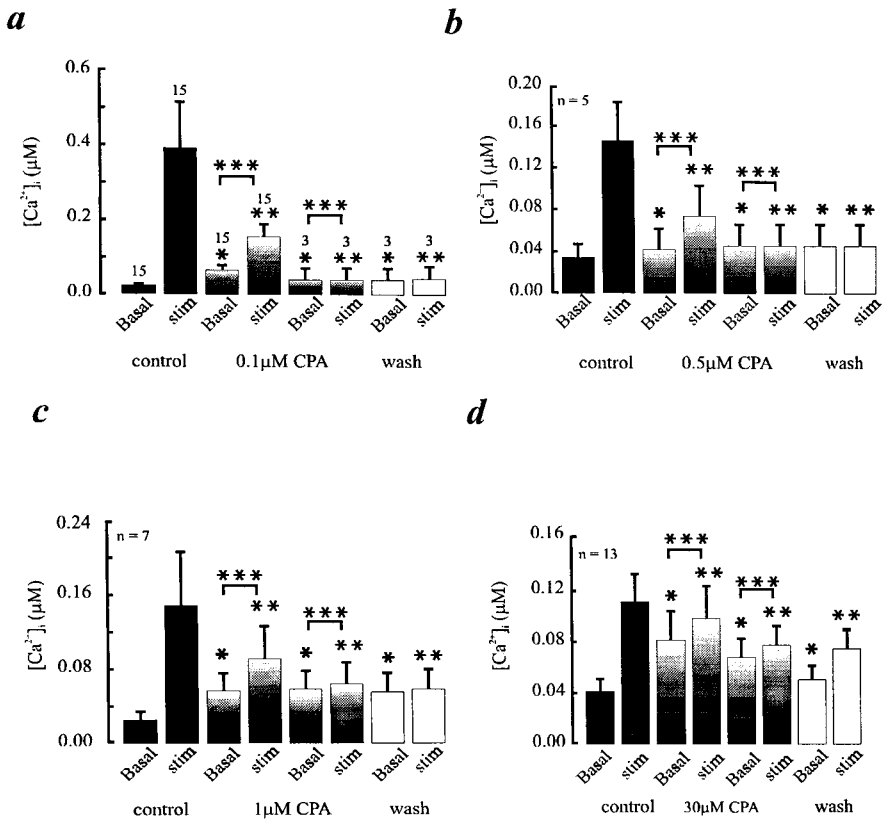


Fig. 3.13. *Mean $[Ca^{2+}]_i$ measured before (basal) and at the peak of the response to NA (stim). CPA (0.1-30 μM) significantly increased basal $[Ca^{2+}]_i$ and in addition significantly reduced NA-stimulated Ca^{2+} levels. * Significantly different from basal control, ** significantly different from stimulated control, *** significant difference between stimulated and basal in comparison to control, $P < 0.05$. Data are expressed as mean ± S.E.M. (n = 3-15). Values above S.E.M. bars indicate the total number of cells tested.*

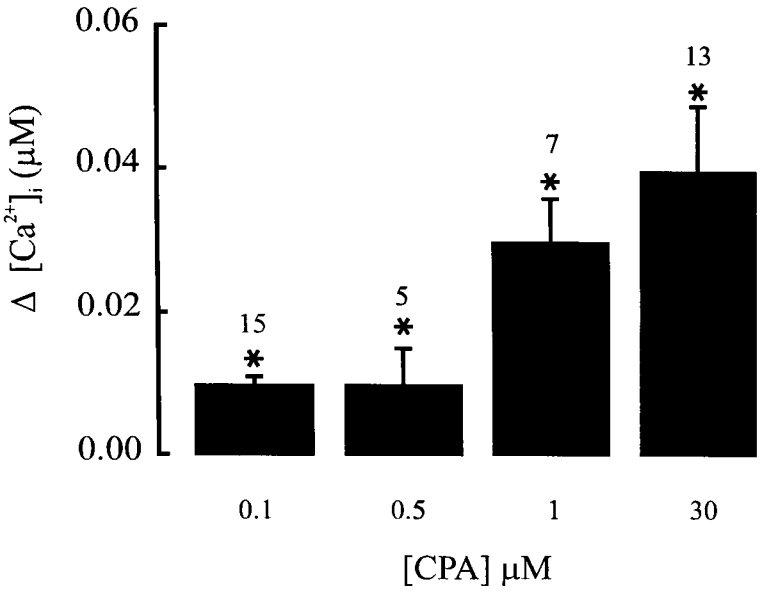


Fig. 3.14. *The concentration dependence of the CPA-induced increase in basal* $[Ca^{2+}]_i$. *Application of CPA at concentrations between 0.1 μM and 30 μM resulted in a progressively larger increase in* $[Ca^{2+}]_i$ *from 17 ± 1 nM (n = 15) to 39 ± 13 nM (n = 13). * Significantly different from control (basal* $[Ca^{2+}]_i$ *in the absence of CPA), P < 0.05. Data are expressed as mean ± S.E.M. (n = 5-15). Values above S.E.M. bars indicate the total number of cells tested.*

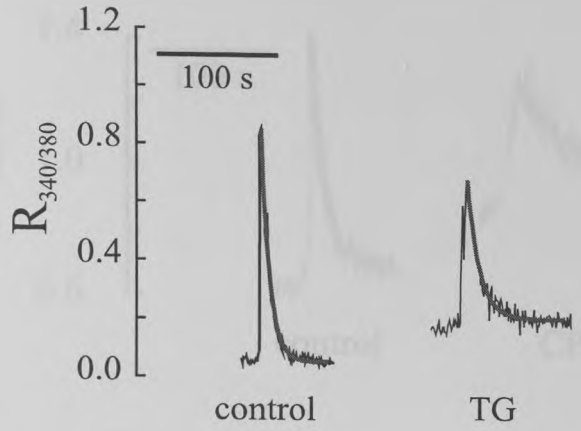
3.3.6 Effects of SR Ca^{2+}-ATPase inhibitors on Ca^{2+} removal

Since inhibitors of SR Ca^{2+}-ATPase are expected to interfere with Ca^{2+} removal from the cell, they might be expected to slow the rate at which $[Ca^{2+}]_i$ declines back to the basal level following Ca^{2+} release from the SR. The effects of TG and CPA on Ca^{2+} removal were therefore investigated by fitting the decline of $[Ca^{2+}]_i$ following the peak response to NA with a single exponential in the absence and presence of TG or CPA. Responses to the first application of NA after applying TG or CPA were fit, because after more prolonged exposure to these drugs the response to NA often disappeared.

Fig. 3.15 summarises the results obtained with TG. A clear slowing of the decline in $R_{340/380}$ is apparent in the records shown in Fig. 3.15a. TG significantly ($P < 0.05$) increased the $[Ca^{2+}]_i$ decay time constant from 15 ± 4 s (n = 7) under control conditions to 110 ± 44 s (n = 7) at 0.1 μM and from 15 ± 4 s (n = 3) to 27 ± 1 s at 1 μM.

Fig. 3.16 illustrates the effect of CPA on the rate of $[Ca^{2+}]_i$ decay following application of NA. CPA had a similar effect to TG, lengthening the time constant in a concentration-dependent manner over 0.1-30 μM. The time constant was increased by 117 ± 6 % (n = 12) at 0.1 μM, from 12 ± 3 s (n = 12) to 26 ± 9 s (n = 12) and 214 ± 8 % (n = 6) at 30 μM from 50 ± 4 s (n = 6) to 157 ± 47 s (n = 6). The concentration dependence of the effect of CPA on the time constant of Ca^{2+} removal is summarised in Fig. 3.17 where it is shown that increasing concentrations of CPA resulted in increasingly slower rates of $[Ca^{2+}]_i$ decay.

a



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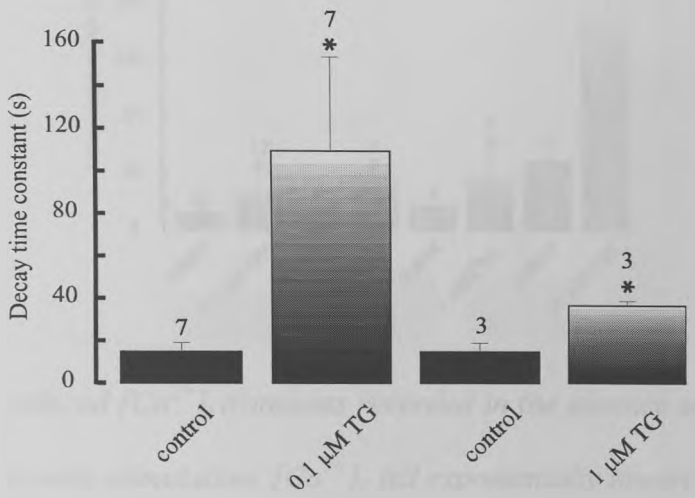

Fig. 3.15. *a NA-induced $[Ca^{2+}]_i$ transients recorded in the absence and presence of 1 μM TG. Following stimulation, $[Ca^{2+}]_i$ fell exponentially towards baseline. The curves superimposed on the decay of the Ca^{2+} transients indicate the best single exponential fit to $R_{340/380}$ decline in each condition, from which decay time constants of 6 s and 14 s were derived. b Bar charts summarising the increase in the mean $[Ca^{2+}]_i$ decay time constant in cells exposed to 0.1 μM and 1 μM TG. * Significantly different from control, $P < 0.05$. Data are expressed as mean ± S.E.M. Values above S.E.M. bars indicate the total number of cells tested.*

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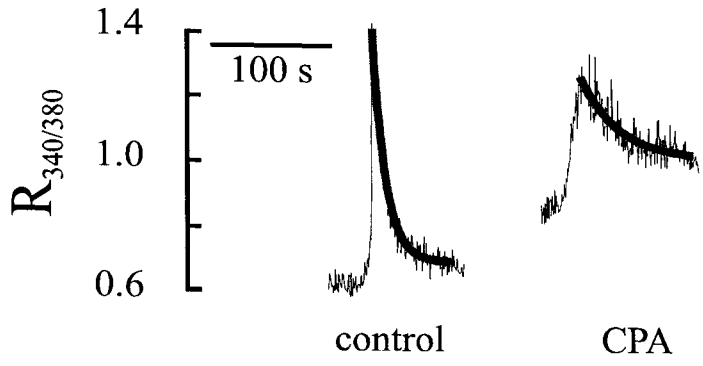

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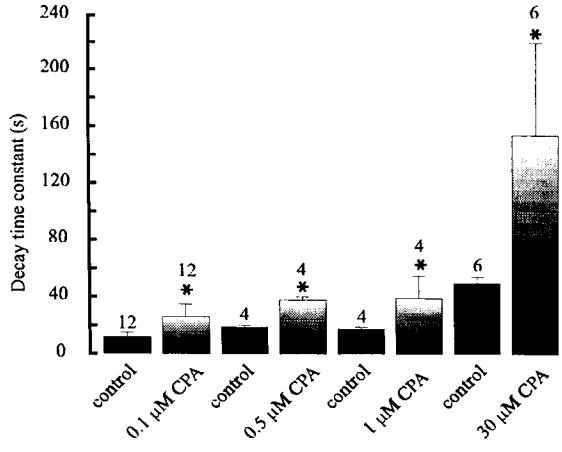


Fig. 3.16. *a NA-induced $[Ca^{2+}]_i$ transients recorded in the absence and presence of 30 μM CPA. Following stimulation, $[Ca^{2+}]_i$ fell exponentially towards baseline. The rate of $[Ca^{2+}]_i$ decay during exposure to CPA was decreased in comparison to control. Superimposed curves indicate the best single exponential fit to $R_{340/380}$ decline in each condition, from which decay time constants of 10 s and 24 s were derived. The maximal effect was obtained with 30 μM CPA. b Bar charts summarising the increase in the mean $[Ca^{2+}]_i$ decay time constant in cells exposed to 0.1-30 μM CPA (n = 4-12). * Significantly different from control, $P < 0.05$. Data are expressed as mean ± S.E.M. Values above S.E.M. bars indicate the total number of cells tested.*

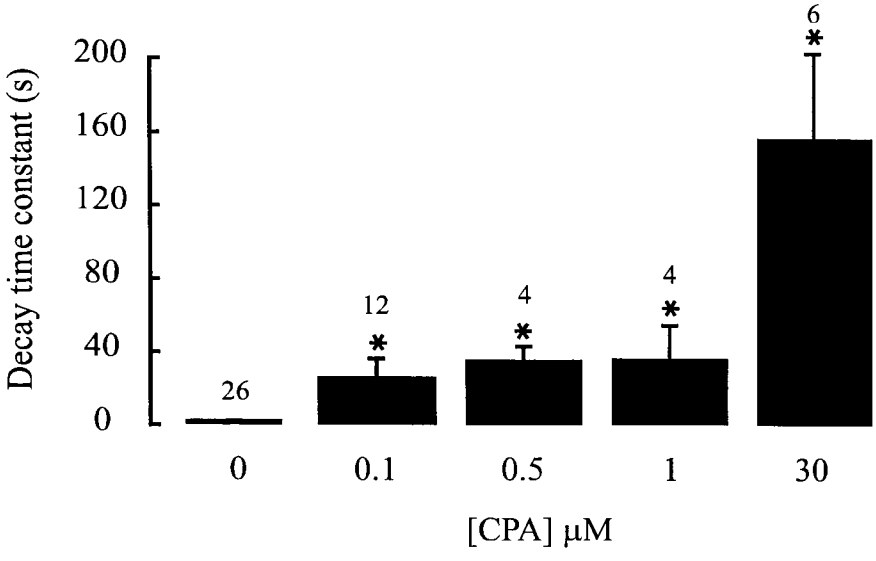


Fig. 3.17. *The concentration dependence of the CPA-induced increase in $[Ca^{2+}]_i$ decay time constant. Application of CPA at concentrations between 0.1 μM and 30 μM resulted in a progressive increase in decay time constant between 26 ± 9 s (n = 12) and 157 ± 47 s (n = 6) in a concentration-dependent manner. * Significantly different from control (absence of CPA), P < 0.05. Data are expressed as mean ± S.E.M. (n = 4-26). Values above S.E.M. bars indicate the total number of cells tested.*

3.4 Discussion

The Ca^{2+} mobilising agonists, caffeine and NA, released Ca^{2+} from the SR resulting in a rapid and transient elevation of $[Ca^{2+}]_i$, as assessed by the change in fura-2 fluorescence. Both basal $[Ca^{2+}]_i$ and the $[Ca^{2+}]_i$ transients produced by caffeine and NA were well maintained over long recording periods and repeated application. The levels of $[Ca^{2+}]_i$ observed at rest and during stimulation with caffeine and NA are consistent with those obtained in a variety of cell types (Takemura et al., 1989; Xuan et al., 1992; Berman et al., 1994; Baro and Eisner, 1995). The time required for Ca^{2+} to reach its peak in response to caffeine was significantly shorter than for NA. This is compatible with the requirements of NA for a time lag for α receptor coupling to PLC and synthesis of IP_3 before it can release Ca^{2+} (Itoh et al., 1992; Pijuan et al., 1993; Lepretre et al., 1994). In contrast, caffeine acts directly on the SR by activating a CICR mechanism (Leijten and van Breemen, 1984; Iino, 1989). With both agents, recovery to basal $[Ca^{2+}]_i$ followed a single exponential time course. This suggests that there may be one dominant Ca^{2+} removal process responsible for removing Ca^{2+} from the cell, or perhaps multiple processes with similar time constants. However, the response to caffeine decayed significantly more quickly than the NA response. This may be accounted for by the fact that caffeine is a phosphodiesterase (PDE) inhibitor. Thus, it is possible that even brief exposure to caffeine may have elevated the concentration of cAMP (Butcher and Sutherland, 1962) or cGMP, sufficiently to stimulate Ca^{2+} removal from the cell (Suematsu et al., 1984). Alternatively, since caffeine acts directly on the SR, but NA releases Ca^{2+} by stimulating IP_3 synthesis Ca^{2+} release may continue following NA removal, thereby prolonging the apparent decline. NA-induced Ca^{2+} release is also accompanied by enhanced Ca^{2+} influx

through VOCs (Nelson et al., 1988; Lepretre et al., 1994). Perhaps Ca^{2+} continues to enter for a time after release is complete, thereby also prolonging the apparent decline.

A lesser proportion of rabbit aortic SMCs responded to caffeine (25 %) in comparison to NA (50 %). The reason for this is unclear, but it may be explained by opposing actions of caffeine. Besides having a contractile effect, which is smaller than that induced by NA (Deth and Casteels, 1977; Leijten and van Breemen, 1984; Ahn et al., 1988), caffeine also has a potent inhibitory effect on smooth muscle, which is partly attributable to a decrease in $[Ca^{2+}]_i$ and partly to a decrease in the sensitivity to Ca^{2+} of the contractile proteins (Ahn et al., 1988; Sato et al., 1988). Since cAMP inhibits smooth muscle contraction (Kerrick and Hoar, 1981), it may be reasonable to assume that the inhibitory affect may in part be mediated by cAMP. Alternatively there may simply be fewer ryanodine receptors in some rabbit aortic SMCs.

3.4.1 Effects of SR Ca^{2+}-ATPase inhibitors

The specific SR Ca^{2+}-ATPase inhibitors, TG and CPA (Seidler et al., 1989; Thastrup, 1990; Lytton et al., 1991), increased basal $[Ca^{2+}]_i$ and reduced agonist-dependent $[Ca^{2+}]_i$ release. The rate of subsequent Ca^{2+} removal from the cytoplasm was also reduced as evidenced by an increase in the Ca^{2+} decay time constant compared to control. This suggests that Ca^{2+} removal from rabbit aortic SMCs depends at least in part on refilling of the SR by the TG/CPA-sensitive SR Ca^{2+}-ATPAse. Since during

prolonged exposure to these agents NA loses its ability to release Ca^{2+}, SR uptake by the ATPase may be vital to store filling and for maintaining responses to NA.

It is evident from the results that the main effects of TG and CPA were on basal $[Ca^{2+}]_i$, which was increased compared to control. Both agents induced a sustained increase in basal $[Ca^{2+}]_i$ and at low concentrations reduced the peak $[Ca^{2+}]_i$ reached in response to NA. This is in accordance with the expectation that TG and CPA would effectively deplete the SR stores, thereby decreasing subsequent Ca^{2+} release from the intracellular store, as seen in rat liver microsomes and guinea-pig ileal and urinary bladder smooth muscle (Thastrup, 1990; Suzuki et al., 1992; Uyama et al., 1992). Studies examining the role of the SR Ca^{2+}-ATPase in rat liver microsomes have shown that inhibition by TG increased basal $[Ca^{2+}]_i$ and abolished the Ca^{2+} releasing ability of IP_3 (Thastrup, 1990). Reduction in Ca^{2+} release from the SR in guinea-pig SMCs was also detected by suppression of TEA-sensitive K_{Ca} current in the presence of CPA (Suzuki et al., 1992). These effects were attributed to a decrease in Ca^{2+} uptake into the SR by inhibition of the Ca^{2+} pump, which decreased the subsequent Ca^{2+} release and reduced K_{Ca} current. Reduction of Ca^{2+} uptake due to CPA-induced inhibition of the SR Ca^{2+}-ATPase has also been observed in skinned intestinal smooth muscle (Uyama et al., 1992), where the functions of the plasma membrane Ca^{2+}-ATPase, Na^+/Ca^{2+} exchange system and ionic currents can be ignored. As a result, CPA in the concentration-range between 0.1 and 10 μM decreased the amplitude of contractions induced by caffeine and IP_3, comparable to the results of the present study.

The increase in basal $[Ca^{2+}]_i$ could be explained by the basal leak of Ca^{2+} from the SR, which would normally be counteracted by reuptake via the SR Ca^{2+}-ATPase. However, both CPA and TG may have additional actions to SR Ca^{2+}-ATPase inhibition, as they enhanced SR Ca^{2+} release in HL-60 cells, resulting in total depletion of the intracellular Ca^{2+} store (Demaurex et al., 1992). Evidence from several other laboratories measuring $[Ca^{2+}]_i$ and $^{45}Ca^{2+}$ flux have also reported that TG discharges SR Ca^{2+} (Thastrup et al., 1990; Mason et al., 1991; Xuan et al., 1992; Kargacin and Kargacin, 1995). This may account for the very large increase in basal $[Ca^{2+}]_i$ seen in rabbit aortic SMCs exposed to the higher concentration of TG (1 μM). Thus, in addition to block of Ca^{2+}uptake, Ca^{2+} may be discharged from the SR in the presence of higher TG concentrations. Two actions have previously been proposed for TG: inhibition of the SR Ca^{2+} pump at low concentrations and induction of Ca^{2+} mobilisation at higher concentrations (Lytton et al., 1991; Kargacin and Kargacin, 1995). This may explain why high concentrations of SR Ca^{2+}-ATPase inhibitors are required for effective depletion of the intracellular store in some tissues (Takemura et al., 1989; Low et al., 1991; Berman et al., 1994; Kargacin and Kargacin, 1995). The nature of the Ca^{2+} efflux pathway that promotes release at high TG concentrations is unknown, but it is possible that TG may induce a direct leak of Ca^{2+} through the SR Ca^{2+} pump itself, by altering the Ca^{2+}-ATPase structure in a manner that allows reversal of the Ca^{2+} flux through the pump, as has been suggested for the interaction of other ligands with the SR Ca^{2+} pump (de Mies, 1991). Under normal circumstances the ATPase is phosphorylated. However, ligands can compete for the same binding site on the enzyme and if this occurs normal regulation of the ATPase may be lost, so that Ca^{2+} leaks through the ATPase when the catalytic site is not

occupied by natural substrates (P_i, Mg^{2+}, ADP, Ca^{2+} and K^+), or occupied by ligands that prevent the binding of natural substrates. Thus, the Ca^{2+}-ATPase can operate either as a pump or as a Ca^{2+} channel. This would explain the large increase in basal $[Ca^{2+}]_i$, to levels usually seen at the peak of the response of rabbit aortic SMCs to NA in control conditions, thereby preventing NA from increasing $[Ca^{2+}]_i$ further. Similar results were reported on cultured aortic SMCs (Berman et al., 1994), where treatment with TG was shown to increase basal $[Ca^{2+}]_i$ and decrease SR Ca^{2+} content by 9-25 %, as manifest by a decreased change in $[Ca^{2+}]_i$ induced by AVP.

$[Ca^{2+}]_i$ declines when the rate of Ca^{2+} release becomes lower than the rate of Ca^{2+} removal from the cell, permitting relaxation. The decline of $[Ca^{2+}]_i$ has been suggested to be primarily attributed to reuptake of Ca^{2+} by the SR Ca^{2+}-ATPase (Kargacin and Fay, 1991; Kargacin and Kargacin, 1995). My results are compatible with a major role for the ATPase in removing Ca^{2+} from rabbit aortic SMCs. Inhibition of SR Ca^{2+}-ATPase with TG and CPA slowed the rate at which $[Ca^{2+}]_i$ declined back to the basal level following Ca^{2+} release evoked by NA, suggesting that it was limited by SR Ca^{2+}-ATPase activity. This supports previous suggestions that the SR Ca^{2+}-ATPase lowers $[Ca^{2+}]_i$ in aorta smooth muscle by stimulating Ca^{2+} uptake into the store following release (Low et al., 1991; Gurney and Allam, 1995). It is likely that more than one removal mechanism was operating to remove Ca^{2+} from the cytosol. This is suggested by the finding that the $[Ca^{2+}]_i$ continued to decline following Ca^{2+} release from the SR in the presence of TG or CPA, albeit at a lower rate. However, the fact that the decay time constants continued to follow a single exponential time course in the presence of TG or CPA demonstrated that the

inhibitors simply prolonged the rate of Ca^{2+} removal from the cell without introducing new components of Ca^{2+} removal. It is therefore likely that Ca^{2+} uptake by the SR makes a major contribution to the lowering of $[Ca^{2+}]_i$ in aortic SMCs under normal circumstances.

The results are largely consistent with contractility studies that reported TG to cause a slowly developing increase in basal tension and to inhibit repletion of the PE-sensitive store in rat aorta and dog mesenteric artery (Low et al., 1991). Inhibition of SR Ca^{2+} sequestration was shown to elevate vascular tone in isolated strip preparations and enhance cell shortening in single VSMCs (Low et al., 1991). SR Ca^{2+}-ATPase inhibitors have been reported to similarly modulate the contractile properties of other smooth muscle preparations (Shima and Blaustein, 1992; Kwan et al., 1994; Takemoto et al., 1998), consistent with their inhibitory effects on the SR Ca^{2+}-ATPase.

The hypothesis that the ATP-dependent Ca^{2+} pump of the SR contributes significantly to the removal of Ca^{2+} from the cytoplasm during transient Ca^{2+} signals is strongly supported by the results of the present chapter. In addition to serving as a source of activator Ca^{2+} for contractile activation, the SR may therefore help to modulate tension in rabbit aorta by supporting agonist-induced rises in $[Ca^{2+}]_i$, through rapid sequestration of Ca^{2+} following evoked release. The proper functioning of the SR Ca^{2+} pump is required for the removal of Ca^{2+} from the cytoplasm of rabbit aortic SMCs and to maintain filling of the SR Ca^{2+} stores.

Chapter 4

Effects of nitrovasodilators on Ca^{2+} transients in rabbit aortic smooth muscle cells: involvement of SR Ca^{2+} stores

4.1 Introduction

The results described in chapter 2 provided evidence that SNP could modulate the amount of Ca^{2+} released from the SR Ca^{2+} stores by caffeine. Several additional lines of evidence also suggest that nitrovasodilators modulate SR Ca^{2+} store function, and this plays an important role in generating vasodilation. Ca^{2+} can be released from stores, either through CICR, which is activated by caffeine, or through activation of IP_3 receptors. Inhibition of SR Ca^{2+} release may be achieved by blocking the ryanodine receptor Ca^{2+} release channel (Meszaros et al., 1996; Aghdasi et al., 1997; Kannan et al., 1997) or by inhibiting PLC and the production of IP_3 (Rapoport et al., 1983; Cheung and MacKay, 1985; Lang and Lewis, 1989; Hirata et al., 1990) or its action. Enhancement of Ca^{2+} release could be produced by facilitating these processes or by stimulating the SR Ca^{2+}-ATPase to promote store filling. The literature contains evidence to support most of these actions in mediating the vasodilator effects of NO. Effects on both caffeine- and IP_3-sensitive Ca^{2+} stores have been proposed. For example, SNAP was shown to reduce $[Ca^{2+}]_i$ by inhibiting SR Ca^{2+} release through both the IP_3 and ryanodine receptors of porcine tracheal smooth muscle (Kannan et al., 1997). However, in the presence of ryanodine, GTN retained most of its ability to relax the coronary artery (Khan et al., 1998), suggesting that the ryanodine receptor/Ca^{2+} release channel of the SR is not its primary site of action. SNP was also found not to influence intracellular Ca^{2+} release through the ryanodine receptor pathway, but instead inhibited Ca^{2+} release stimulated by IP_3 in rat aortic VSM (Ji et al., 1998). Similar reports using authentic NO have been reported in the pulmonary artery (Yuan et al., 1997).

The inhibition of Ca^{2+} release from IP_3-sensitive stores is a well-established function of NO in various cell systems (Cheung and MacKay, 1985; Collins et al., 1986; Lang and Lewis, 1989; Hirata et al., 1990). For example, Cheung and MacKay (1985) showed the effects of SNP on neurally evoked responses in the rat tail artery to be consistent with a mechanism whereby it inhibits intracellular Ca^{2+} release from the IP_3 receptor/channel. SNP had no effect on the action potential during inhibition of contraction, indicating that it did not interfere with the influx of external Ca^{2+}, but inhibited Ca^{2+} release. Collins et al. (1986) also proposed such an action for EDRF and nitrovasodilators in the rabbit aorta. However, $^{45}Ca^{2+}$ flux and contractile studies suggested that in addition to inhibition of Ca^{2+} release, NO also inhibited Ca^{2+} influx, thereby reducing the $[Ca^{2+}]_i$ available for contraction. Subsequent reports on aortic smooth muscle have shown that agents that increase cGMP levels inhibit agonist-stimulated IP_3 production (Lang and Lewis, 1989; Hirata et al., 1990), which provides an explanation for the previously observed reduction in Ca^{2+} release from the SR. However, Sumimoto et al. (1987) reported that GTN had no effect on the hydrolysis of PIP_2 in arterial smooth muscle contracted by NA and Fujino et al. (1991) failed to observe a reduction of the ATP-induced increase in $[Ca^{2+}]_i$ in the presence of GTN.

Further definition of the mechanism by which cGMP-generating nitrovasodilators produce their effects on the SR comes from contractile studies in the rat aorta, where SNP and 8-Br-cGMP were suggested to antagonise increases in $[Ca^{2+}]_i$ by enhancing Ca^{2+} sequestration into the SR, thus, decreasing contractile activity (Lincoln, 1983). In this study, 8-Br-cGMP completely abolished tension development to NA in Ca^{2+}-

free solution and in the presence of Ca^{2+} antagonists, suggesting that cGMP interfered with cellular activation of Ca^{2+} rather than Ca^{2+} influx. Similar evidence for an effect of SNP on Ca^{2+} sequestration in rat aorta was provided by Karaki et al. (1988). Consistent with these findings, cGMP increased the removal of ~ 20 % of total Ca^{2+} into the SR of aortic cells (Twort and van Breemen, 1988; van Breemen et al., 1988). It is also supported by electrophysiological evidence in isolated SMCs as previously described (Komori and Bolton, 1989; Clapp and Gurney, 1991a) and by the effects of ATPase inhibitors on the relaxant response to NO donors (Luo et al., 1993; Andriantsitohaina et al., 1995; Raymond and Wendt, 1996; Khan et al., 1998). Furthermore, KCl-induced contraction was less markedly inhibited by SNP and 8-Br-cGMP than NA-induced contraction (Lincoln, 1983), implying an action on a pathway selectively activated in the presence of NA.

Since there is evidence for modulation by NO of Ca^{2+} stores through actions on ryanodine receptors, IP_3 receptors and the SR Ca^{2+}-ATPase, the current chapter explored the contribution of these actions to the effects of NO donors on $[Ca^{2+}]_i$ transients in rabbit aortic SMCs generated by the application of caffeine or NA. $[Ca^{2+}]_i$ was measured in single SMCs loaded with fura-2, using the membrane-permeant, fura-2/AM, as described in chapter 3.

4.2 Materials and Methods

The experiments reported in this chapter employed the methods described in chapter 3 (pages 83-95).

4.2.1 List of drugs

The following drugs were used: caffeine (Sigma), noradrenaline (arterenol, bitartrate salt, Sigma), sodium nitroprusside (SNP, Sigma), glyceryl trinitrate (nitronal, Lipha Pharmaceuticals Ltd., West Drayton, Middlesex, U.K.), cyclopiazonic acid (CPA, Calbiochem). Drugs prepared on the day of each experiment were routinely dissolved in water as stock solutions, and further dilutions were made in the same solution bathing the cells, with the exception of caffeine, which was dissolved directly in bath solution. SNP was stored in a dark flask at 4 °C as a 20 mM stock solution and used on the same day as it was prepared. CPA was dissolved as a stock solution in DMSO and further diluted in bath solution. All solutions were routinely filtered before use through a pore size of 0.2 μm.

4.3 Results

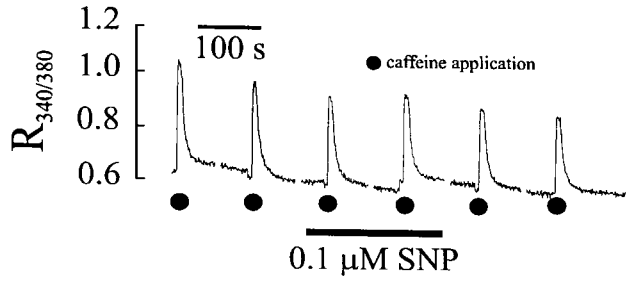
4.3.1 Effect of nitrovasodilators on caffeine-induced $[Ca^{2+}]_i$ mobilisation

4.3.1.1 SNP

Caffeine (20 mM) was applied to individual SMCs at 5 min intervals and caused a transient elevation of $[Ca^{2+}]_i$ that returned to baseline levels within a few seconds and was reproducible, as shown in chapter 3. After obtaining two similar responses to caffeine, SNP was added to the bathing solution for 5 min and caffeine applications were repeated. After obtaining two responses in the presence of SNP, the drug was removed and caffeine was tested again in drug-free solution. Fig. 4.1 illustrates the effect of SNP on caffeine-induced $[Ca^{2+}]_i$ transients, shown as the $R_{340/380}$ signal reported by fura-2. At 0.1 μM SNP, application of caffeine induced a transient

increase in $[Ca^{2+}]_i$ to a level similar to that attained without SNP (Fig. 4.1a). Elevating SNP to 1 μM also failed to consistently modify the response to caffeine. Although, in the example shown in Fig. 4.1b, the response to caffeine was increased, it did not return to the control level following washout of SNP. Furthermore, mean basal $[Ca^{2+}]_i$ and caffeine-stimulated Ca^{2+} levels, measured for 7-8 cells, were not significantly different ($P > 0.05$) in the presence of SNP at 0.1 μM (Fig. 4.2a) or 1 μM (Fig. 4.2b) compared with control. Gaps between the records in Fig. 4.1 represent gaps in the recording when fura-2 excitation was stopped to limit photobleaching of the probe during the experiment, while the SR Ca^{2+} stores refilled. When the SNP concentration was increased further to 10 μM, caffeine responses were significantly ($P < 0.05$) reduced. Fig. 4.3a illustrates the effect of 10 μM SNP on the caffeine-induced $[Ca^{2+}]_i$ transients reported by fura-2 followed by full recovery upon washout. SNP was applied 5 min prior to the addition of caffeine and this immediately reduced the caffeine response. While addition of 10 μM SNP reduced the caffeine-induced $[Ca^{2+}]_i$ transients, it had no effect on resting $[Ca^{2+}]_i$ as illustrated in Fig. 4.3b. The peak $[Ca^{2+}]_i$ induced by caffeine was significantly ($P < 0.05$) reduced by 43 ± 1 % ($n = 9$). Prior to the application of SNP, caffeine increased $[Ca^{2+}]_i$ from 61 ± 1 to 190 ± 10 nM ($n = 9$), whereas in the presence of 10 μM SNP, $[Ca^{2+}]_i$ was increased from 62 ± 1 to 110 ± 10 nM ($n = 9$).

a



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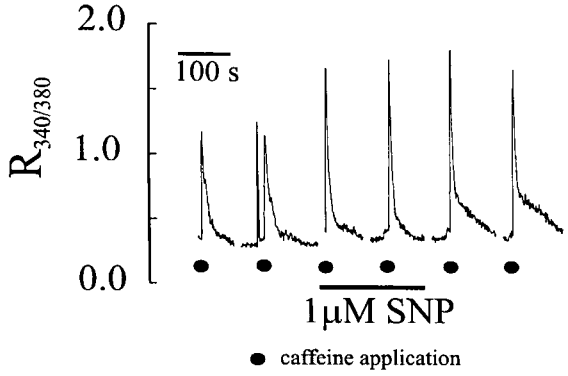
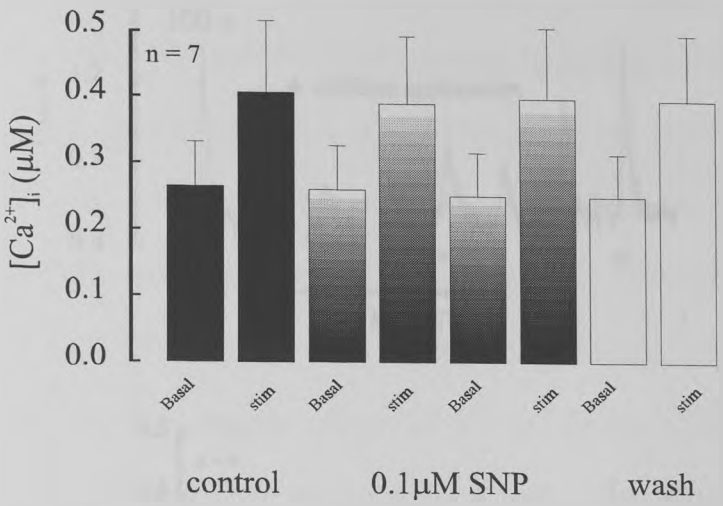


Fig. 4.1. *Caffeine-induced $[Ca^{2+}]_i$ transients in the presence and absence of SNP. At 0.1 μM (a) and 1 μM (b), SNP did not affect resting $[Ca^{2+}]_i$ or caffeine-induced increases in $[Ca^{2+}]_i$ as indicated by $R_{340/380}$. Gaps in the records represent 5 min recovery periods. Caffeine was applied at the times indicated (•). SNP was applied to the cells at a concentration of 0.1 μM or 1 μM for the duration indicated by the horizontal bars. SNP was added for 5 min before testing caffeine in its presence.*

a



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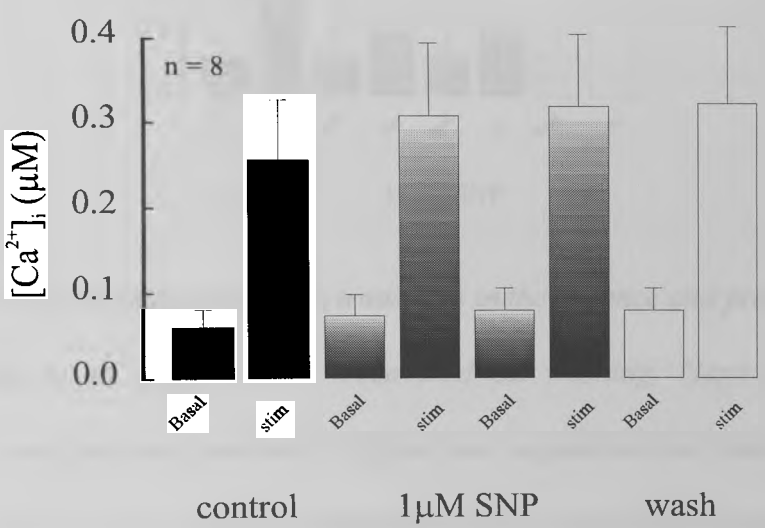
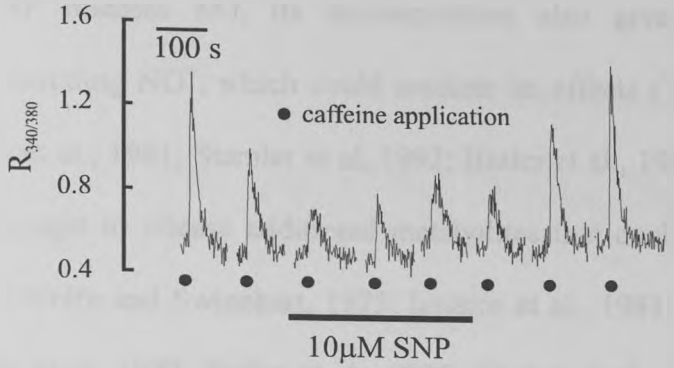


Fig. 4.2. *Mean $[Ca^{2+}]_i$ measured before (basal) and at the peak of the response to caffeine (stim). At 0.1 μM (a) and 1 μM (b), SNP did not significantly affect mean basal $[Ca^{2+}]_i$ or caffeine-stimulated Ca^{2+} levels. Data are expressed as mean ± S.E.M. (n = 7 at 0.1 μM and 8 at 1 μM SNP).*

a



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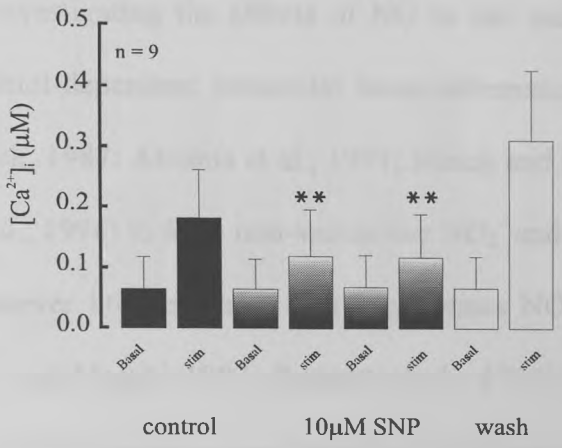


Fig. 4.3. *a Caffeine-induced $[Ca^{2+}]_i$ transients in the absence and presence of 10 μM SNP. Responses to caffeine were recovered on washing. Gaps in the records represent 5 min recovery periods. Caffeine was applied at the times indicated (•). SNP was applied to the cell at a concentration of 10 μM for the duration indicated by the horizontal bar and for 5 min before testing caffeine. b Mean $[Ca^{2+}]_i$ measured before (basal) and at the peak of the response to caffeine (stim) in the absence and presence of SNP. At 10 μM, SNP significantly reduced caffeine-induced $[Ca^{2+}]_i$ transients, but had no effect on basal $[Ca^{2+}]_i$. ** Significantly different from stimulated control, $P < 0.05$. Data are expressed as mean ± S.E.M. ($n = 9$).*

4.3.1.2 GTN

Although SNP releases NO, its decomposition also gives rise to additional byproducts, including NO^+, which could mediate its effects (Wolfe and Swinehart, 1975; Ignarro et al., 1981; Stamler et al, 1992; Butler et al., 1995). In fact, most NO donors are thought to release additional metabolites that could potentially mediate their actions (Wolfe and Swinehart, 1975; Ignarro et al., 1981; Noack and Feelisch, 1991; Stamler et al, 1992; Butler et al., 1995; Chabot et al., 1997). It is therefore, important when investigating the effects of NO to use multiple NO donors. GTN releases NO by thiol-dependent metabolic biotransformation (Ignarro et al., 1981; Feelisch and Noack, 1987; Abrams et al., 1991; Noack and Feelisch, 1991; Feelisch, 1991; Bennett et al., 1994) to form non-vasoactive NO_2^- and subsequent formation of NO. There is however little evidence that it generates NO^+ or other redox related species (Feelisch and Noack, 1987; Bennett et al., 1994). The effect of GTN on caffeine-induced $[Ca^{2+}]_i$ transients was tested at 1 μM, because at this concentration it causes maximal relaxation of rabbit aortic smooth muscle (Martin et al., 1985; Luo et al., 1993). Fig. 4.4 illustrates the effect of 1 μM GTN on basal $[Ca^{2+}]_i$ and caffeine-induced $[Ca^{2+}]_i$ transients. Representative records of the increase in $R_{340/380}$ evoked by fast application of caffeine (20 mM) are shown in the absence and presence of 1 μM GTN in Fig. 4.4a. Application of caffeine in the presence of GTN induced a transient increase in $[Ca^{2+}]_i$ to a level similar to that attained without GTN. Furthermore, mean basal $[Ca^{2+}]_i$ and NA-stimulated Ca^{2+} levels measured in the presence of GTN (Fig. 4.4b) did not differ significantly ($P > 0.05$) from control ($n = 7$). GTN was applied 5 min prior to the addition of caffeine.

4.3.2 Influence of nitrovasodilators on the rate of Ca^{2+} removal from the cytoplasm following caffeine application

As outlined previously, the elevated $[Ca^{2+}]_i$ observed after application of caffeine fell back to baseline along an exponential time course. This was also true when caffeine was applied in the presence of the nitrovasodilators, SNP and GTN. Fig. 4.5a illustrates the exponential fits to the decline of $[Ca^{2+}]_i$ following a caffeine-induced $[Ca^{2+}]_i$ transient, in the absence and presence of 0.1 μM SNP. The control decay time constant was measured as 7 s, compared with a $[Ca^{2+}]_i$ decay time constant during SNP treatment of 8 s. The mean results showed that, at 0.1 μM, SNP did not significantly alter the rate of Ca^{2+} removal from the cell (Fig. 4.5b). The $[Ca^{2+}]_i$ decay time constant measured in the presence of higher SNP concentrations also did not differ significantly from control (n = 7-9), as shown in Fig. 4.5c for 1 μM SNP and Fig. 4.5d for 10 μM SNP. Thus, 10 μM SNP reduced the amplitude of the $[Ca^{2+}]_i$ transient without altering its rate of decay. GTN also had no consistent effect on the Ca^{2+} decay time constant as shown in Fig. 4.5e. As with SNP, the decline of $[Ca^{2+}]_i$ remained exponential in the presence of GTN, with a decay time constant of 41 ± 7 s (n = 7) compared with 44 ± 8 s (n = 7) before its application.

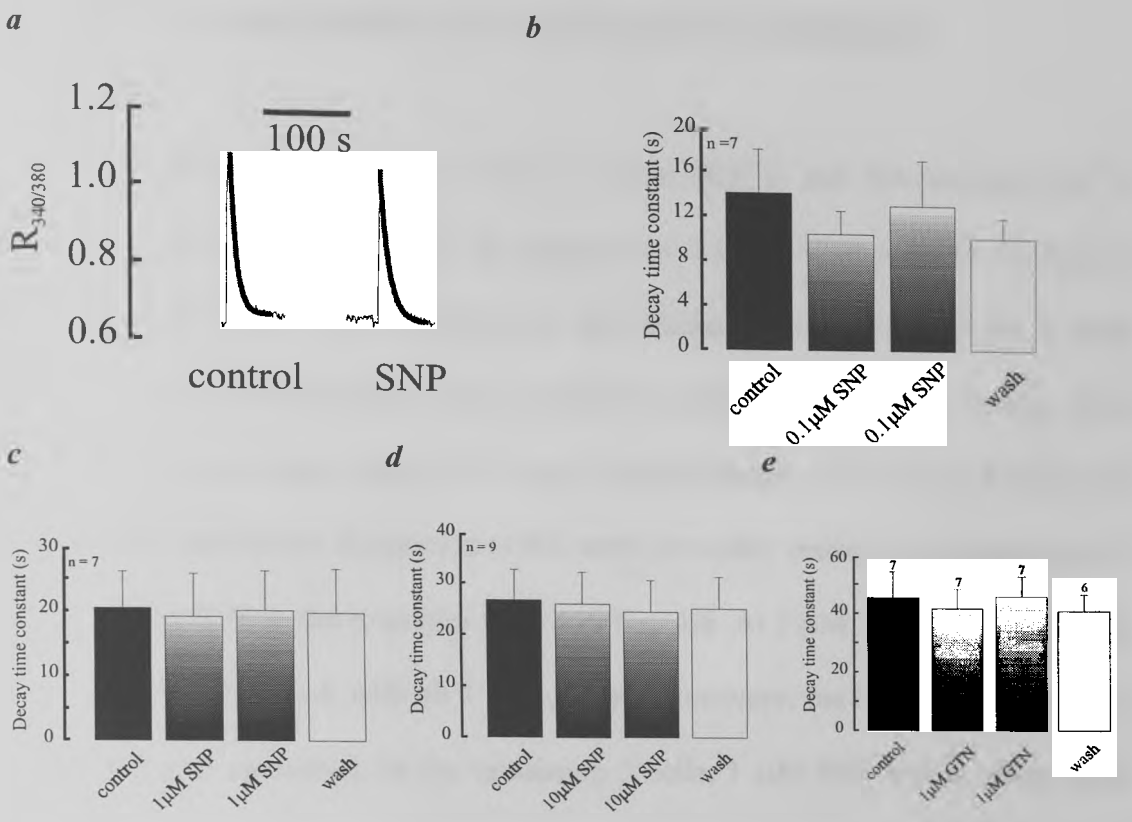


Fig. 4.5. *a Caffeine-induced $[Ca^{2+}]_i$ transients showing the decay of $[Ca^{2+}]_i$ in the absence and presence of 0.1 μM SNP. The curves superimposed on the decay of the Ca^{2+} transients indicate the best single exponential fit to the decline in $R_{340/380}$ with a time constant of 7 s under control conditions and 8 s after applying SNP. b-e Mean $[Ca^{2+}]_i$ decay time constants from 6-9 cells exposed to SNP at 0.1 μM (b), 1 μM (c), 10 μM (d) or 1 μM GTN (e). Responses were recorded 5 min apart. SNP or GTN was added for 5 min or washed off for 5 min before testing caffeine. There were no significant differences between $[Ca^{2+}]_i$ decay time constants measured at any concentration of SNP or GTN compared with control. Data are expressed as mean ± S.E.M. Values above S.E.M. bars indicate the total number of cells tested.*

4.3.3 Effect of nitrovasodilators on NA-induced $[Ca^{2+}]_i$ mobilisation

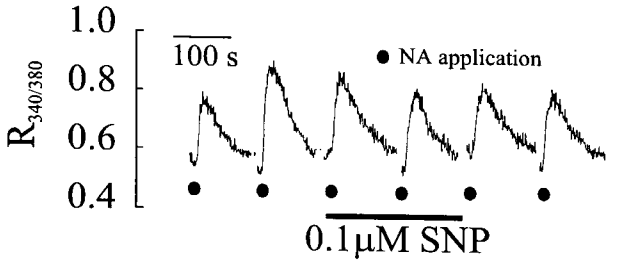
4.3.3.1 SNP

Fig. 4.6 illustrates the effect of SNP on basal $[Ca^{2+}]_i$ and NA-induced $[Ca^{2+}]_i$ transients. Application of NA in the presence of 0.1 μM SNP induced a transient increase in $[Ca^{2+}]_i$ to a level similar to that attained without SNP in all 7 cells examined. The lack of effect of 0.1 μM SNP on these cells is clear in Fig. 4.7a, which shows the mean basal $[Ca^{2+}]_i$ and NA-stimulated $[Ca^{2+}]_i$ level before and during SNP application. Responses to NA were reversibly reduced in the presence of 1 and 10 μM SNP in the examples shown in Fig. 4.6. At 1 μM, SNP reduced $[Ca^{2+}]_i$ in 3 out of 9 cells tested, with all 3 cells showing recovery, but it increased $[Ca^{2+}]_i$ in 1 cell that also recovered. In the remaining 5 cells, 1 μM SNP had no discernible effect. At 10 μM, SNP reduced $[Ca^{2+}]_i$ in 3 out of 7 cells tested, with 2 cells showing recovery, and increased $[Ca^{2+}]_i$ in 2 cells, with 1 cell recovering. Basal $[Ca^{2+}]_i$ did not appear to be reversibly changed in any cell tested. Despite these observations, the mean basal $[Ca^{2+}]_i$ and NA-stimulated Ca^{2+} levels measured in the presence of 1 or 10 μM SNP did not differ significantly ($P > 0.05$) from control (Fig. 4.7). SNP was applied 5 min prior to the addition of NA and washed off for 5 min before testing for recovery of the NA response.

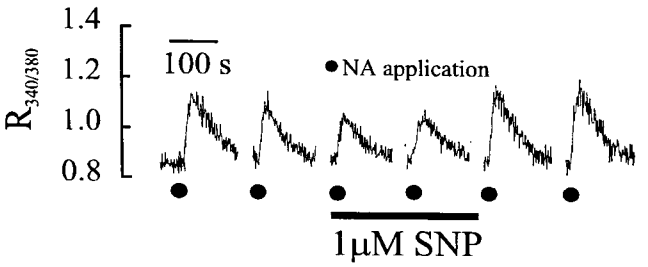
4.3.3.2 GTN

Fig. 4.8a illustrates the effect of 1 μM GTN on basal $[Ca^{2+}]_i$ and NA-induced $[Ca^{2+}]_i$ transients. In the example shown, application of NA in the presence of 1 μM GTN induced a transient increase in $[Ca^{2+}]_i$, which became gradually reduced and did not

a



b



c

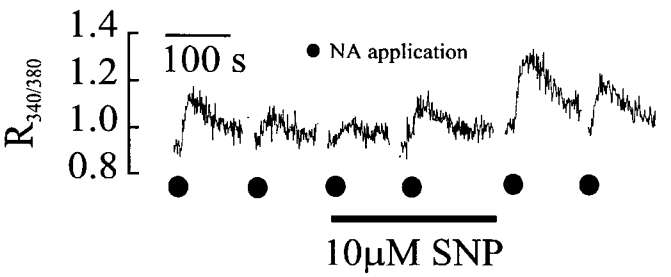


Fig. 4.6. *NA-induced $[Ca^{2+}]_i$ transients in the absence and presence of SNP at 0.1 μM (a), 1 μM (b) and 10 μM (c). NA-induced increases in $[Ca^{2+}]_i$ were reversibly decreased in (b) and (c). This effect was seen in 3 cells at 1 μM SNP and 2 cells at 10 μM SNP. Gaps in the records represent 5 min recovery periods. NA was applied at the times indicated (•). SNP was applied to the cells at a concentration of 0.1-10 μM for the duration indicated by the horizontal bars and for 5 min before testing NA.*

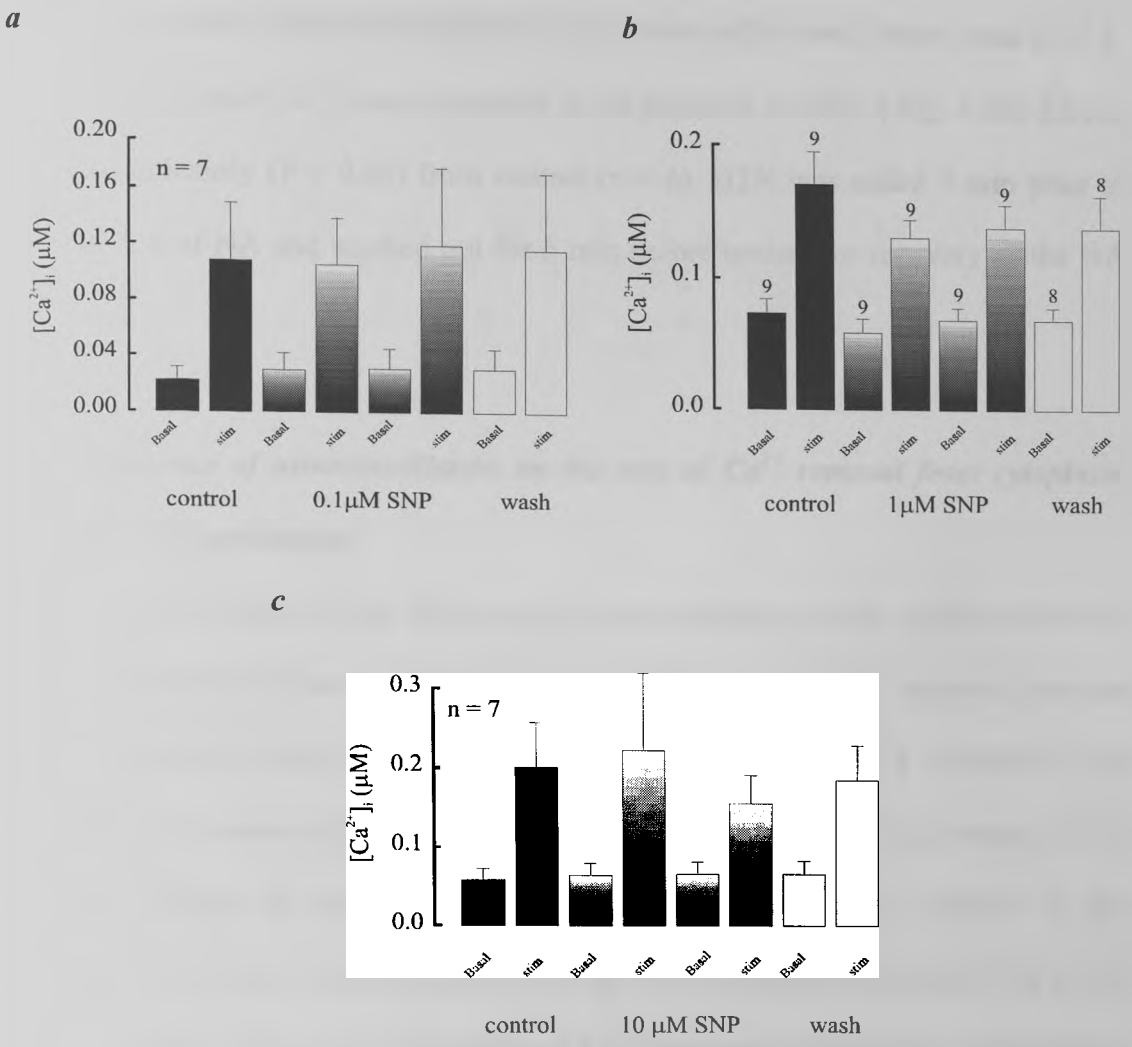


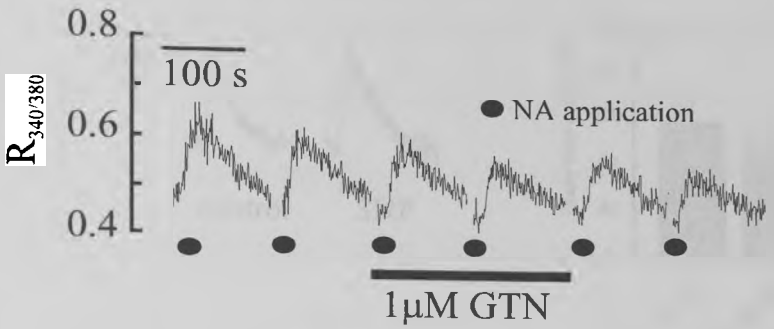
Fig. 4.7. *Mean $[Ca^{2+}]_i$ measured before (basal) and at the peak of the response to NA (stim) in the absence and presence of SNP at 0.1 μM (a), 1 μM (b) and 10 μM (c). SNP did not significantly affect mean basal $[Ca^{2+}]_i$ or NA-stimulated Ca^{2+} levels at any concentration tested. Data are expressed as mean ± S.E.M. (n = 7-9). Values above S.E.M. bars indicate the total number of cells tested.*

recover on washout. This was representative of most cells tested. Mean basal $[Ca^{2+}]_i$ and NA-stimulated Ca^{2+} levels measured in the presence of GTN (Fig. 4.8b) did not differ significantly ($P > 0.05$) from control ($n = 6$). GTN was added 5 min prior to the addition of NA and washed out for 5 min before testing for recovery of the NA response.

4.3.4 Influence of nitrovasodilators on the rate of Ca^{2+} removal from cytoplasm following NA application

As well as investigating the influence of nitrovasodilators on the peak response to NA, their potential effect was also investigated on the rate of Ca^{2+} removal from the cell, by comparing the declining phase of the NA-induced $[Ca^{2+}]_i$ transient in the absence and presence of nitrovasodilator drugs. In the presence of both drugs, $[Ca^{2+}]_i$ declined towards the basal level in an exponential manner, as observed in the absence of the drug (control conditions). Fig. 4.9a illustrates exponential fits to the decline of $[Ca^{2+}]_i$ following a NA-induced Ca^{2+} transient in the absence and presence of 1 μM SNP. The control decay time constant of $[Ca^{2+}]_i$ prior to the application of 1 μM SNP was measured as 52 s, compared with 51 s in its presence. The mean $[Ca^{2+}]_i$ decay time constant measured in the presence of SNP at 3 concentrations did not differ significantly ($P > 0.05$) from control ($n = 7$-9) as shown in Fig. 4.9b-d (0.1, 1 and 10 μM). Thus, even at the highest SNP concentration, the profile of Ca^{2+} removal from the cell was unaltered. GTN also failed to alter the profile of Ca^{2+} removal. The $[Ca^{2+}]_i$ decay time constant measured in the presence of GTN (1 μM) did not differ significantly ($P > 0.05$) from control ($n = 6$) as shown in Fig. 4.9e.

a



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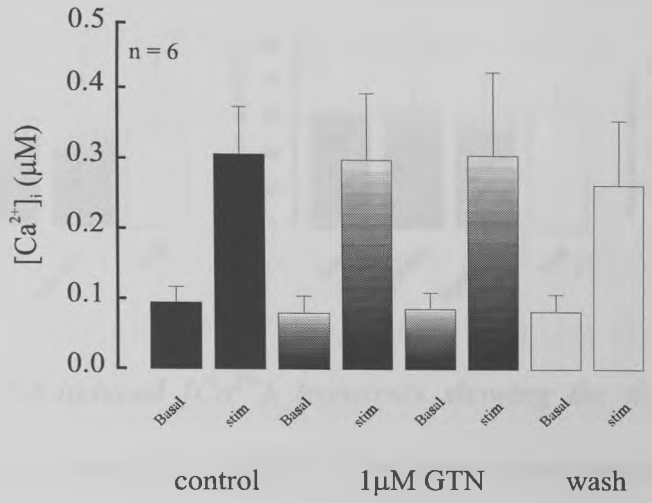


Fig. 4.8. *a NA-induced $[Ca^{2+}]_i$ transients in the absence and presence of 1 μM GTN. Gaps in the records represent 5 min recovery periods. NA was applied at the times indicated (•). GTN was applied to the cell for the duration indicated by the horizontal bar and for 5 min before testing NA. b Mean $[Ca^{2+}]_i$ measured before (basal) and at the peak of the response to NA (stim) in the absence and presence of GTN. At 1 μM, GTN did not significantly affect mean basal $[Ca^{2+}]_i$ or NA-stimulated Ca^{2+} levels. Data are expressed as mean ± S.E.M. (n = 6).*

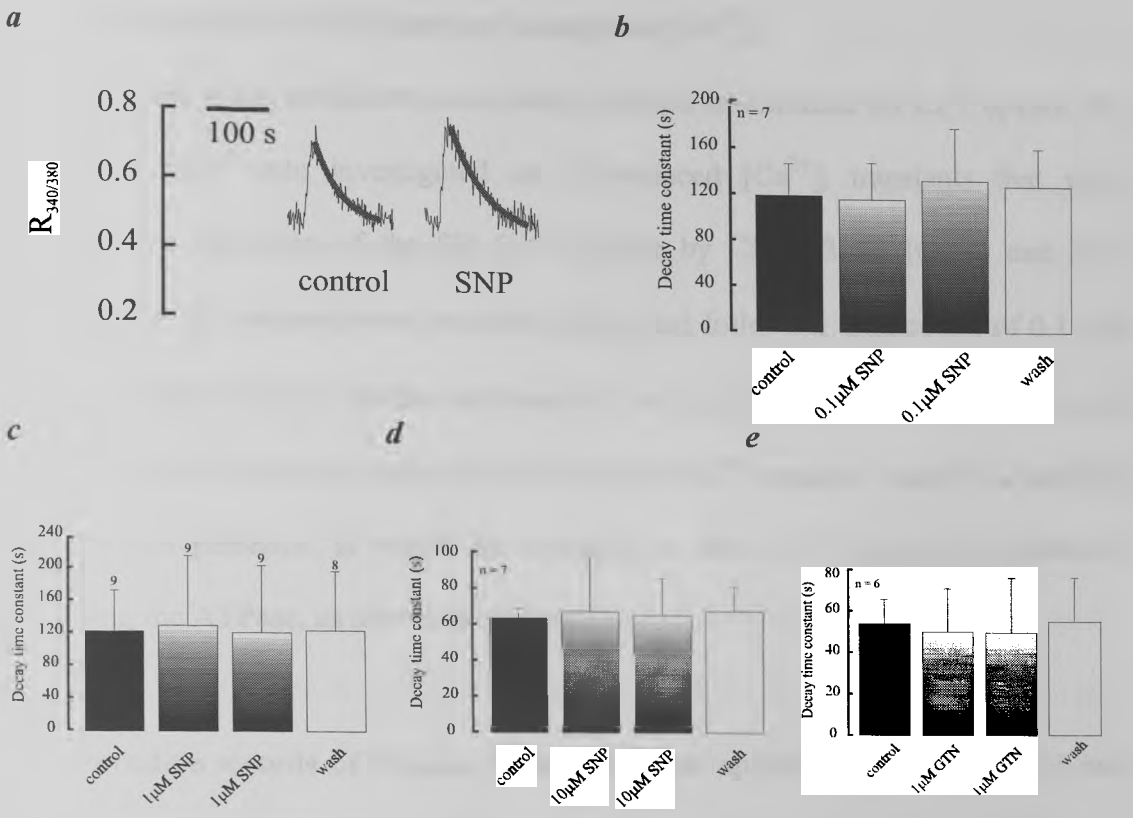


Fig. 4.9. *a NA-induced $[Ca^{2+}]_i$ transients showing the decay of $[Ca^{2+}]_i$ in the absence and presence of 1 μM SNP. The curves superimposed on the decay of the Ca^{2+} transients indicate the best single exponential fit to the decline in $R_{340/380}$ with a time constant of 52 s under control conditions and 51 s in the presence of SNP. b-e Mean $[Ca^{2+}]_i$ decay time constant from 6-9 cells exposed to SNP at 0.1 μM (b), 1 μM (c), 10 μM (d) or 1 μM GTN (e). There were no significant differences between $[Ca^{2+}]_i$ decay time constants measured at any concentration of SNP or GTN compared with control. Data are expressed as mean ± S.E.M. (n = 6-9). Values above S.E.M. bars indicate the total number of cells tested.*

4.3.5 Effect of SNP on CPA-induced changes in $[Ca^{2+}]_i$

Since NO and nitrovasodilators have been proposed to stimulate SR Ca^{2+} uptake, the effects of SNP were investigated on NA-induced $[Ca^{2+}]_i$ transients that were modified by inhibition of the SR Ca^{2+}-ATPase by CPA. Basal $[Ca^{2+}]_i$ and NA-induced $[Ca^{2+}]_i$ transients were recorded before and following application of 0.1 μM CPA, and again after the further addition of 1 or 10 μM SNP. This concentration of CPA reduced but did not abolish the NA-induced Ca^{2+} transient when NA was first tested in its presence. It would be expected to slow Ca^{2+} removal by partially inhibiting the ATPase, as shown in chapter 3.

Representative records of $R_{340/380}$, evoked by brief application of NA, before and during the application of CPA, then after additional application of 10 μM SNP, are shown in Fig. 4.10a. As before, the response to NA was decreased and basal $[Ca^{2+}]_i$ was increased by CPA. Under these conditions, 10 μM SNP almost abolished the response to NA, but reversed the CPA-induced increase in basal $[Ca^{2+}]_i$ as can be seen in the representative example shown in Fig. 4.10a. Fig. 4.10b and c summarise the effects of 1 μM and 10 μM SNP, respectively, applied in the presence of CPA. The CPA-induced reduction in NA-stimulated Ca^{2+} levels was further enhanced following addition of SNP at both concentrations. However, since prolonged application of CPA on its own caused increased inhibition, it is not clear if this represents an additive effect of SNP. However, SNP clearly influenced basal $[Ca^{2+}]_i$. At 1 μM, SNP had no effect on basal $[Ca^{2+}]_i$ compared with CPA alone, but it reversed the CPA-induced increase in basal $[Ca^{2+}]_i$ at 10 μM (Fig. 4.10c). Basal $[Ca^{2+}]_i$ was reduced from 0.08 ± 0.02 μM (n = 6) in the presence of CPA alone to

0.03 ± 0.01 μM (n = 6) following addition of 10 μM SNP. This compares with basal $[Ca^{2+}]_i$ of 0.05 ± 0.02 μM (n = 3) under control conditions. This reversal of the CPA effect was caused by SNP, because during prolonged CPA exposure in the absence of SNP no recovery was observed. CPA was added 12 min and SNP was added 5 min prior to application of NA in the presence of both drugs.

4.4 Discussion

The Ca^{2+} mobilising agonists, caffeine and NA, have previously been shown to stimulate Ca^{2+} release from the SR through a ryanodine receptor/channel and an IP_3 receptor/channel, respectively. The commonly used NO donor drugs SNP and GTN were investigated as examples of nitrovasodilators that release NO through different mechanisms. Neither drug had any effect on basal $[Ca^{2+}]_i$, even though tested at relatively high concentrations sufficient to produce maximal vasodilation. They also had no consistent effect on the Ca^{2+} transients evolved by caffeine or NA, when applied at 1 μM.

4.4.1 Evidence for modulation of SR Ca^{2+} release in the regulation of $[Ca^{2+}]_i$ by SNP

Overall, the peak $[Ca^{2+}]_i$ induced by caffeine or NA was unaffected by either 0.1 or 1 μM SNP or 1 μM GTN. However, when the SNP concentration was increased further to 10 μM, peak $[Ca^{2+}]_i$ induced by caffeine was significantly reduced, but the response to NA was not. These results suggest that at relatively high concentrations SNP selectively inhibited Ca^{2+} release through the ryanodine receptor/channel. SNP

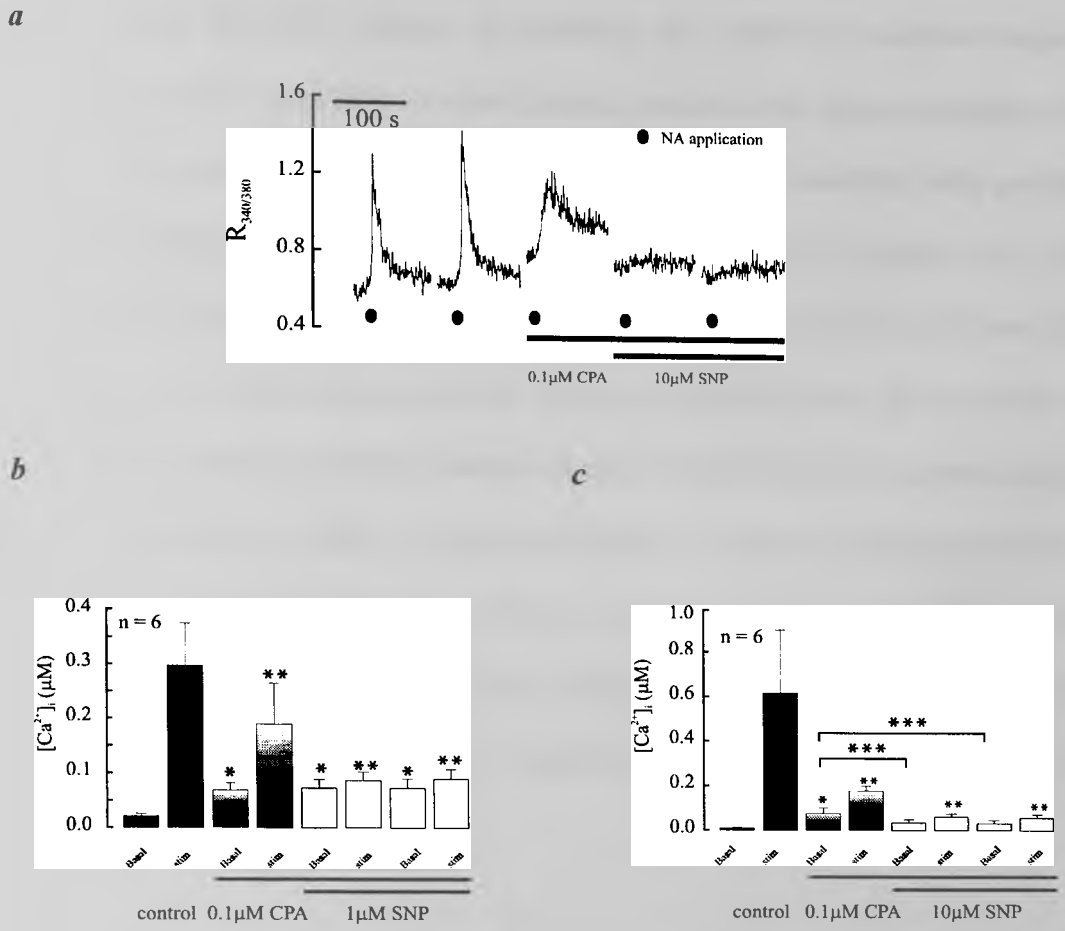


Fig. 4.10. *Influence of SNP on NA-induced $[Ca^{2+}]_i$ transients modified by CPA. a NA was applied at the times indicated (•). CPA and SNP were applied to the cell at 0.1 μM and 10 μM, respectively, for the durations indicated by the horizontal bars and for 5 min before testing NA. Gaps in the records represent 5 min recovery periods. b-c Mean $[Ca^{2+}]_i$ measured before (basal) and at the peak of the response to NA (stim), before and during the application of CPA and again after adding 1 μM SNP (b) or 10 μM SNP (c). * Significantly different from basal control, ** significantly different from stimulated control, *** Significantly different from basal (CPA), $P < 0.05$. Data are expressed as mean ± S.E.M. (n = 6).*

could inhibit SR Ca^{2+} release, by blocking the ryanodine receptor/channel or reducing the Ca^{2+} sensitivity of the channel, such that the open probability of the channel was reduced at physiological $[Ca^{2+}]_i$. This is consistent with previously published studies (Meszaros et al., 1996; Aghdasi et al., 1997; Kannan et al., 1997). For example, in isolated skeletal muscle SR, the NO donors SNAP, SNP and SIN-1, were shown to reduce the rate of Ca^{2+} release by approximately 65 % and the open probability of single ryanodine channels in planar lipid bilayers by approximately 60 % (Meszaros et al., 1996). In support of both the present findings and those of Meszaros et al. (1996), SNAP was shown to reduce the intracellular Ca^{2+} response to caffeine and prevent activation of the ryanodine receptor in porcine tracheal SMCs and skeletal muscle SR membranes, respectively (Kannan et al., 1997; Aghdasi et al., 1997).

Consistent with the present study, inhibition of SR Ca^{2+} release was found at relatively high concentrations of NO donors in isolated skeletal muscle (Meszaros et al., 1996). However, previous work in other laboratories has shown that NO and NO donors can inhibit Ca^{2+} release through the ryanodine receptor of the SR at lower concentrations (Aghdasi et al., 1997; Kannan et al., 1997). This discrepancy may arise from (1) NO donor reaction by-products and/or (2) vessel/species differences. However, it cannot be discounted that this effect occurred in rabbit aortic SMCs at lower SNP concentrations, but was masked by additional mechanisms that were more prominent.

Although on average SNP did not affect responses to NA, a reversible reduction was seen in some cells at 1 μM and 10 μM SNP. This is consistent with multiple mechanisms influencing the level of stored Ca^{2+}. Perhaps in these cells an inhibitory effect of SNP on Ca^{2+} release was more prominent than in other cells. Since ryanodine receptors are likely to play a small role in mediating responses to NA, compared with caffeine, an inhibitory effect on these receptors would not be expected to influence the NA response strongly. The inhibition of NA-induced Ca^{2+} release observed in this subset of cells could therefore be due to inhibition of the IP_3 pathway. Since in other cells, 1 or 10 μM SNP appeared to reversibly enhance the response to NA, any inhibitory action could be offset by an opposing stimulatory effect. Thus SNP may regulate $[Ca^{2+}]_i$ by stimulating Ca^{2+} release and/or Ca^{2+} uptake as well as inhibiting Ca^{2+} release. As a result of the opposing effects they would have on stored Ca^{2+}, inconsistent responses might be expected. Nevertheless, both actions would tend to reduce $[Ca^{2+}]_i$ during stimulation and would ultimately promote relaxation.

Although the only consistent effect on Ca^{2+} release that was observed was inhibition of the caffeine response at high SNP concentrations, such a mechanism could contribute significantly to NO-induced relaxation only if ryanodine receptors contribute significantly to VSM contraction *in vivo*. The caffeine-sensitive store of aortic smooth muscle has been shown to contain sufficient Ca^{2+} to produce tension development when released (Leijten and van Breemen, 1984). Ca^{2+} itself has been demonstrated to trigger Ca^{2+} release from the ryanodine receptor/channel under physiological conditions (Saida, 1981; Saida, 1982; Saida and van Breemen, 1983;

Bezprozvanny et al., 1991) and induce contraction (Saida and van Breemen, 1984a). Studies by Bezprozvanny et al. (1991) have demonstrated that maximal activity of the ryanodine receptor was maintained between 1 and 100 μM Ca^{2+}. Thus, within the physiological range of $[Ca^{2+}]_i$ reached during cell activation, the ryanodine receptor/channel can behave as a calcium-activated channel in smooth muscle. Investigators have also proposed that arterial smooth muscle contraction may be due initially to IICR, which subsequently initiates CICR from the SR (Saida and van Breemen, 1984a; Saida and van Breemen, 1984b).

4.4.2 Does SNP stimulate Ca^{2+} sequestration by the SR?

As mentioned previously, SNP (1 and 10 μM) reversibly enhanced the NA-induced $[Ca^{2+}]_i$ transient in a subset of cells. While it is clear that stimulation of SR Ca^{2+} sequestration was not a major action of SNP or GTN, in modulating these responses, it may make a greater contribution in some cells. Combined with inhibition of Ca^{2+} release, such a mechanism would help to maintain the low $[Ca^{2+}]_i$ needed to promote relaxation. My results provide additional evidence both for and against a role for stimulation of the SR Ca^{2+}-ATPase by SNP and GTN. Evidence against a stimulatory effect on the ATPase was provided by the lack of effect of SNP or GTN, at any concentration, on the rate of Ca^{2+} removal from the cell following a stimulus. This is in agreement with previous studies that have shown SNAP to have no effect on the rate of Ca^{2+} uptake (Meszaros et al., 1996) or the magnitude of agonist-induced $[Ca^{2+}]_i$ transients following store depletion and subsequent refilling (Kannan et al., 1997). However, in the absence of CPA, perhaps the rate of Ca^{2+} uptake into the SR is maximal in rabbit aortic SMCs. In these conditions SNP would be unable to

further increase the rate at which Ca^{2+} was removed from the cell. However, when Ca^{2+} sequestration was partially blocked by CPA, there was no evidence that SNP could overcome this block and enhance the response to NA.

Evidence in favour of a stimulatory action of SNP on the SR Ca^{2+}-ATPase was provided by the observation that at 10 μM it reversed the increase in basal $[Ca^{2+}]_i$, produced in the presence of CPA. The rise in $[Ca^{2+}]_i$ was caused by the release of Ca^{2+} from the SR and/or Ca^{2+} entering the cell as a result of store depletion (Mason et al., 1991; Demaurex et al., 1992; Shima and Blaustein, 1992), as discussed in chapter 3. Thus, SNP appeared able to stimulate the removal of Ca^{2+} from the cell when it was elevated as a consequence of store depletion. This could reflect stimulation of either SR or plasma membrane Ca^{2+} uptake, or possibly inhibition of Ca^{2+} influx, mediated by store-depletion activated channels. SNP was shown to inhibit store-depletion activated Ca^{2+} influx in mouse anococcygeus smooth muscle, but as a consequence of inhibition of the SR Ca^{2+}-ATPase (Wayman et al., 1996b).

That SNP may enhance Ca^{2+} sequestration by the SR Ca^{2+}-ATPase is consistent with studies by Lincoln (1983) in rat aortic smooth muscle, where SNP and 8-Br-cGMP were suggested to reduce $[Ca^{2+}]_i$ by enhancing Ca^{2+} sequestration into the SR, thus, decreasing contractile activity. Moreover, Karaki et al. (1988) demonstrated that SNP could augment agonist-induced transient contractions in Ca^{2+}-free medium, following store refilling after a period of Ca^{2+} depletion. Further evidence from rabbit portal vein (Komori and Bolton, 1989) and rat aortic smooth muscle (Cornwell et al., 1991) led to the proposal of a similar action for intracellular cGMP, because it

enhanced the response to caffeine after prior depletion of the store. This effect appeared to be mediated via PKG, which phosphorylates the SR Ca^{2+}-ATPase regulatory protein, phospholamban (Raeymeakers at al., 1988; Cornwell et al., 1991; Lincoln and Cornwell, 1993). Using immunocytochemical techniques, Cornwell et al. (1991) additionally showed that PKG was localised to the same cellular region as phospholamban. cGMP-dependent stimulation of SR Ca^{2+} uptake has also been measured directly in permeabilised aorta (Twort and van Breemen, 1988). Nevertheless, despite the clear evidence from earlier studies and from my results in chapter 2, that NO and cGMP stimulate SR Ca^{2+}-ATPase activity, the results in this chapter do not convincingly demonstrate a role for such an action in the regulation of stored Ca^{2+} by SNP or GTN.

In conclusion, modulation of SR Ca^{2+} store function could contribute to the lowering of $[Ca^{2+}]_i$ by nitrovasodilators. Inhibition of SR Ca^{2+} release was demonstrated at high SNP concentrations, but not with GTN. It may therefore be most significant at high NO concentrations or it is specific to SNP and therefore unlikely to mediate the vasodilation action of NO. I also provided limited evidence that stimulation of Ca^{2+} sequestration could contribute to the regulation of $[Ca^{2+}]_i$ by nitrovasodilators although my results do not support it as a major mechanism. The results of the present chapter demonstrate that (1) selective inhibition of Ca^{2+} release from the ryanodine receptor/channel of the SR and (2) augmented removal of Ca^{2+} from the cytoplasm may contribute to the lowering of $[Ca^{2+}]_i$ by SNP in rabbit aortic smooth muscle. GTN, even at a maximally effective concentration with regards to vasomotor tone, had no effect on $[Ca^{2+}]_i$ transients evoked by NA or caffeine, suggesting either

that these mechanisms contribute little to NO vasodilation, or that concurrent inhibition of SR Ca^{2+} release and stimulation of Ca^{2+} uptake mask each others actions under these conditions.

Chapter 5

Influence of cGMP on Ca^{2+} mobilisation

5.1 Introduction

VSM relaxation induced by SNP or the organic nitrates is accompanied by an elevation of cGMP levels (Arnold et al., 1977; Katsuki et al., 1977; Murad et al., 1985; Rapoport et al., 1985a; Rapoport et al., 1985b; Taylor et al., 1988; Magliola and Jones, 1990; Kukovetz et al., 1991). It has therefore been thought for a long time that intracellular cGMP may mediate the effects of these drugs, as well as the effects of the endogenous vasodilator NO (Rapoport et al., 1982; Rapoport et al., 1983; Murad et al., 1985; Ignarro et al., 1986; Jones et al., 1994). In each case, the elevation of cGMP was found to correlate well with the associated decrease in contractile tension of VSM (Gruetter et al., 1981; Lincoln, 1983; Murad et al., 1985; Rapoport et al., 1985b; Rashatwar et al., 1987; Kukovetz et al., 1991; Jones et al., 1994). Consistent with these observations, certain membrane permeant analogues of cGMP have been shown to mimic the effects of NO donors in VSM, including aorta (Lincoln, 1983; Collins et al., 1986; Francis et al., 1988; Fujino et al., 1991; Minowa et al., 1997), as does intracellular application of cGMP in rabbit portal vein (Komori and Bolton, 1989).

A role for cGMP in mediating smooth muscle relaxation by nitrovasodilators is further supported by studies utilising inhibitors of various processes in the cGC/cGMP pathway. For example, inhibition of NO-stimulated effects by ODQ, a selective cGC inhibitor, has provided a valuable tool in identifying cGMP-mediated effects (Garthwaite et al., 1995; Schrammel et al., 1996; Wayman et al., 1996b). ODQ is without effects on particulate GC, so does not block actions of NO unrelated to cGC. It also penetrates tissues quickly (Garthwaite et al., 1995). MB, a less-

selective inhibitor of NO-induced activation of GC, has also been shown to prevent elevated levels of cGMP and relaxation induced by several nitrovasodilators in a variety of VSM preparations, including rabbit aorta (Martin et al., 1985; Rapoport et al., 1985b; Gryglewski et al., 1992; Jones et al., 1994). However, MB can generate superoxide anions, and inhibits NOS at concentrations 100-fold lower than those that inhibit cGC (Marczin et al., 1992; Mayer et al., 1993). Inhibitors of the cGMP-metabolizing enzyme, PDE, such as zaprinast, cause elevation of cGMP levels and potentiate relaxation in response to nitrovasodilators (Katsuki et al., 1977; Lorenz and Wells, 1983; Wood and Owen, 1989; Zhou and Torphy, 1991).

In view of the central role played by $[Ca^{2+}]_i$ in the regulation of smooth muscle tension, experiments aimed at determining the cellular mechanisms of cGMP-dependent relaxation have strongly suggested that agents that increase cGMP levels relax VSM by primarily reducing $[Ca^{2+}]_i$ (Murad, 1986; Karaki et al., 1988). $[Ca^{2+}]_i$ was first reported to be decreased by 8-Br-cGMP in fura-2 loaded, cultured VSMCs from rat aorta (Kai et al., 1987). 8-Br-cGMP decreased $[Ca^{2+}]_i$ regardless of whether VSMCs were at rest, depolarised by K^+ or in Ca^{2+}-free medium. The reduction in $[Ca^{2+}]_i$ was maintained as long as 8-Br-cGMP was present, and was dose-dependent. Similar studies have shown nitrovasodilators to attenuate stimulus induced increases in $[Ca^{2+}]_i$ in isolated SMCs and permeabilised arteries (Rashatwar et al., 1987; Word et al., 1991; Andriantsitohaina et al., 1995). However, the mechanism by which cGMP modulates $[Ca^{2+}]_i$ appears to be complex. For example, cGMP has been found to: (1) inhibit Ca^{2+} influx (Collins et al., 1986; Andriantsitohaina et al., 1995), (2) enhance Ca^{2+} sequestration into the SR (Twort and van Breemen, 1988; Komori and

Bolton, 1989; Andriantsitohaina et al., 1995), (3) enhance Ca^{2+} extrusion across the plasma membrane (Kobayashi et al., 1985; Popescu et al., 1985a; Popescu et al., 1985b; Vrolix et al., 1988; Magliola and Jones, 1990), (4) inhibit Ca^{2+} release from the SR (Collins et al., 1986; Lang and Lewis, 1989; Meszaros et al., 1996; Kannan et al., 1997) and (5) activate K_{Ca} channels, to inhibit Ca^{2+} influx through VOCs (Archer et al., 1994; Carrier et al., 1997). The apparent different effects of cGMP that have been reported may be explained by a variation with species, tissue and localisation, or it may cause vasodilation through multiple mechanisms.

In the previous chapters it was proposed that inhibition of SR Ca^{2+} release and augmented Ca^{2+} sequestration may contribute to the lowering of $[Ca^{2+}]_i$ by SNP in rabbit aortic smooth muscle. This chapter therefore investigated the role of the cGMP pathway in this inhibitory effect employing the cGC inhibitor ODQ, and the cGMP-specific PDE inhibitor zaprinast, and 3-isobutyl-1-methyl xanthine (IBMX), a non-specific PDE inhibitor. The effect of these agents were tested on $[Ca^{2+}]_i$ transients in single rabbit aortic SMCs produced by NA or caffeine, in the absence or presence of modulation by SNP.

5.2 Materials and Methods

The experiments reported in this chapter employed the methods described in chapter 3 (pages 83-95).

5.2.1 List of drugs

The following drugs were used: caffeine (Sigma), noradrenaline (arterenol, bitartrate salt, Sigma), 8-bromoguanosine, 3':5'-cyclic monophosphate (sodium salt, Sigma), 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, Calbiochem), zaprinast (M&B 22948, Sigma), 3-isobutyl-1-methylxanthine (IBMX, Sigma) and sodium nitroprusside (Sigma). Drugs prepared on the day of each experiment were routinely dissolved in water as stock solutions, and further dilutions were made in the same solution bathing the cells, with the exception of caffeine, which was dissolved directly in bath solution. SNP was stored in a dark flask at 4 °C as a 20 mM stock solution and used on the same day as it was prepared. ODQ, zaprinast and IBMX were stored at –20 °C as stock solutions in DMSO. All solutions were routinely filtered before use through a pore size of 0.2 μm.

5.3 Results

5.3.1 Effect of 8-Br-cGMP on $[Ca^{2+}]_i$ transients

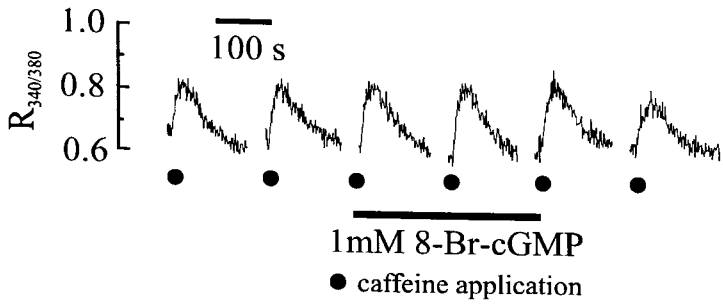
The influence of 8-Br-cGMP on $[Ca^{2+}]_i$ transients was tested in the same manner as that described in chapter 4 for SNP and GTN. As previously described, brief application of 20 mM caffeine evoked transient elevations of $[Ca^{2+}]_i$ that were reproducible upon repeated application. After obtaining two reproducible responses to caffeine, 8-Br-cGMP was applied for 5 min prior to applying caffeine once again, and then washed out. Representative records of increases in $R_{340/380}$ evoked by fast application of caffeine (20 mM) in the absence and presence of 1 mM 8-Br-cGMP are shown in Fig. 5.1a. Application of caffeine in the presence of 1 mM 8-Br-cGMP

induced a transient increase in $[Ca^{2+}]_i$, to a similar level to that attained without 8-Br-cGMP. Mean basal $[Ca^{2+}]_i$ and caffeine-stimulated Ca^{2+} levels measured in the presence of 8-Br-cGMP did not differ significantly ($P > 0.05$) from control (Fig. 5.1b; $n = 9$). 8-Br-cGMP also failed to modulate NA-induced $[Ca^{2+}]_i$ responses as shown in Fig. 5.2. Both mean and basal NA-stimulated levels of $[Ca^{2+}]_i$ were not significantly altered.

5.3.1.1 Influence of 8-Br-cGMP on the rate of Ca^{2+} removal from the cytosol following agonist application

Results from the previous chapter indicated that the cGMP-associated nitrovasodilators, SNP (0.1-10 μM) and GTN (1 μM), did not significantly affect the rate of $[Ca^{2+}]_i$ removal from the cell following caffeine or NA stimulation. Experiments were repeated in the presence of 8-Br-cGMP, to determine whether cGMP could affect the rate of Ca^{2+} removal. Fig. 5.3 illustrates exponential fits to the decline of $[Ca^{2+}]_i$ following a caffeine- (a) and NA- (b) induced Ca^{2+} transient, before and during 8-Br-cGMP (1 mM) application. The $[Ca^{2+}]_i$ decay time constants prior to application of 1 mM 8-Br-cGMP were 35 s (caffeine) and 78 s (NA). During 8-Br-cGMP treatment they were 46 s and 86 s, respectively, which were not significantly different from control. Thus, 8-Br-cGMP did not alter the rate of Ca^{2+} removal from the cell, as seen from the mean data from several cells, summarised in Fig. 5.3c and d. This was true whether the stimulant was caffeine or NA.

a



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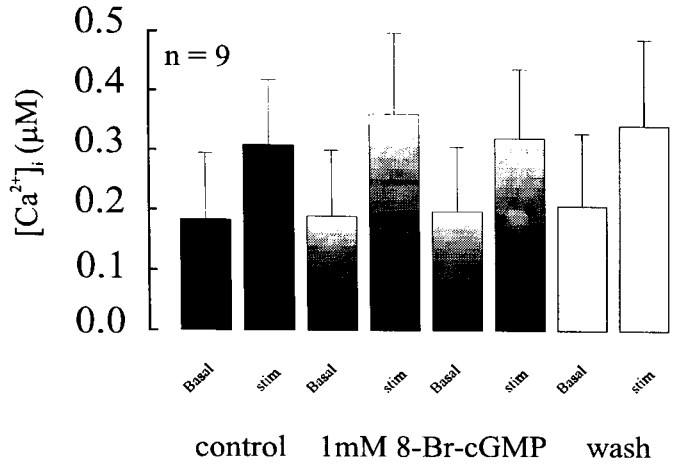
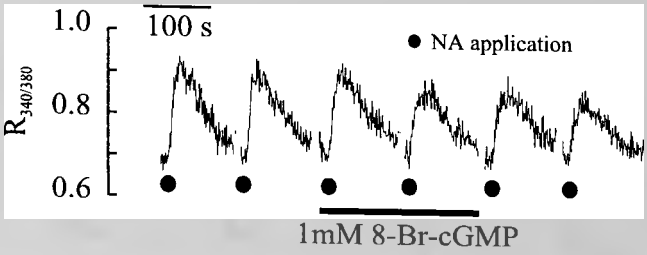


Fig. 5.1. *a Caffeine-induced $[Ca^{2+}]_i$ transients in the absence and presence of 1 mM 8-Br-cGMP as indicated by $R_{340/380}$. Gaps in the records represent 5 min recovery periods. Caffeine was applied at the times indicated (•). 8-Br-cGMP was applied to the cell for the duration indicated by the horizontal bar and for 5 min before testing caffeine. b Mean $[Ca^{2+}]_i$ measured before (basal) and at the peak of the response to caffeine (stim) in the absence and presence of 8-Br-cGMP. At 1 mM, 8-Br-cGMP did not significantly affect mean basal $[Ca^{2+}]_i$ or caffeine-stimulated Ca^{2+} levels. Data are expressed as mean ± S.E.M. (n = 9).*

a



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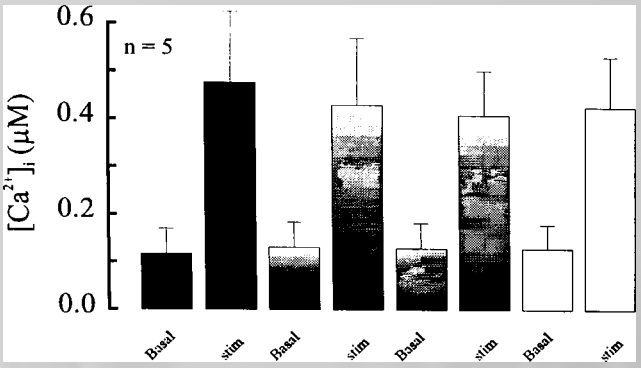

Fig. 5.2. *a NA-induced $[Ca^{2+}]_i$ transients in the absence and presence of 1 mM 8-Br-cGMP as indicated by $R_{340/380}$. Gaps in the records represent 5 min recovery periods. NA was applied at the times indicated (•). 8-Br-cGMP was applied to the cell for the duration indicated by the horizontal bar and for 5 min before testing NA. b Mean $[Ca^{2+}]_i$ measured before (basal) and at the peak of the response to NA (stim) in the absence and presence of 8-Br-cGMP. At 1 mM, 8-Br-cGMP did not significantly affect mean basal $[Ca^{2+}]_i$ or NA-stimulated Ca^{2+} levels. Data are expressed as mean ± S.E.M. (n = 5).*

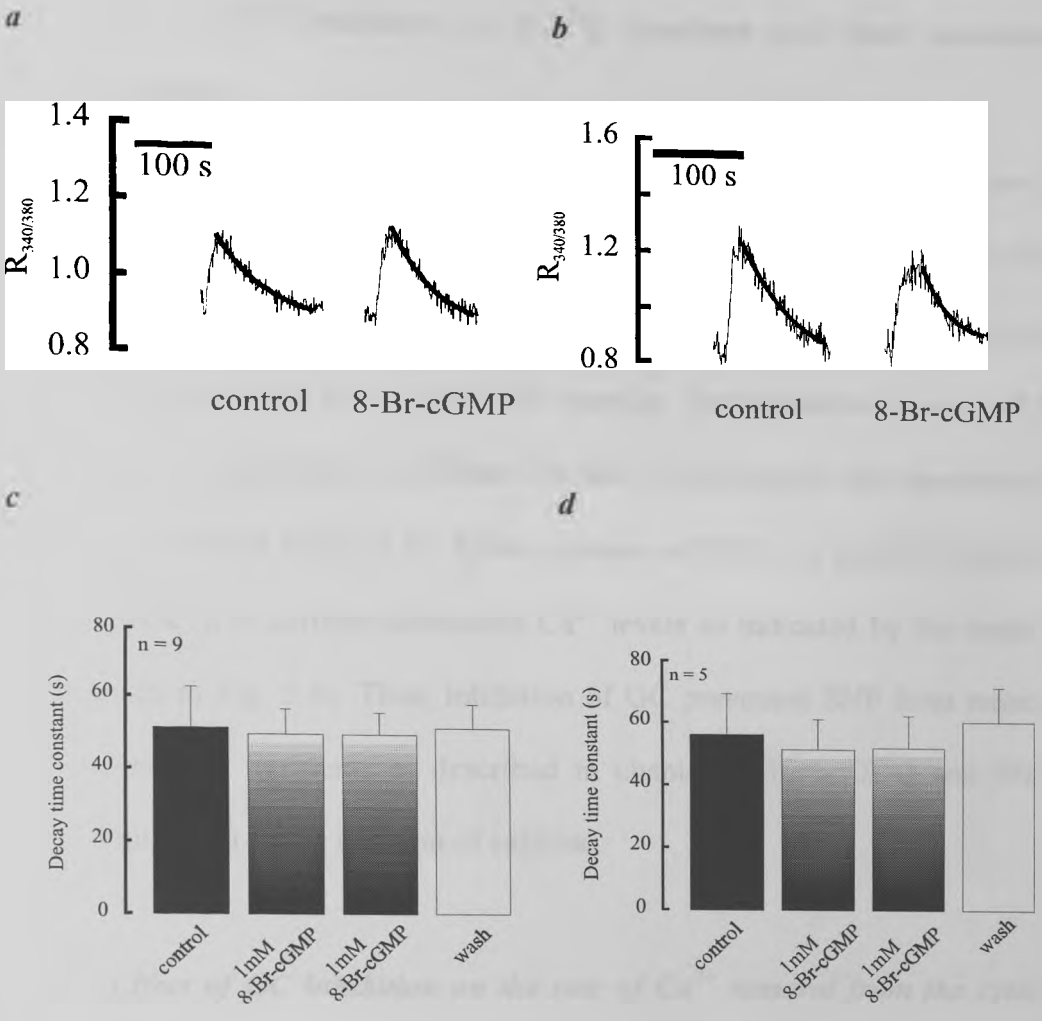

Fig. 5.3. *Caffeine-induced (a) and NA-induced (b) $[Ca^{2+}]_i$ transients showing the decay of $[Ca^{2+}]_i$ in the absence and presence of 1 mM 8-Br-cGMP. The curves superimposed on the decay of the Ca^{2+} transients indicate the best single exponential fit to the decline in $R_{340/380}$. c-d Mean $[Ca^{2+}]_i$ decay time constants from cells stimulated by caffeine (c) or NA (d), following the peak of the $[Ca^{2+}]_i$ transient in the absence and presence of 1 mM 8-Br-cGMP. There were no significant differences between $[Ca^{2+}]_i$ decay time constants measured in the presence of 8-Br-cGMP and control. Data are expressed as mean ± S.E.M. (n = 9 (c) and 5 (d)).*

5.3.2 Effect of GC inhibition on $[Ca^{2+}]_i$ transients and their modulation by nitrovasodilators

Neither the basal $[Ca^{2+}]_i$ nor the peak $[Ca^{2+}]_i$ induced by caffeine were altered significantly ($P > 0.05$) by ODQ applied at 10 μM (Fig. 5.4a; $n = 8$). The ability of ODQ to block the effect of SNP on caffeine-induced $[Ca^{2+}]_i$ transients was tested by applying 10 μM ODQ and 10 μM SNP together. Representative records of $R_{340/380}$ evoked by fast application of caffeine (20 mM) in the absence and presence of ODQ and SNP are shown in Fig. 5.4b. In the presence of ODQ, 10 μM SNP had no effect on basal $[Ca^{2+}]_i$ or caffeine-stimulated Ca^{2+} levels as indicated by the mean results summarised in Fig. 5.4c. Thus, inhibition of GC prevented SNP from reducing the caffeine-induced transients as described in chapter 4. Both ODQ and SNP were added 5 min prior to the addition of caffeine.

5.3.2.1 Effect of GC inhibition on the rate of Ca^{2+} removal from the cytosol was not modulated by nitrovasodilators

In addition to testing the ability of ODQ to modulate the amplitude of $[Ca^{2+}]_i$ transients, its effect on the rate of Ca^{2+} removal from the cytosol following a caffeine stimulus was investigated. ODQ applied alone did not significantly ($P > 0.05$) alter the $[Ca^{2+}]_i$ decay time constant (Fig. 5.5a). SNP (10 μM) did not alter the $[Ca^{2+}]_i$ decay time constant when applied with ODQ (Fig. 5.5b).

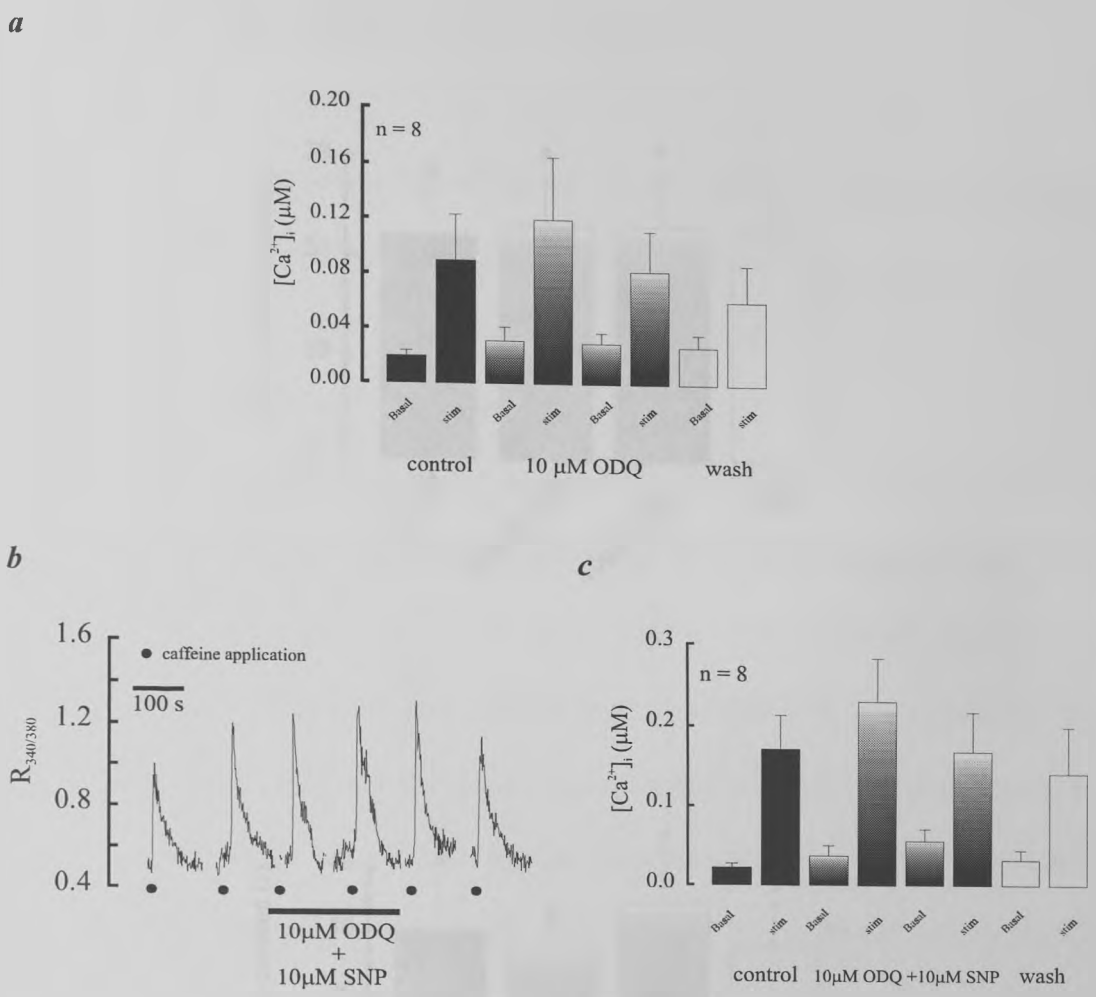

Fig. 5.4. *a Mean $[Ca^{2+}]_i$ measured before (basal) and at the peak of the response to caffeine (stim) in the absence and presence of ODQ. At 10 μM, ODQ did not significantly affect mean basal $[Ca^{2+}]_i$ or caffeine-stimulated Ca^{2+} levels. (b) Caffeine-induced $[Ca^{2+}]_i$ transients in the absence and presence of both SNP & ODQ. Gaps in the records represent 5 min recovery periods. Caffeine was applied at the times indicated (•). ODQ and SNP were both applied to the cell at a concentration of 10 μM for the duration indicated by the horizontal bar and for 5 min before testing caffeine. (c) Mean $[Ca^{2+}]_i$ measured before (basal) and at the peak of the response to caffeine (stim) in the absence and presence of ODQ and SNP. Data are expressed as mean ± S.E.M. (n = 8).*

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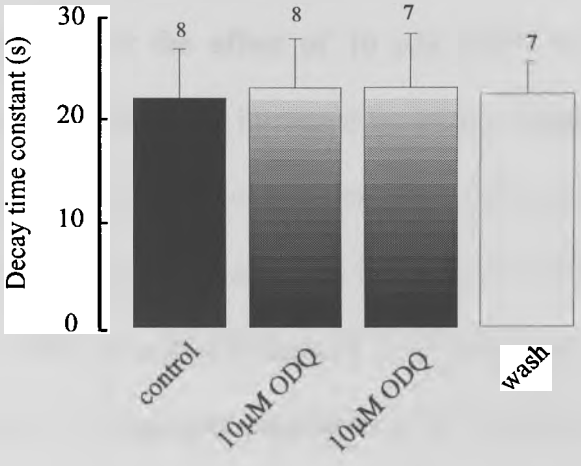

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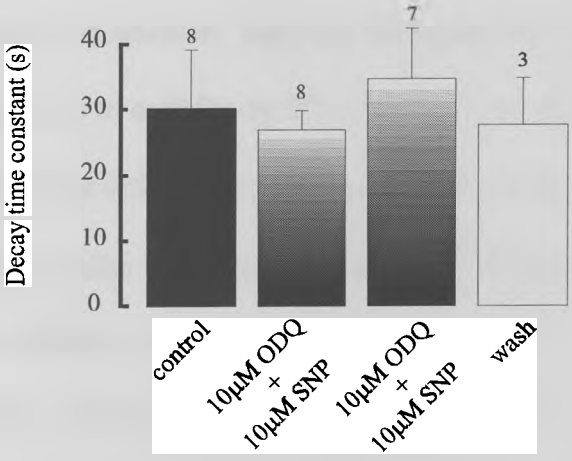

Fig. 5.5. *Mean $[Ca^{2+}]_i$ decay time constants in cells exposed to ODQ (10 μM) (a) and ODQ (10 μM) and SNP (10 μM) (b) following caffeine stimulation. There were no significant differences between $[Ca^{2+}]_i$ decay time constants measured in treated and control cells. Data are expressed as mean ± S.E.M. (n = 3-8). Values above S.E.M. bars indicate the total number of cells tested.*

5.3.3 Effect of PDE inhibitors on $[Ca^{2+}]_i$ transients

5.3.3.1 Zaprinast

To further clarify whether the effect of 10 μM SNP was mediated by cGMP, endogenous levels of cGMP were increased by PDE inhibitors to test whether this would potentiate the effect of SNP on a caffeine-induced response. Fig. 5.6 illustrates the effect of 200 μM zaprinast, a selective inhibitor of cGMP degradation, (Beavo and Reifsnyder, 1990), on caffeine-induced $[Ca^{2+}]_i$ transients. The mean data are summarised in Fig. 5.7. Zaprinast significantly ($P < 0.05$) increased basal $[Ca^{2+}]_i$ from 0.03 μM ± 0.01 (n =10) to 0.08 μM ± 0.02 (n =10) at 20 μM and from 0.01 μM ± 0.004 (n = 6) to 0.05 μM ± 0.008 (n = 6) at 200 μM. The increase in basal $[Ca^{2+}]_i$ following zaprinast addition was maintained throughout the experiment, even after 15 min washout. In addition, zaprinast decreased the levels of Ca^{2+} reached at the peak of the response to caffeine by 20 ± 2 % (n = 10) at 20 μM and by 53 ± 3 % (n = 6) at 200 μM. This effect was however delayed, being seen only after 15 min exposure to zaprinast. After only 5 min exposure to zaprinast at either concentration, the response to caffeine was unchanged, but basal $[Ca^{2+}]_i$ was increased. The reduction in caffeine-induced Ca^{2+} transients following zaprinast addition was maintained throughout the experiment. This is consistent with contractile studies, which have shown that zaprinast vasodilation could not be recovered following a 1 hr wash period (Wood and Owen, 1989).

In addition to reducing the amplitude of the $[Ca^{2+}]_i$ transients, zaprinast also altered the rate of recovery of $[Ca^{2+}]_i$ following the response to caffeine. $[Ca^{2+}]_i$ decayed back to baseline more slowly after applying zaprinast (Fig. 5.8). As shown in Fig. 5.9

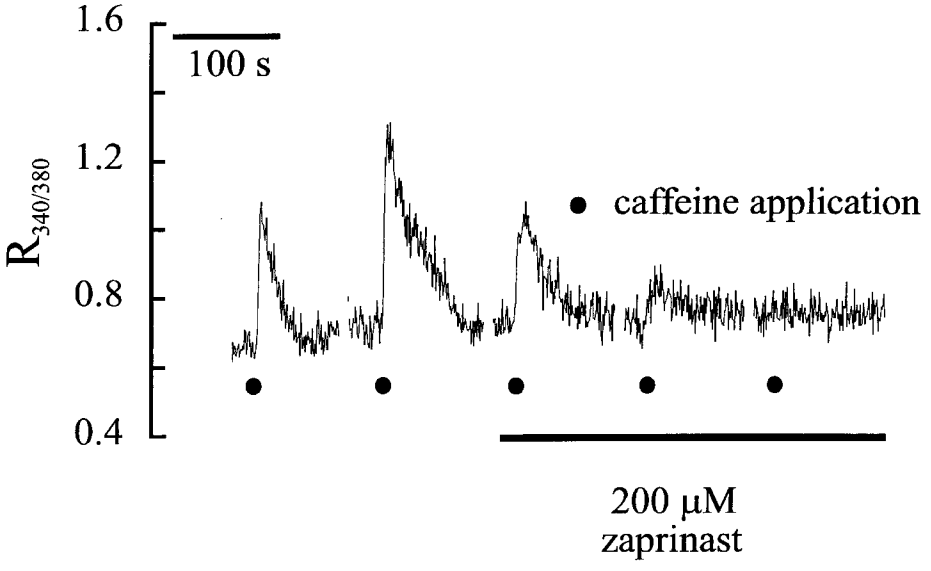

Fig. 5.6. *Effect of 200 μM zaprinast on caffeine-induced $[Ca^{2+}]_i$ transients. Zaprinast increased basal $[Ca^{2+}]_i$ in addition to reducing caffeine-stimulated Ca^{2+} levels. Gaps in the records represent 5 min recovery periods. Caffeine was applied at the times indicated (•). Zaprinast was applied to the cell for the duration indicated by the horizontal bar and for 5 min before testing caffeine.*

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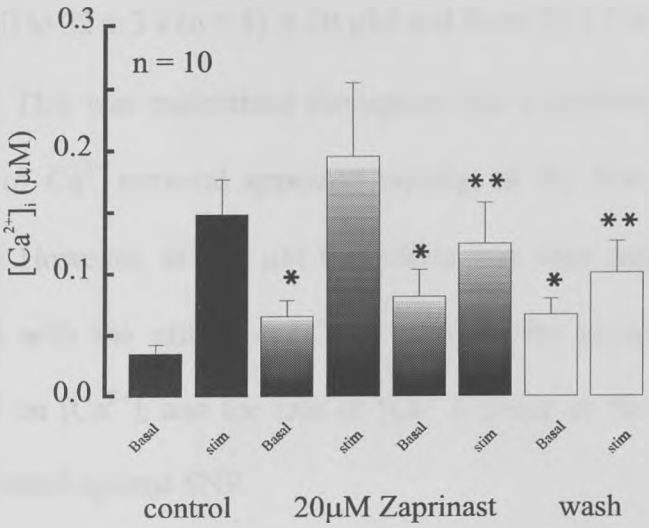

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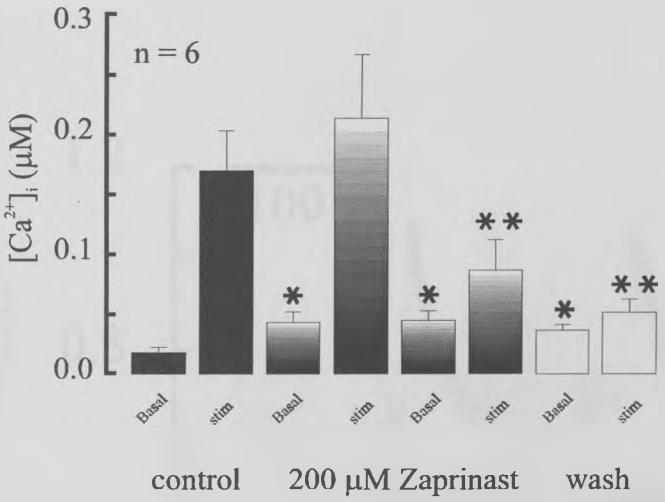


Fig. 5.7. *Mean $[Ca^{2+}]_i$ measured before (basal) and at the peak of the response to caffeine (stim) in the absence and presence of zaprinast. At 20 μM (a) and 200 μM (b), zaprinast significantly increased basal $[Ca^{2+}]_i$ and in addition significantly reduced caffeine-stimulated Ca^{2+} levels. * Significantly different from basal control, ** significantly different from stimulated control, $P < 0.05$. Data are expressed as mean ± S.E.M. (n = 10 (a) and 6 (b)).*

zaprinast significantly ($P < 0.05$) increased the $[Ca^{2+}]_i$ decay time constant from 23 ± 4 s (n = 5) to 32 ± 3 s (n = 5) at 20 μM and from 22 ± 5 s (n = 3) to 32 ± 5 s (n = 3) at 200 μM. This was maintained throughout the experiment. At 20 μM zaprinast, the slowing of Ca^{2+} removal appeared rapidly, at the first caffeine application in its presence. However, at 200 μM this effect was seen only after 15 min exposure as observed with the effect on $[Ca^{2+}]_i$. Due to the pronounced inhibitory effect of zaprinast on $[Ca^{2+}]_i$ and the rate of $[Ca^{2+}]_i$ decay in the absence of SNP, zaprinast was not tested against SNP.

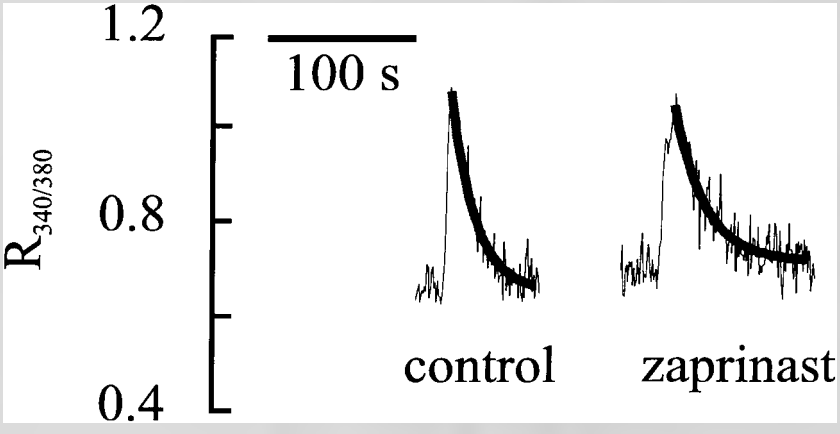
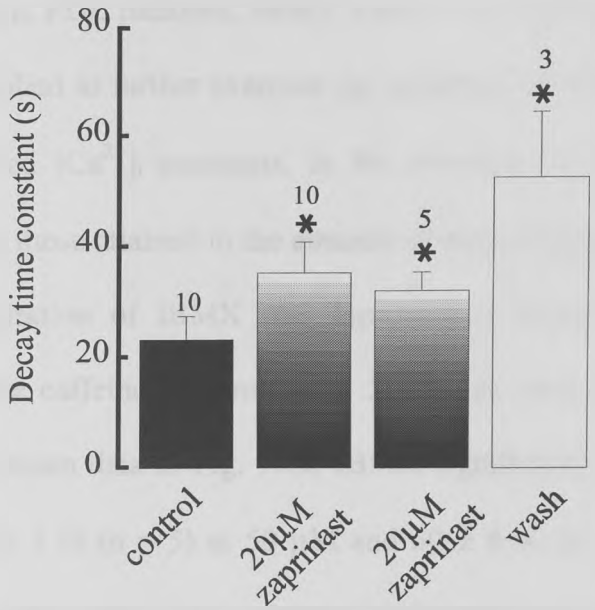


Fig. 5.8. *Caffeine-induced $[Ca^{2+}]_i$ transients showing the decay of $[Ca^{2+}]_i$ in the absence and presence of zaprinast (200 μM). The curves superimposed on the decay of the Ca^{2+} transients indicate the best single exponential fit to the decline in $R_{340/380}$ with a time constant of 15 s under control conditions and 39 s after applying zaprinast.*

a



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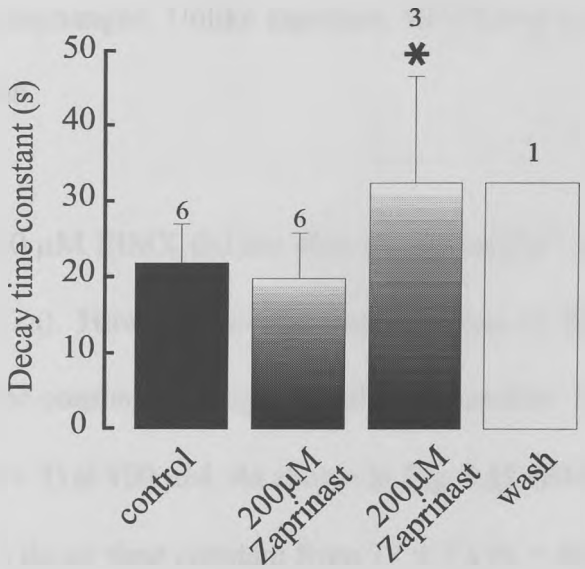

Fig. 5.9. *Bar charts summarising the significant increase in mean $[Ca^{2+}]_i$ decay time constants in cells exposed to 20 μM (a) and 200 μM (b) zaprinast. * Significantly different from control, $P < 0.05$. Data are expressed as mean ± S.E.M. (n = 3-10). Values above S.E.M. bars indicate the total number of cells tested.*

5.3.3.2 IBMX

The non-specific PDE inhibitor, IBMX (Beavo and Reifsnyder, 1990; Shahid et al., 1991), was applied to further examine the influence of cGMP on $[Ca^{2+}]_i$ transients. Caffeine-induced $[Ca^{2+}]_i$ transients, in the presence of 10 μM IBMX, reached a similar level to those attained in the absence of drug exposure (Fig. 5.10a). However, as the concentration of IBMX was increased it began to show an irreversible reduction of the caffeine response (Fig. 5.10b), as seen with zaprinast. As can be seen from the mean data in Fig. 5.11, IBMX significantly ($P < 0.05$) reduced peak $[Ca^{2+}]_i$ by 55 ± 2 % (n = 5) at 50 μM and 60 ± 6 % (n = 6) at 100 μM. As with zaprinast, this effect was slow to develop, being seen only after 15 min exposure to IBMX. After only 5 min exposure to IBMX at either 50 μM or 100 μM the response to caffeine was unchanged. Unlike zaprinast, IBMX had no effect on basal $[Ca^{2+}]_i$ at any concentration.

Application of 10 μM IBMX did not alter the rate of Ca^{2+} removal from rabbit aortic SMCs (Fig. 5.12a). However, as the concentration of IBMX was increased, the $[Ca^{2+}]_i$ decay time constant was significantly increased by 24 ± 8 % (n = 4) at 50 μM and 86 ± 6 % (n = 3) at 100 μM. As shown in Fig. 5.12 IBMX significantly increased the mean $[Ca^{2+}]_i$ decay time constant from 17 ± 2 s (n = 4) to 21 ± 2 s (n = 4) at 50 μM and from 29 ± 2 s (n = 3) to 54 ± 6 s (n = 3) at 100 μM. The increase in the $[Ca^{2+}]_i$ decay time constant was slow to develop, being observed only after 15 min exposure to IBMX, but was maintained throughout the remainder of the experiment, even after 15 min washout.

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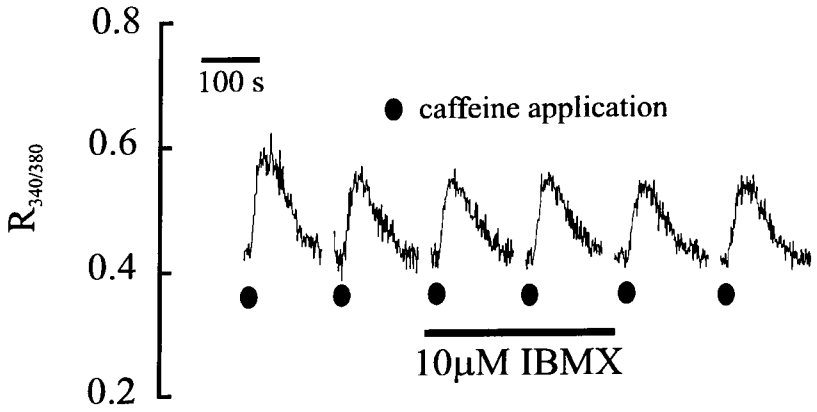

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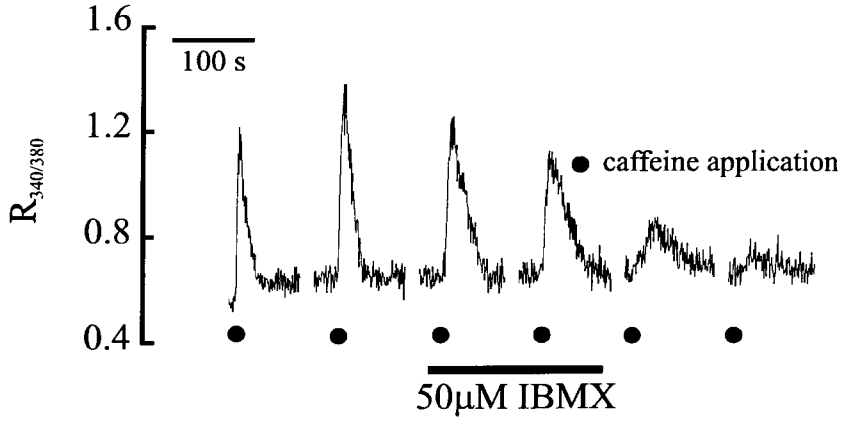

Fig. 5.10. *Caffeine-induced $[Ca^{2+}]_i$ transients in the absence and presence of IBMX. $[Ca^{2+}]_i$ remained unchanged in the presence of 10 μM (a) IBMX but at 50 μM (b), IBMX reduced peak Ca^{2+} levels. Gaps in the records represent 5 min recovery periods. Caffeine was applied at the times indicated (•). IBMX was applied to the cell at a concentration of 10 μM (a) and 50 μM (b) for the duration indicated by the horizontal bars and for 5 min before testing caffeine.*

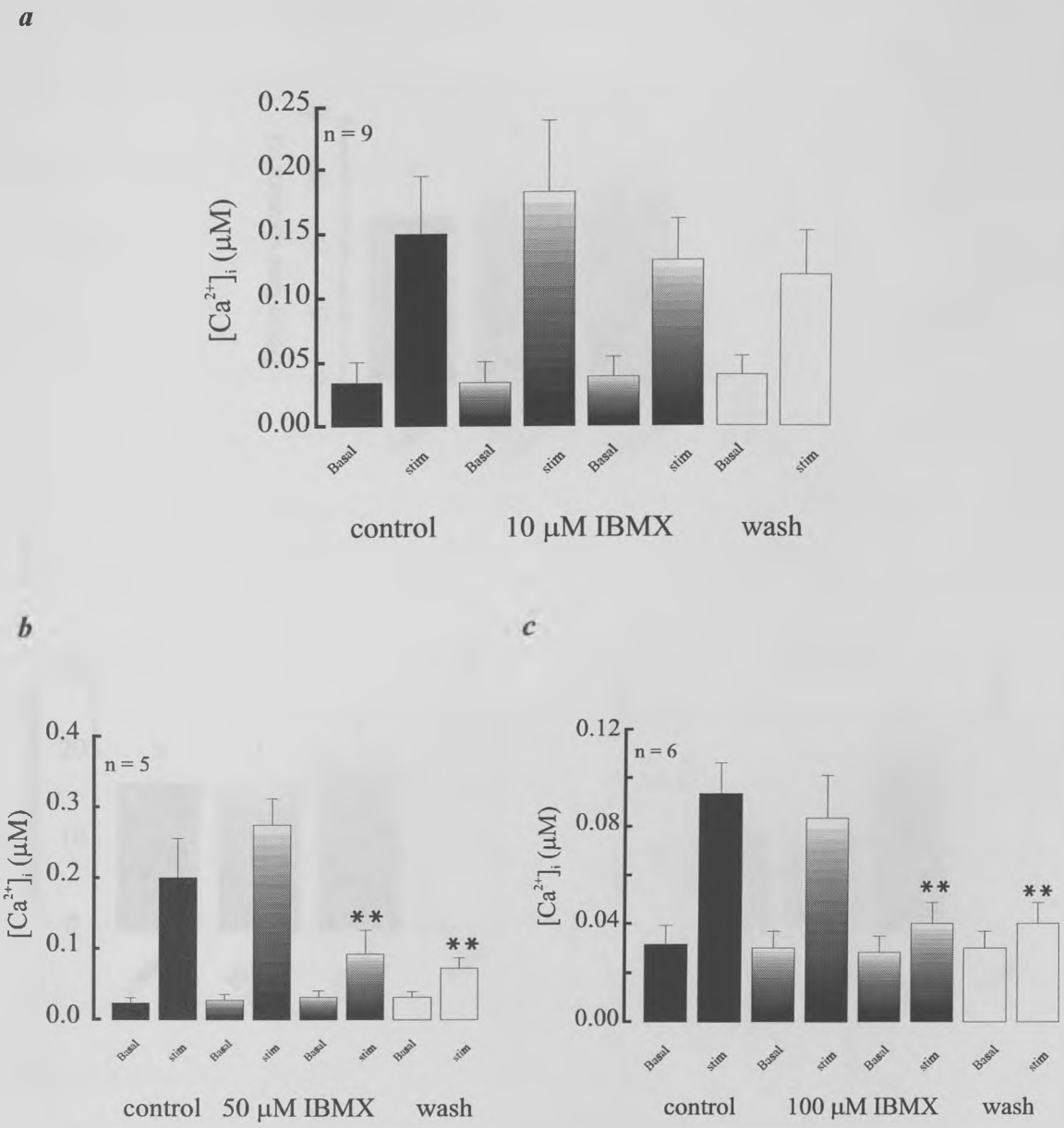

Fig. 5.11. *Mean $[Ca^{2+}]_i$ measured before (basal) and at the peak of the response to caffeine (stim) in the absence and presence of IBMX. At 10 μM (a), IBMX did not significantly affect mean basal or stimulated $[Ca^{2+}]_i$. At 50 μM (b) and 100 μM (c), IBMX significantly reduced caffeine-stimulated Ca^{2+} levels, but had no effect on basal $[Ca^{2+}]_i$. ** Significantly different from stimulated control, $P < 0.05$. Data are expressed as mean ± S.E.M. (n = 5-9).*

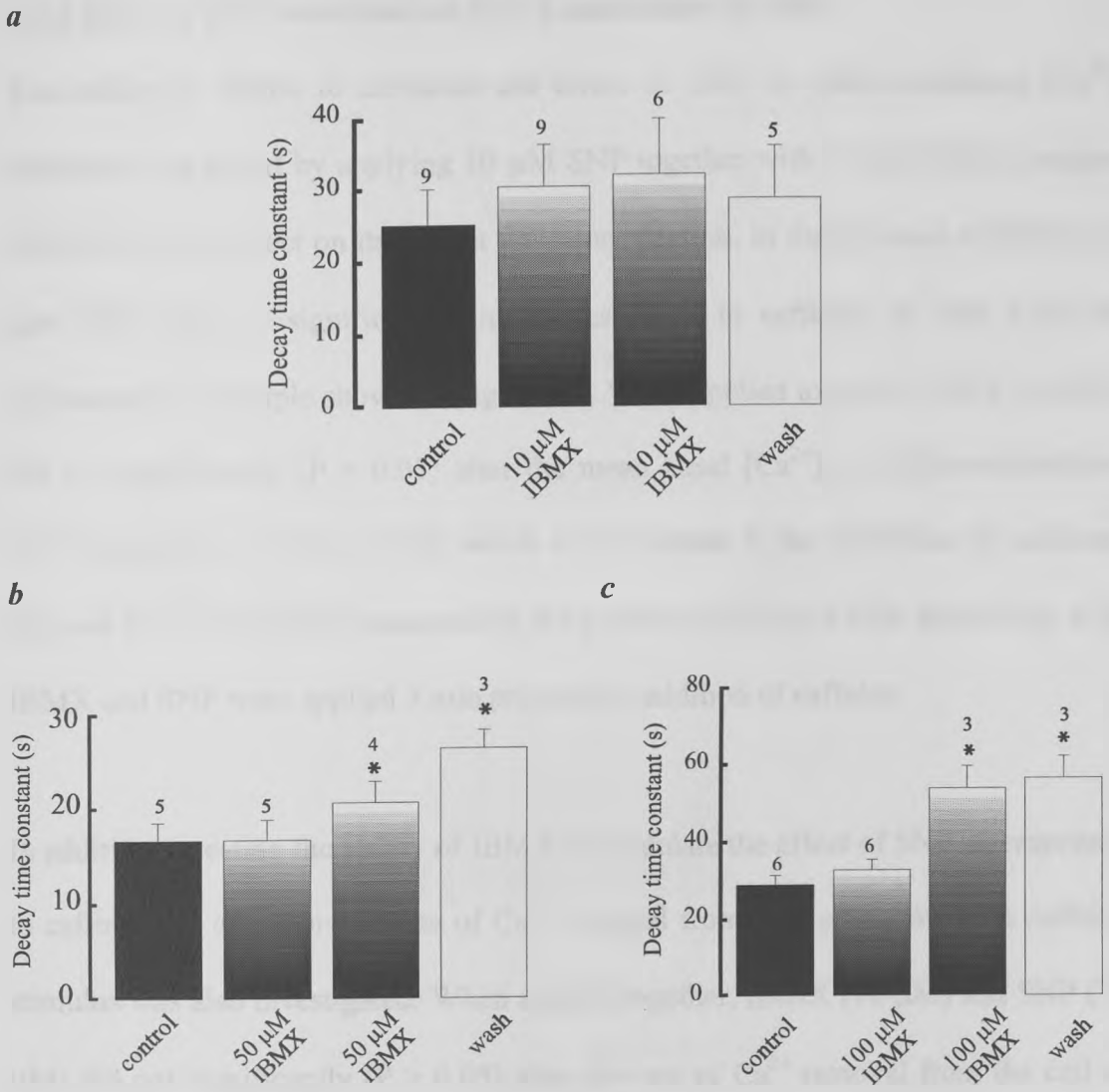

Fig. 5.12. *Mean $[Ca^{2+}]_i$ decay time constants in cells exposed to 10 μM (a), 50 μM (b) and 100 μM (c) IBMX. * Significantly different from control, $P < 0.05$. Data are expressed as mean ± S.E.M. (n = 3-9). Values above S.E.M. bars indicate the total number of cells tested.*

5.3.4 Effect of PDE inhibition on $[Ca^{2+}]_i$ modulation by SNP

The ability of IBMX to modulate the effect of SNP on caffeine-induced $[Ca^{2+}]_i$ transients was tested by applying 10 μM SNP together with 10 μM IBMX, because IBMX had little effect on its own at this concentration. In the presence of IBMX, 10 μM SNP failed to significantly inhibit responses to caffeine, as seen from the representative example shown in Fig. 5.13a. When applied together, IBMX and SNP did not significantly ($P > 0.05$) alter the mean basal $[Ca^{2+}]_i$ or caffeine-stimulated Ca^{2+} levels (Fig. 5.13b; $n = 9$), which is in contrast to the inhibition of caffeine-induced $[Ca^{2+}]_i$ transients measured in the presence of 10 μM SNP alone (Fig. 4.3). IBMX and SNP were applied 5 min prior to the addition of caffeine.

In addition to testing the ability of IBMX to modulate the effect of SNP on responses to caffeine, its effect on the rate of Ca^{2+} removal from the cell following a caffeine stimulus was also investigated. When applied together, IBMX (10 μM) and SNP (10 μM) did not significantly ($P > 0.05$) alter the rate of Ca^{2+} removal from the cell as shown in Fig. 5.14 ($n = 9$).

5.4 Discussion

It was shown in chapter 4 that a supramaximal (10 μM) concentration of SNP selectively inhibited responses to caffeine, which would lower the $[Ca^{2+}]_i$ available for contraction. If this effect was mediated by intracellular cGMP, it would be expected to be mimicked by membrane permeant analogues of cGMP. However, 8-Br-cGMP did not affect $[Ca^{2+}]_i$ or the rate of Ca^{2+} removal from the cell. On the

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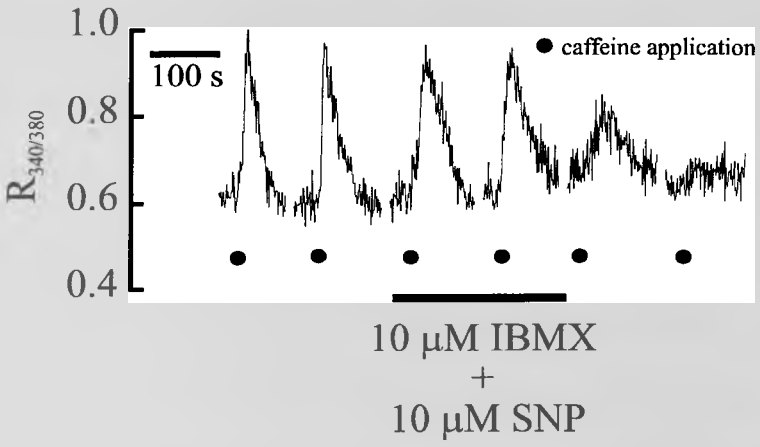
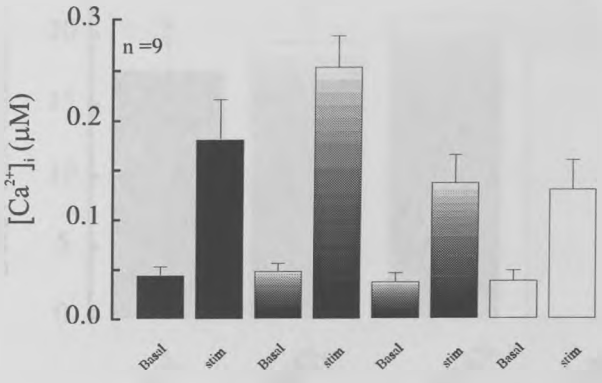


10 μM IBMX
+
10 μM SNP

b



control 10 μM IBMX + 10 μM SNP wash

Fig. 5.13. *a Effect of IBMX (10 μM) and SNP (10 μM) on caffeine-induced* $[Ca^{2+}]_i$ *transients. Gaps in the records represent 5 min recovery periods. Caffeine was applied at the times indicated (•). IBMX and SNP were applied to the cell for the duration indicated by the horizontal bar and for 5 min before testing caffeine. b Mean* $[Ca^{2+}]_i$ *measured before (basal) and at the peak of the response to caffeine (stim) in the absence and presence of IBMX and SNP. Data are expressed as mean ± S.E.M. (n = 9).*

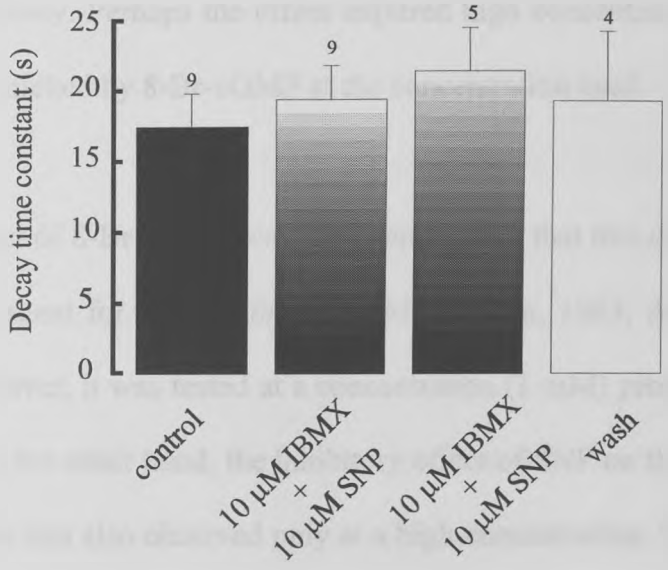


Fig. 5.14. *Mean $[Ca^{2+}]_i$ decay time constants in 9 cells exposed to IBMX (10 μM) & SNP (10 μM). There were no significant differences between $[Ca^{2+}]_i$ decay time constants measured in the presence of IBMX and SNP and control. Data are expressed as mean ± S.E.M. (n = 4-9). Values above S.E.M. bars indicate the total number of cells tested.*

other hand, the cGC inhibitor, ODQ, blocked the inhibitory effect of SNP on the response to caffeine suggesting that it required an active cGC, and therefore cGMP. This is also supported by the effects of zaprinast and IBMX on caffeine-induced Ca^{2+} transients. Both are expected to elevate intracellular cGMP levels by preventing its metabolism by PDE, and both mimicked the effect of 10 μM SNP in that they reduced the response to caffeine. On balance, these results indicate that SNP inhibited caffeine-induced Ca^{2+} release in rabbit aortic smooth muscle via the cGC/cGMP pathway. Perhaps the effect required high concentrations of cGMP that could not be mimicked by 8-Br-cGMP at the concentration used.

The lack of effect of 8-Br-cGMP was surprising, given that this cGMP analogue is a potent relaxing agent for pre-contracted VSM (Lincoln, 1983; Andriantsitohaina et al., 1995). Moreover, it was tested at a concentration (1 mM) producing pronounced vasodilation. On the other hand, the inhibitory effect of SNP on the caffeine-induced $[Ca^{2+}]_i$ transients was also observed only at a high concentration. Thus, although this inhibitory effect may be mediated by cGMP, it probably occurs only at high levels of intracellular cGMP, and may not be important for relaxation. As well as having no effect on caffeine-induced $[Ca^{2+}]_i$ transients, 8-Br-cGMP did not inhibit the responses to NA. Although these results contradict studies that suggest 8-Br-cGMP acts to reduce $[Ca^{2+}]_i$ (Kai et al., 1987; Rashatwar et al., 1987; Lincoln et al., 1988; Andriantsitohaina et al., 1995), they are in accordance with several other studies that argue against modulation of SR Ca^{2+} as the mechanism of NO or cGMP-dependent vasodilation. For example, a reduction in the Ca^{2+} sensitivity of the contractile apparatus at constant $[Ca^{2+}]_i$ can mediate VSM relaxation (Nishimura and van

Breemen, 1989; Bolz et al., 1999). Lincoln et al. (1991) also identified mechanisms independent of changes in $[Ca^{2+}]_i$ that could contribute to the relaxation of smooth muscle by cGMP. It was also shown in VSM that SNP-induced relaxation may not be associated with proportional decreases in $[Ca^{2+}]_i$ (Karaki et al., 1988; Nishimura and van Breemen, 1989; McDaniel et al., 1992). A lowering of $[Ca^{2+}]_i$ could not fully account for the relaxation induced by SNP in anococcygeus muscle, where mechanisms independent of changes in $[Ca^{2+}]_i$ have also been proposed (Raymond and Wendt, 1996). Moreover, cGMP was shown to relax VSM without any drop in $[Ca^{2+}]_i$ in rat mesenteric arteries (Lagaud et al., 1996; Martinez et al., 1996). In each case, inhibition of NA-induced sensitisation of contractile proteins to Ca^{2+} probably accounted for the effect seen.

5.4.1 cGC inhibition

It is likely that the inhibition of SNP action by ODQ was due to inhibition of cGC, because when used at 10 μM, it is a potent and selective cGC inhibitor (Garthwaite et al., 1995; Schrammel et al., 1996; Dong et al., 1998). It does not inhibit particulate GC (Garthwaite et al., 1995) and unlike other inhibitors of cGC, such as MB, it has no known effects on NOS (Mayer et al., 1993; Garthwaite et al., 1995), making it the most specific inhibitor of cGC in present use. Moreover, it had no effect on caffeine or NA-induced $[Ca^{2+}]_i$ transients in the absence of SNP. The results of the ODQ experiments therefore provide strong evidence that SNP exerted its inhibitory effect through the production of cGMP.

5.4.2 PDE inhibition

As expected for an effect mediated by cGMP, the inhibitory effect of 10 μM SNP on caffeine-induced Ca^{2+} transients was mimicked by the PDE inhibitors, zaprinast and IBMX. Zaprinast is a PDE V selective inhibitor and is therefore selective for the metabolism of cGMP (Kukovetz et al., 1979; Lugnier et al., 1983; Lugnier et al., 1986; Beavo and Reifsnyder, 1990; Shahid et al., 1991) whereas IBMX is a non-specific PDE inhibitor (Lugnier et al., 1986; Shahid et al., 1991). Both would elevate cGMP levels, but IBMX would also be expected to raise cAMP levels. Despite IBMX having been observed to preferentially potentiate cAMP-dependent relaxant responses (Fujimoto and Matsuda, 1990; Barbier and Lefebvre, 1992), it is difficult to distinguish cGMP effects from cAMP effects. Although both agents inhibited the caffeine-induced $[Ca^{2+}]_i$ transient in a similar manner, their effects differed. Zaprinast also increased the basal $[Ca^{2+}]_i$ and slowed the decline of $[Ca^{2+}]_i$ back to baseline following stimulation. IBMX did not have these effects. Nor did SNP. Perhaps zaprinast has other actions, in addition to PDE inhibition (Wells and Kramer, 1981), which can account for these effects.

Three major isoforms of cyclic nucleotide PDE (cGMP-PDE, cAMP-PDE and calmodulin-sensitive (CaM-PDE)) have been isolated from aortic tissue (Hagiwara et al., 1984; Lugnier et al., 1986; Souness et al., 1989). cGMP-PDE (type V) exhibits minor hydrolytic activity towards cAMP, but preferentially hydrolyses cGMP (Lugnier et al., 1986). cAMP-PDE (type III) selectively hydrolyses cAMP (Lugnier et al., 1986; Souness et al., 1989). CaM-PDE (type I) hydrolyses both cAMP and

cGMP (Lugnier et al., 1986), but preferentially hydrolyses cGMP (Hagiwara et al., 1984; Souness et al., 1989).

At the concentrations used, zaprinast was able to inhibit the CaM-PDE in addition to cGMP-PDE (Lugnier et al., 1986; Souness et al., 1989). Therefore, zaprinast may cause a larger increase in cGMP levels than the other agents, leading to additional effects. For example zaprinast can stimulate Ca^{2+} influx in rat pituitary GH_3 cells, which is possibly regulated by the state of filling of the store (Willmott et al., 1996b). Nevertheless, PDE inhibitors directly relax bovine tracheal and coronary arterial smooth muscle as a result of cGMP accumulation (Katsuki and Murad, 1977; Holzmann, 1983; Lorenz and Wells, 1983), so actions common to zaprinast and IBMX are likely to reflect the rise in cGMP that is responsible for vasodilation.

Although the evidence so far cited provides compelling support for the role of cGMP as a mediator of SNP-induced inhibition of caffeine-activated $[Ca^{2+}]_i$ transients in rabbit aortic smooth muscle, not all of the data obtained in the present study is consistent with this proposal. Especially perplexing was the observation that IBMX inhibited the effect of SNP in a similar manner to ODQ. If cGMP is involved in the mechanism of action of SNP, inhibitors of cGMP-PDE activity would be expected to modulate it in the opposite direction to ODQ. Thus, IBMX should have selectively potentiated the ability of SNP to increase cGMP levels and reduce the $[Ca^{2+}]_i$ transient (Lincoln, 1983; Lorenz and Wells, 1983; Zhou and Torphy, 1991). However, the result is consistent with observations made by others in cat gastric fundus, showing IBMX to decrease the peak relaxant response to NO (Barbier and

Lefebvre, 1995). Relaxation elicited by concentrations of SNP greater than 0.1 μM in aortic smooth muscle has also been shown to be inhibited following PDE inhibition, although in this case by zaprinast (Schoeffter et al., 1987). The inhibitory effect of IBMX is not easy to explain. One possibility is that IBMX may have inhibited the effect of SNP as a result of a non-specific increase in cAMP levels (Beavo et al., 1970). Although this idea appears novel, there is evidence in the literature to support it. In the same way as suggested by my results for low SNP concentrations, cAMP has been proposed to increase Ca^{2+} uptake into the SR (Saida and van Breemen, 1984; ZhuGe et al., 1999) and to enhance Ca^{2+} spark frequency through ryanodine-receptor channels in the SR of myocytes isolated from rat cerebral and coronary arteries (Porter et al., 1998). The increased Ca^{2+} uptake would tend to increase the SR load (Porter et al., 1998; ZhuGe et al., 1999). This would increase the amount of stored Ca^{2+} available for subsequent release and, by adding to the stimulatory effect of SNP on Ca^{2+} uptake, could counteract its inhibitory effect on the response to caffeine. Alternatively, cAMP, which has been shown to enhance CICR in skinned arterial smooth muscle (Saida and van Breemen, 1984), could enhance Ca^{2+} release in response to caffeine. This could be mediated via PKA, which was shown to phosphorylate the cardiac ryanodine-receptor channel in microsomal preparations (Takasago et al., 1989). In this way, IBMX could enhance the open state probability of the ryanodine-receptor channel in aorta smooth muscle.

In the lung, studies have inferred that cross-talk between cAMP and cGMP signalling processes may exist (Burns et al., 1992). The type V cGMP-PDE can be phosphorylated by PKA, which results in activation of PDE activity and the

subsequent increase in cGMP hydrolysis (Burns et al., 1992). Thus, upon elevation of intracellular cAMP levels, the levels of intracellular cGMP are reduced. The physiological significance of this is uncertain but it has been proposed that two pools of cGMP exist. One elicits relaxation, whereas the other inhibits cAMP accumulation. Thus, when the levels of cAMP reach the threshold for PKA activation, the type V cGMP-PDE hydrolyses the inhibitory cGMP pool (Burns et al., 1992). IBMX may therefore have inhibited the effect of SNP by stimulating cGMP hydrolysis via PKA-induced activation of the cGMP-PDE (Fig. 5.15), as was shown in the guinea-pig lung (Burns et al., 1992), supporting the role of cGMP as a mediator of SNP-induced inhibition of caffeine-activated $[Ca^{2+}]_i$ transients in aorta smooth muscle.

The type III cGMP-inhibited cAMP-PDE is also a substrate for PKA and PI 3-kinase (Loten et al., 1978; Kilgour et al., 1989; Rahn et al., 1994). cAMP-dependent phosphorylation of the PDE leads to increased cAMP-PDE activity and subsequent reduction in cAMP levels. This may be of physiological importance in regulating intracellular levels of cAMP and cGMP where increased cAMP levels may then activate the cAMP-PDE (Fig. 5.15). As previously discussed, suppressed levels of cGMP are produced upon elevation of cAMP, which will also activate the cAMP-PDE.

The xanthines, including IBMX, have some effects that do not appear to result from the inhibition of PDE activity. For example, using a series of xanthines, including IBMX, no relationship was found between their ability to increase tension in directly

stimulated rat diaphragms or to inhibit cGMP or cAMP hydrolysis, or to change cyclic nucleotide levels (Kramer and Wells, 1980). Perhaps the concentration of IBMX used in the present study was subthreshold for inhibition of cGMP-PDE, but sufficient to interfere with other mechanisms possibly involved in smooth muscle relaxation. In bovine coronary artery, Lorenz and Wells (1983) could not see any potentiating effect of IBMX at concentrations less than 15 μM on SNP relaxant responses. Alternatively, IBMX may have the ability to inhibit cyclic nucleotide synthesis in addition to breakdown, as has been reported for methylxanthines in other tissues (Sheppard, 1970; Strinden and Stellwagon, 1984), which may sometimes lower cyclic nucleotide levels instead of raising them. For these reasons it is difficult to interpret data that is generated by the use of non-selective PDE inhibitors.

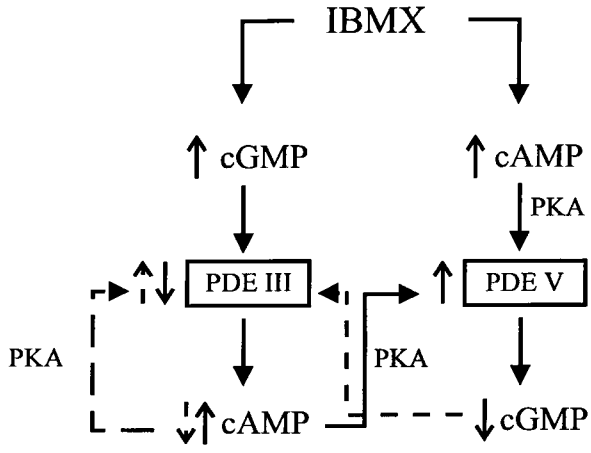


Fig. 5.15 *Opposing regulation of cAMP and cGMP levels in tissues expressing both cAMP-PDE (III) and cGMP-PDE (V).*

It is important to recognise that a common consequence of using high concentrations of pharmacological agents is a loss of specificity. It is therefore of concern that inhibition of caffeine-induced Ca^{2+} release was observed only in response to PDE

inhibitors and high concentrations of SNP, but not in the presence of 8-Br-cGMP. It may therefore be independent of cGMP or require high concentrations. In any case it appears to occur at concentrations above those needed to produce relaxation of rabbit aorta smooth muscle.

In conclusion, this chapter provides evidence in support of the hypothesis that lowering of $[Ca^{2+}]_i$ by SNP is associated with a cGMP-dependent pathway, although, some of the results were not consistent with this proposal. Particularly difficult to explain is the observation that IBMX inhibited rather than potentiated the effect of SNP. This may reflect changes in cAMP, which promotes store filling and activates cGMP-PDE. However, it is clear that mechanisms independent of the lowering of $[Ca^{2+}]_i$ also contribute to SNP and cGMP-mediated vasorelaxation.

Chapter 6

Discussion

It is well established that Ca^{2+} plays an important role as a second messenger in most cell types, including VSM. In smooth muscle, the regulation of $[Ca^{2+}]_i$ is accomplished by transport systems situated in the plasma membrane and intracellular membranes, which transport Ca^{2+} between four different pools: ECS, cytoplasm, mitochondria and SR. NO and its donors have been suggested to influence both Ca^{2+}-influx and Ca^{2+}-removal pathways in the relaxation of smooth muscle.

The apparent association between the relaxant effects of NO donors and their ability to increase intracellular cGMP has led to the suggestion that cGMP may mediate the effects of these drugs (Gruetter et al., 1981; Lincoln, 1983; Murad et al., 1985; Rapoport et al., 1985b; Kukovetz et al., 1991; Jones et al., 1994), as well as the effects of the endogenous vasodilator, NO. Despite their widespread use, the precise mechanisms responsible for these drugs causing vasodilation remain uncertain. Nevertheless, many of the suggested mechanisms centre on the lowering of $[Ca^{2+}]_i$ (Karaki et al., 1988; Jones et al., 1994; Kannan et al., 1997; Ji et al., 1998). These include acceleration of Ca^{2+} extrusion from the cell, reduction of Ca^{2+} influx into the cell, increased intracellular Ca^{2+} sequestration, reduced Ca^{2+} release from the SR and activation of K^+ channels. This study attempted to establish the relative contribution of SR Ca^{2+} accumulation and mobilisation as sites of action of exogenous cGMP-generating nitrovasodilators in lowering $[Ca^{2+}]_i$.

6.1 K^+ channel modulation

This study confirmed the presence of voltage-activated K^+ currents in SMCs of the rabbit aorta. Approximately 60 % of the current activated at potentials positive to -30

mV was blocked by TEA and CTX (Giminez-Gallego et al., 1988; Nelson and Quayle, 1995), suggesting that it was carried through BK_{Ca} channels. The cells also expressed currents attributable to K_v channel activation. Under the recording conditions employed, SNP consistently failed to modulate either K_v or BK_{Ca} current at any potential studied, suggesting that K^+ channel activation is not central to SNP-induced relaxation in the rabbit aorta. This is despite the suggestion from many studies that opening of these K^+ channels in the cell membrane increases K^+ efflux, which results in membrane hyperpolarisation by drawing the membrane potential closer to the K^+ equilibrium potential and closure of voltage-dependent L-type Ca^{2+} channels. This would decrease Ca^{2+} entry, which ultimately leads to vasodilation.

It is feasible that K^+ channels were already maximally activated during depolarisation, due to the low Ca^{2+} buffering employed in this study. However, at 1 μM, SNP potentiated the K^+ currents activated in response to SR Ca^{2+} release by caffeine. In addition, BK_{Ca} currents have been shown to be enhanced by NO in VSMCs studied with the perforated patch technique. This method uses anti-fungal drugs to puncture small holes in the membrane patch, which are small enough to prevent all but small ions from exchanging between the pipette and cell. Thus, there would be less cytosolic Ca^{2+} buffering than in the present study (Mistry and Garland, 1998). Thus, the lack of effect of SNP on voltage-activated K^+ current most likely reflects a lack of effect of NO on K^+ channels. Although enhancement of K^+ channel activity has been shown in various preparations, including aorta, with a range of NO donors (Hamaguchi et al., 1992; Bolotina et al., 1994; Archer et al., 1994; Yuan et al., 1996; Lang and Watson, 1998; Mistry and Garland, 1998) these studies generally

used rather high NO donor concentrations. Some studies used a concentration of SNP as high as 100 μM (Lang and Watson, 1998; Mistry and Garland, 1998), which is ten-fold higher than studied here and 100-fold higher than required for maximal vasorelaxation (Rapoport et al., 1985a; Karaki et al, 1988). In view of this, it is possible that higher concentrations of NO are required for K^+ channel activation than muscle relaxation or that high concentrations of the donor drugs have non-specific effects.

There are several other possible explanations for the discrepancy between the present results and those of others using the patch-clamp technique to investigate NO regulation of BK_{Ca} channel activity. It is possible that an important signalling molecule, such as cCG or PKG, required for the activation of BK_{Ca} channels, may have been lost from the cell during standard whole-cell recording. Studies employing the perforated patch configuration would have more effectively retained signal transduction systems in the cell (Mistry and Garland, 1998). However, the fact that SNP altered caffeine-induced membrane currents implies that the signal transduction mechanisms were intact. Alternatively, as previously discussed, reported actions of NO may depend on the source of NO or the different vessels/species tested. Then again, perhaps effects in other studies were due to NO^+ or $OONO^-$, the redox related forms of NO.

Several other investigators agree that K^+ channel activation is not a major mechanism of NO-induced relaxation. The present results are therefore consistent with those of Hamaguchi et al. (1992) and Bialecki and Stinson-Fisher (1995), who

showed that CTX and iberiotoxin had little effect on the relaxant effects of NO donors on rabbit aorta, although they did reduce the response of other smooth muscles (Hamaguchi et al., 1992). Since SNP had no effect on voltage-activated K^+ currents in rabbit aorta cells at concentrations causing maximal relaxation, an alternative mechanism(s) must explain modulation of vascular tone by NO and its donors in this vessel.

6.2 Enhanced sarcoplasmic reticulum accumulation

My results suggest a marked dissociation between the effects of SNP on global $[Ca^{2+}]_i$ and $[Ca^{2+}]_s$. $[Ca^{2+}]_s$, represented by membrane currents activated in the presence of caffeine, was enhanced by 1μM SNP, although at the same concentration SNP had no effect on the caffeine-induced $[Ca^{2+}]_i$ transient reported by fura-2. This is consistent with previously published data that have shown intracellular Ca^{2+} gradients (Williams et al., 1985) and local domains of high $[Ca^{2+}]_i$ that can be regulated independently (Chen et al., 1992; Stehno-Bittel and Sturek, 1992; Etter et al., 1994; Nelson et al., 1995; Etter et al., 1996). Thus, local increases in $[Ca^{2+}]_s$, due to spontaneous Ca^{2+} release from the SR via the ryanodine receptor, can activate BK_{Ca} channels to initiate STOCs, while having little direct effect on spatially averaged $[Ca^{2+}]_i$ (van Breemen and Saida, 1989; Stehno-Bittel and Sturek, 1992; Nelson et al., 1995). The selective enhancing effects of 1 μM SNP on the caffeine-induced rise in $[Ca^{2+}]_s$, suggest that it may either increase the amount of Ca^{2+} stored in the SR and/or enhance SR Ca^{2+} release from SR located close to the plasma membrane. Such a mechanism could perhaps ensure that Ca^{2+} is extruded from the cell, without increasing global $[Ca^{2+}]_i$ (van Breemen and Saida, 1989; Stehno-Bittel

and Sturek, 1992). Although on average SNP did not affect global $[Ca^{2+}]_i$, a reversible increase in $[Ca^{2+}]_i$ was seen in some cells, suggesting that the effect on the SR may be sufficiently large or widespread in some cells to influence global $[Ca^{2+}]_i$. As discussed in chapter 4, the enhancing effect of SNP on caffeine-induced $[Ca^{2+}]_i$ transients is likely to reflect enhanced sequestration of Ca^{2+} by the SR, rather than enhanced release. This suggests that the Ca^{2+}-ATPase, which sequesters Ca^{2+}, may be preferentially located on the SR located near the plasma membrane, i.e. the peripheral SR. This is consistent with evidence that much of the SR in SMCs is located close to the plasma membrane (Devine et al., 1972; Moore et al., 1993) and the suggestion that sequestration by the peripheral SR buffers Ca^{2+} entry (van Breemen, 1977; van Breemen et al., 1988; van Breemen and Saida, 1989; Sturek et al., 1992; van Breemen et al., 1995).

6.3 Reduced sarcoplasmic reticulum Ca^{2+} release

A decline in both the $[Ca^{2+}]_s$ and global $[Ca^{2+}]_i$ transient induced by Ca^{2+} released through the ryanodine receptor/channel was observed at a higher concentration (10 μM) of SNP. At this concentration, inhibition of SR Ca^{2+} release appeared to become important. This is consistent with several previous observations suggesting such an action for NO donors (Meszaros et al., 1996; Aghdasi et al., 1997; Kannan et al., 1997). Therefore, it can be postulated that at high concentrations, SNP may inhibit SR Ca^{2+} release, possibly by blocking the receptor/channel or reducing the Ca^{2+} sensitivity of the channel, such that the channel is less easily activated at physiological $[Ca^{2+}]_i$. Since both $[Ca^{2+}]_s$ and global $[Ca^{2+}]_i$ transients were inhibited, ryanodine receptors may be distributed equally in peripheral and central SR. This

agrees with immunohistochemical studies that investigated the distribution of ryanodine receptors in aorta (Lesh et al., 1998). At 10 μM, SNP also increased the rate of STOC discharge in the rabbit aorta as previously found in rabbit pulmonary artery (Clapp and Gurney, 1991a). This possibly reflected increased spontaneous release of Ca^{2+} from the SR near the plasma membrane (Nelson et al., 1995), and most likely resulted from enhanced uptake of Ca^{2+} by the stores, leading to overloading, which would also be promoted by the simultaneous inhibition of Ca^{2+} release through ryanodine receptors. Thus, it may be reasonable to suggest that at high concentrations of NO or NO donors, increased Ca^{2+} uptake and reduced release co-operate with each other to maintain the low $[Ca^{2+}]_i$ needed to promote relaxation.

Studies with the SR Ca^{2+}-ATPase inhibitors, TG (Thastrup, 1990) and CPA (Seidler, 1989), showed that the functioning ATPase was important for maintaining store filling and a low basal $[Ca^{2+}]_i$. As discussed in chapter 3, this implies that Ca^{2+} removal from rabbit aortic SMCs depends at least in part on refilling of the SR by the TG/CPA-sensitive SR Ca^{2+}-ATPase. If, as suggested, SNP acts in part by stimulating the activity of the SR Ca^{2+}-ATPase, it might be expected to increase the rate of Ca^{2+} removal from the cell following stimulation. However, SNP failed to modify the rate of Ca^{2+} removal from the cell following stimulation by caffeine or NA. It is of course possible that following stimulation, when the $[Ca^{2+}]_i$ was high, the rate of Ca^{2+} uptake into the SR was already maximal. However, SNP also failed to modify the rate of Ca^{2+} removal after the SR Ca^{2+}-ATPase was partially inhibited by CPA. A possible explanation is that the influence of SNP on the ATPase is restricted to regions close to the plasma membrane, through preferential localisation of

intracellular signalling molecules (e.g. PKG) close to the ATPase on peripheral SR. A restricted action of SNP on the ATPase in peripheral SR might be unable to influence the global $[Ca^{2+}]_i$ transient or the overall rate of Ca^{2+} removal from the cytoplasm. However, SNP, at 10 μM, was able to counteract the raised basal $[Ca^{2+}]_i$ observed in the presence of CPA. Although this is consistent with SNP lowering $[Ca^{2+}]_i$ by stimulating the SR Ca^{2+}-ATPase, it could also be explained by an effect on the plasma membrane Ca^{2+}-ATPase, or possibly on Ca^{2+} influx caused by store depletion (Wayman et al., 1996b).

GTN behaved in a similar way to SNP in that, at 1 μM, it had no effect on caffeine or NA-induced $[Ca^{2+}]_i$ transients recorded with fura-2. This concentration of GTN causes a similar degree of relaxation of aorta as 1 μM SNP (Martin et al., 1985; Luo et al., 1993; Pagani et al., 1993). Since it was not tested at higher concentrations or against caffeine-activated membrane currents, it is not possible to comment on its possible effects on SR Ca^{2+} sequestration or release.

The present findings support a role for the SR in VSM as a barrier, so that fluctuations in $[Ca^{2+}]$ near the plasma membrane do not affect global $[Ca^{2+}]_i$ (Benham and Bolton, 1986; Stehno-Bittel and Sturek, 1992; Sturek et al., 1992). Thus, the peripheral SR would buffer Ca^{2+} entering the cell and inhibit bulk cytoplasmic concentrations from rising, thereby promoting relaxation. This adds weight to the idea that $[Ca^{2+}]_s$ reaches higher levels and changes more quickly than global $[Ca^{2+}]_i$ (van Breeman and Saida, 1989; Stehno-Bittel and Sturek, 1992; Etter et al., 1994; Nelson et al., 1995; Etter et al., 1996), and this may influence the

regulation of membrane potential and thus, Ca^{2+} entry and excitability. According to fura-2 fluorescence images, $[Ca^{2+}]_i$ is not uniformly distributed within the SMC, but is higher in the nucleus, SR and beneath the plasma membrane, than in the cytoplasm (Williams et al., 1985). This is further supported by the fact that BK_{Ca} channels of smooth muscle may be spontaneously activated without contractile activity being apparent (Behnam and Bolton, 1986) and by measurement of $[Ca^{2+}]_s$ using membrane-selective Ca^{2+} indicators (Etter et al., 1994; Etter et al., 1996). Ca^{2+} release from the SR can therefore come into close apposition with the plasma membrane. In fact, it has been suggested from observations of fura-2 fluorescence in saponin-skinned SMCs, that sub-plasmalemmal regions reflect $[Ca^{2+}]$ inside the SR (Williams et al., 1985). Thus, it is not unreasonable to propose a selective action of NO or NO donors on $[Ca^{2+}]$ regulation in this region.

6.4 Role of cGMP

The inhibitory effect of SNP on $[Ca^{2+}]_i$ transients was blocked by the selective guanylate cyclase inhibitor, ODQ (Garthwaite et al., 1995; Schrammel et al., 1996; Dong et al., 1998), confirming an essential role for cGMP. This result is in agreement with the proposed role of cGMP as a mediator of NO-induced VSM relaxation (Gruetter et al., 1981; Lincoln, 1983; Rapoport et al., 1983; Murad et al., 1985; Rapoport et al., 1985b; Rashatwar et al., 1987; Kukovetz et al., 1991; Jones et al., 1994). In contrast, 8-Br-cGMP was unable to mimic this effect at a concentration known to promote vasodilation. Although this argues against cGMP as the mediator of the inhibitory effect of 10 μM SNP, the lack of effect of 8-Br-cGMP matches the observations at 1 μM SNP, which produces near maximal vasodilation. Perhaps

cGMP mediates responses to high concentrations of SNP, but not low concentrations and high concentrations of cGMP are needed for the inhibitory effect. Experiments with higher concentrations of 8-Br-cGMP should clarify this. 8-Br-cGMP can, however, mimic some of the actions of SNP on local increases in $[Ca^{2+}]_s$ (Nelson et al., 1995) and more recently cyclic nucleotides were found to increase the frequency of STOC discharge and Ca^{2+} sparks by two- to three-fold (Porter et al., 1997; Porter et al., 1998). To further investigate the potential role of cGMP, inhibitors of PDE activity were investigated. These would be expected to mimic and potentiate any effects of SNP mediated by cGMP. Both the specific cGMP-PDE inhibitor, zaprinast, and the non-selective inhibitor, IBMX, mimicked SNP in that they reduced the $[Ca^{2+}]_i$ transient evoked by caffeine. However, zaprinast had additional effects, increasing basal $[Ca^{2+}]_i$ and slowing the rate of Ca^{2+} removal from the cytoplasm. As a result of these actions, its ability to potentiate SNP could not be investigated. These effects of zaprinast are surprising, given that it promotes vasodilation (Katsuki and Murad, 1977; Holzmann, 1983; Lorenz and Wells, 1983; Souness et al., 1989), which is not consistent with elevated basal $[Ca^{2+}]_i$. IBMX also behaved in an unexpected manner, by blocking the inhibitory effect of SNP. As discussed in chapter 5, this effect may be attributed to a non-specific increase in cAMP levels (Beavo et al., 1970; Shahid et al., 1990; Barbier and Lefebvre, 1992) resulting in a complex response reflecting changes in both cAMP and cGMP levels. Overall, the experiments designed to test the role of cGMP in mediating the effects of SNP were inconclusive. Further investigation will be required to clarify any role of the cGMP signalling pathway.

The present study provides pharmacological evidence that modulation of SR Ca^{2+} store function contributes to the lowering of $[Ca^{2+}]_i$ by nitrovasodilators in rabbit aorta smooth muscle and that it may involve cGMP. It appears, however, that more than one cellular mechanism is employed. Inhibition of SR Ca^{2+} release and stimulation of Ca^{2+} sequestration appear to contribute to the lowering of $[Ca^{2+}]_i$ to facilitate relaxation. The evidence is, however, strongest for an effect on SR Ca^{2+} release through ryanodine receptors, although it appears to occur at relatively high concentrations of SNP, being clear only at concentrations above those required to cause maximal vasodilation.

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