**A VOLTAGE-DEPENDENT NORADRENALINE-
SENSITIVE INTRACELLULAR CALCIUM STORE IN
SMOOTH MUSCLE CELLS OF GUINEA PIG AORTA**

by

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Dedicated to

my late mother and
my father, my sister and my brother,
whom I miss day and night.

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Abstract

In vascular smooth muscle noradrenaline (NA) and caffeine (CAF) release Ca^{2+} from a common intracellular Ca^{2+} store, referred to as the NA/CAF-sensitive store, of the sarcoplasmic reticulum (SR). Effects of high K^+ on NA and CAF-induced Ca^{2+} release in guinea pig aorta were studied in this project.

In regular Ca^{2+} -free Krebs solution (KS) the first application of either $1\text{ }\mu\text{M}$ NA or 10 mM CAF elicited a contraction. The second CAF stimulation failed to induce a contraction, and repetitive NA stimulations induced progressively declining responses. The progressive decline in the response to repeated NA-, but not CAF-stimulations was not only reversed, but also enhanced by bathing tissue in high K^+ Ca^{2+} -free KS. The enhanced response to NA was still evident when 2 mM EGTA was present in this high K^+ solution. These results suggest that, under high K^+ conditions, Ca^{2+} was mobilized from a voltage-dependent and NA-sensitive (VDNAS) store, instead of the NA/CAF-sensitive one. Cyclopiazonic acid (CPA, $10\text{ }\mu\text{M}$), a putative SR Ca^{2+} -ATPase inhibitor, did not affect NA-induced contraction in 105 mM K^+ Ca^{2+} -free KS. However, a subsequent NA stimulation was significantly inhibited in this high K^+ solution, suggesting that Ca^{2+} recycling by SR Ca^{2+} ATPase contributes to the enhanced NA response. Furthermore, pinacidil, which hyperpolarizes cell membrane, reduced the contractile response to NA in the presence of high K^+ . Based on these results it is proposed that guinea pig aortic smooth muscle contains two intracellular Ca^{2+} stores: one is the well-known NA/CAF-sensitive store, and the other is a VDNAS store with efficient recycling capability, which is highly susceptible to inhibition by CPA.

Abbreviations

μm	micrometer
$[\text{Ca}^{2+}]$	calcium ion concentration
$[\text{Ca}^{2+}]_i$	cytosolic calcium ion concentration
1 st	the first
2 nd	the second
AA	arachidonic acid
ACh	acetylcholine
Ang II	angiotension II
ATP	adenosine 5'-triphosphate
AVP	arginine vasopressin
CAF	caffeine
cAMP	cyclic adenosine 3':5'-monophosphate
cGMP	cyclic guanosine 3':5'-monophosphate
CICR	Ca^{2+} -induced Ca^{2+} release
CPA	cyclopiazonic acid
DAG	diacylglycerol
DMSO	dimethyl sulfoxide

DNP	dinitrophenol
ECS	extracellular space
EGTA	ethylene glycol bis (β -amino-ethyl-ether)-N, N'-tetra-acetic acid
Fig.	figure
g	gram
GTP	guanosine 5'-triphosphate
IICR	IP ₃ -induced Ca ²⁺ release
IP ₃	inositol 1,4,5-trisphosphate
IP ₃ -R	IP ₃ receptor
IP ₄	inositol 1,3,4,5-tetrakisphosphate
KS	Krebs solution
LC ₂₀	the 20-kDa regulatory light chain of myosin
mg	milligram
min.	minutes
mM	millimolar
MLC ₂₀	20-kDa myosin light chain
MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
MP	membrane potential
mV	millivolt
NA	noradrenaline
NA/CAF-	noradrenaline and caffeine

nm	nanometer
nM	nanomolar
nPKC	novel protein kinase C
n th	the nth time
p	probability
pCa	$-\log_{10}[\text{Ca}^{2+}]$
PE	phenylephrine hydrochloride
pH	$-\log_{10}[\text{H}^+]$
PTN	pinacidil
PIP ₂	phosphatidylinositol 4,5-bisphosphate
P _K	cell membrane permeability to K ⁺
PKC	protein kinase C
PLC	phospholipase C
P _{Na}	cell membrane permeability to Na ⁺
PSS	physiologic saline solution
ROC	receptor-operated Ca ²⁺ channel
RYA	ryanodine
RYA-R	ryanodine receptor
SEM	standard error of mean
VDNAS	voltage-dependent noradrenaline-sensitive
VOC	voltage-operated Ca ²⁺ channel
VSG	voltage sensitive gate

Publication and conference paper

Parts of this thesis have been published and presented at conference.

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1. Introduction

This thesis focuses on the role of intracellular calcium (Ca^{2+}) stores in tension development by guinea pig aortic smooth muscle. This introduction provides a brief description of the morphology and existing information on the cellular mechanisms involved in the contraction of vascular smooth muscle cells.

1.1. Basic mechanisms of smooth muscle contraction

1.1.1. Contractile filaments

A smooth muscle fiber is an elongated spindle-shaped cell, typically 2 ~ 5 μm in diameter and 100 ~ 400 μm long and has a single nucleus. Its cytoplasm is largely filled with thin filaments and a small number of thick filaments (Fig. 1). Thin filaments are 5 ~ 8 nm in diameter containing actin and tropomyosin. Thick filaments, containing myosin, are usually 12 ~ 18 nm in diameter (Chamley-campbell et al, 1979). The thick and thin filaments are loosely arranged with the actin anchored either to the plasma membrane or to dense bodies which are analogous to the Z-disks of striated muscle, containing the same proteins. The dense bodies are held together in a three-dimensional network by intermediate filaments which serve to maintain the shape of the smooth muscle cell

Figure 1. Illustration of physical structure of smooth muscle cell. (Taken from Guyton, 1991).

(Hathaway et al, 1991). The organization of thin, thick and intermediate filament gives vascular myocytes an elongated shape.

The interaction between the major contractile proteins, actin and myosin, is essential for the tone-generating property of vascular smooth muscle. It is thought that their interaction is similar to the sliding-filament model of striated muscle (Hathaway et al, 1991). The heads of myosin molecules (cross bridges) in thick filaments bind to actin in a reaction driven by ATP hydrolysis, moving from an angle of 90° to 45° with respect to the long axis of actin and myosin containing filaments. The translocation of cross bridges propel the thin and thick filaments past each other, causing shortening of the smooth muscle.

1.1.2. Sarcoplasmic reticulum

The contractile capacity of vascular smooth muscle has long been associated with its sarcoplasmic reticulum (SR) (Somlyo et al, 1971; Devine et al, 1972). The SR is an interconnecting system of tubules distributed at both the periphery and central region of the smooth muscle fiber. The tubules of central SR course through the cell interior and communicate with rough endoplasmic reticulum and perinuclear space. The peripheral, or superficial, SR tubules run between surface vesicles to form fenestrated loops around them (Somlyo et al, 1971; Devine et al, 1972). The peripheral SR makes close contact (approximately 10 ~ 20 nm) with the surface membrane (Somlyo et al, 1971; Devine et

al, 1972). It is believed that the SR does not form obvious junctions with the cell membrane (Forssmann and Girardier, 1970), but some periodic structures crossing the gap between the surface membrane and the SR are observed (Fig. 2) (Somlyo and Franzine-Armstrong, 1985). The total volume of SR varies in different smooth muscles, ranging about 1.5 ~ 7.5% of the cell volume, e.g., about 5% for the main pulmonary artery and thoracic aorta of rabbit, and 2.2% for portal-anterior mesenteric vein of rabbit.

The importance of SR is attributed to its regulation of cytosolic Ca^{2+} , the trigger factor for contraction (see the section below). The content of calcium in SR has been estimated to be 1 mM (Walsh, 1994). Smooth muscles containing a relatively large volume of SR still respond to drugs in a Ca^{2+} -free medium, while those that have less SR do not (Somlyo et al, 1971; Devine et al, 1972). Using isolated fragmented SR from the microsomal preparation of cow uterus, Carsten (1969) provided direct evidence that the SR in smooth muscle is a Ca^{2+} binding and storage site, as occurs in skeletal and heart muscles, and adenosine 5'-triphosphate (ATP) is necessary for the Ca^{2+} uptake by SR. These findings were also confirmed in rabbit aortic smooth muscles (Fitzpatrick et al, 1972). Furthermore, electron microprobe studies on subcellular compositions of relaxed and noradrenaline (NA)-contracted blood vessels showed that NA released Ca^{2+} from the peripheral SR (Bond et al, 1984) as well as central SR (Kowarski et al, 1985).

Figure 2. Electron micrograph of a portion of a deep-etched smooth muscle cell from rabbit portal anterior mesenteric vein, showing periodic structures crossing the gap between the surface membrane and the SR, indicated by arrows. ecs, extracellular space; SR, sarcoplasmic reticulum; SV, surface vesicle. Taken from Somlyo and Franzini-Armstrong (1985).

1.1.3. Ca^{2+} as the trigger factor for contraction of smooth muscle

The resting cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) of smooth muscle cells ranges from 80 ~ 200 nM (Somlyo and Himpens, 1989), whereas the Ca^{2+} concentration is about 1.5 mM in the extracellular space. It is estimated to be 0.5 ~ 1 mM in the lumen of SR (Somlyo and Himpens, 1989; Walsh 1994). Thus an increase in permeability to Ca^{2+} at the cell membrane and the SR membrane leads to an increase in $[\text{Ca}^{2+}]_i$. The importance of Ca^{2+} in activating vascular smooth muscle contraction was discovered more than 30 years ago. Filo et al. (1965) observed that in demembranated fibers an increase in the free $[\text{Ca}^{2+}]$ in the bathing solution induced contraction. Furthermore, smooth muscle contraction induced by neuronal or hormonal stimulation is known to be accompanied by an increase in $[\text{Ca}^{2+}]_i$ to 500 ~ 700 nM (Defeo and Morgan, 1977; Taylor et al, 1989).

1.1.4. Myosin phosphorylation in regulation of smooth muscle contraction

The key effect of Ca^{2+} on contraction is phosphorylation of myosin and the resultant binding of actin with myosin (Walsh, 1994). This mechanism, depicted in Fig. 3, shows the central importance of the phosphorylation and dephosphorylation of myosin in regulating smooth muscle contraction. A rise of $[\text{Ca}^{2+}]_i$ results in the binding of calcium to calmodulin to form a calcium-calmodulin complex. This complex then binds to myosin light chain kinase (MLCK) (Dabrowska et al, 1978). The activated MLCK uses ATP to phosphorylate myosin globular heads (cross bridges), which then bind to actin filaments (Williams and Fay, 1986; Walsh 1994). The movement of cross bridges propels

Figure 3. Regulation of smooth muscle contraction-relaxation by myosin phosphorylation-dephosphorylation. CaM, calmodulin; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; myosin-p: phosphorylated myosin. Taken from Walsh (1994).

the thin and thick filaments moving past each other, causing shortening of the smooth muscle and tension development.

When cytosolic calcium decreases, MLCK becomes inactive, and thus myosin phosphorylation ceases. At the same time myosin becomes dephosphorylated by myosin light chain phosphatase (MLCP). Therefore the amount of phosphorylated myosin gradually decreases, producing relaxation (Shimizu et al, 1994; Shirazi et al. 1994; Walsh et al, 1995)

1.2. Excitation-contraction coupling in smooth muscle

Tension development is largely dependent on the processes that regulate cytosolic Ca^{2+} level. The mechanisms of Ca^{2+} regulation can be divided into (a) electromechanical coupling and (b) pharmacomechanical coupling. Under the former circumstances contraction is accompanied by cell membrane potential change. During the later circumstance, agonist-induced contraction occurs without affecting the cell membrane potential. As indicated in the preceding section, increase in permeability to Ca^{2+} at either the cell membrane or the SR membrane results in contraction. The following sections give a brief description of the mechanisms of Ca^{2+} regulation involved at these two levels.

1.2.1. Ca^{2+} regulation by cell membrane

In resting vascular smooth muscle cells the electrochemical gradient of ions produces a membrane potential of about -40 to -55 mV (Hathaway et al, 1991).

Depolarization of smooth muscle, induced by electrical stimulation, high potassium (K^+) and various neurotransmitters, activate voltage-operated Ca^{2+} channels (VOC) (Fig. 4) (Nelson et al, 1990). Activation of K^+ channels by β -adrenergic agonists, presumably via cAMP-dependent phosphorylation, increases K^+ permeability and then hyperpolarizes cell membrane which results in inactivation of VOC (Nelson et al, 1990; Walsh et al, 1995).

Agonists, such as NA, histamine and angiotensin, increase $[Ca^{2+}]_i$ through pharmacomechanical coupling with little effect on the cell membrane potential. Ca^{2+} influx stimulated by these chemicals is additive to the maximal high K^+ -induced Ca^{2+} influx in smooth muscle cells, but much less sensitive to Ca^{2+} channel blockers than that induced by cell membrane depolarization (Zschauer et al, 1987; van Breemen et al, 1980; Cauvin et al, 1983). These observations support the idea that these agonists activate receptor operated Ca^{2+} channels (ROCs) (Bolton, 1979). The mechanisms for regulation of ROC remain unclear. Cell membrane proteins such as guanosine 5'-triphosphate (GTP)-binding proteins and second messengers such as inositol 1,4,5-trisphosphate (IP_3), inositol 1,3,4,5-tetrakisphosphate (IP_4), as well as cytosolic Ca^{2+} may be involved in the activation of ROC (van Breemen and Saida, 1989).

At the cell membrane level, Ca^{2+} influx occurs predominantly through the VOC and ROC (Hurwitz, 1986). Opening of VOC or neuronal and hormonal stimulation of the muscle results in an increase in cytosolic free Ca^{2+} concentration from 140 to 500 ~ 700 nM (Walsh, 1994). This stimulates the sarcolemma Ca^{2+} pump and Na^+/Ca^{2+} exchanger to extrude excessive Ca^{2+} out of the cell so that the Ca^{2+} gradient across the cell

Figure 4. Schematic diagram of the major processes of Ca^{2+} regulation in vascular smooth muscle cell. CICR, Ca^{2+} -induced Ca^{2+} release; DAG, 1,2-diacylglycerol; G_q , members of the subfamily of stimulatory G proteins of phosphoinositide-specific phospholipase C β isoenzymes (PLC- β); IICR, IP_3 -induced Ca^{2+} release; IP_3 , inositol 1,4,5-trisphosphate; $\text{IP}_3\text{-R}$, IP_3 receptor; PIP_2 , phosphatidylinositol 4,5-bisphosphate; R, agonist receptor; RYA: ryanodine; RYA-R, ryanodine receptor; ROC, receptor-operated Ca^{2+} channel; VOC, voltage-operated Ca^{2+} channel.

membrane is maintained (Missiaen et al, 1992). Cyclic adenosine 3':5'-monophosphate (cAMP) and cyclic guanosine 3':5'-monophosphate (cGMP) activate Ca^{2+} efflux via the sarcolemmal Ca^{2+} pump. The relative contribution of $\text{Na}^+/\text{Ca}^{2+}$ exchange to Ca^{2+} transport in smooth muscle cell is still controversial and appears to vary from tissue to tissue (van Breemen and Saida, 1989; Gillespie et al, 1992a).

1.2.2. Ca^{2+} regulation by SR

In addition to causing Ca^{2+} influx through ROC on the cell membrane, agonists can alter $[\text{Ca}^{2+}]_i$ and the contractile state of smooth muscle by releasing Ca^{2+} from SR. The mechanisms for this process are gradually unfolded. Michell (1975) proposed that inositide metabolism on the cell membrane is involved in the action of agonists. Later IP_3 , one of the product of phospholipase C (PLC)-mediated hydrolysis of phosphatidylinositol-4,5-bisphosphat (PIP_2), was found to be the messenger that links not only calcium and inositides, but also the cell membrane and internal Ca^{2+} stores (Berridge and Irvine, 1984; Ehrlich and Watras, 1988). The signal transduction pathway involved in this regulation are illustrated in Fig. 4. The binding of agonist to its receptors activates the coupling of the receptors to phosphoinositide-specific phospholipase C, by way of members of the G_q subfamily of GTP-binding proteins, resulting in the activation of phospholipase C β isoenzymes ($\text{PLC-}\beta$). This enzyme then hydrolyses membrane phosphoinositide PIP_2 to generate two second messengers: IP_3 and 1,2-diacylglycerol (DAG), which activates protein kinase C (PKC). IP_3 diffuses through the cytosol and binds to the IP_3 receptor ($\text{IP}_3\text{-R}$) on the SR membrane (Walsh, 1994), activating this

receptor to induce Ca^{2+} release from SR. This mechanism is referred to as IP_3 -induced Ca^{2+} release. This receptor and the ryanodine receptor (RYA-R) on the SR are two principle receptor families responsible for mobilizing stored calcium (Berridge, 1993). Both receptors have four subunits combining to form functional calcium channels. When stimulated, these receptors undergo a conformational change which results in channel opening, allowing Ca^{2+} to diffuse down its concentration gradient from the SR lumen into the cytosol, thereby triggering contraction (Berridge, 1993). So far, no other chemical is known to activate IP_3 receptors. RYA-R can be activated by micromolar Ca^{2+} and the chemicals caffeine and ryanodine. It is inhibited by ruthenium red (Ehrlich et al, 1994). Activation of RYA-R by cytosolic Ca^{2+} is referred to as Ca^{2+} -induced Ca^{2+} release (CICR). Caffeine greatly enhances the sensitivity of RYA-R to Ca^{2+} .

Calcium uptake from the cytosol into SR is accomplished by Ca^{2+} -ATPase on the SR membrane, a 105,000-dalton protein that undergoes Ca^{2+} -dependent phosphorylation (Sumida et al, 1984). The SR Ca^{2+} -ATPase is regulated by phospholamban, a small protein that functions normally to inhibit the pump. Phosphorylation of phospholamban by second messenger cAMP- or cGMP-dependent protein kinases reduces this inhibition, unmasking the activity of this pump to result in a decrease in $[\text{Ca}^{2+}]_i$ and thus myocyte relaxation (Walsh, 1994). This may be one mechanism of vascular relaxation induced by β -adrenergic agonists and nitric oxide; these chemicals increase cAMP and cGMP respectively (Mueller and van Breemen, 1979; Suematsu et al, 1984; Popescu et al, 1985; Walsh et al, 1995).

1.2.3. Tension regulation by sensitization/desensitization of myosin phosphorylation

Ca^{2+} is the primary chemical which elicits smooth muscle contraction. However, the contractile state of smooth muscle can also be regulated pharmacologically without changing $[\text{Ca}^{2+}]_i$. One mechanism for this to occur is through sensitization or desensitization of myosin phosphorylation to Ca^{2+} (Walsh, 1994; Walsh et al, 1995). This occurs in vascular smooth muscle permeabilized with α -toxin in response to α_1 -adrenergic and muscarinic agonists (Nishimura et al, 1988; Kitazawa et al, 1989). When vascular smooth muscles permeabilized with α -toxin contract at a constant high $[\text{Ca}^{2+}]_i$ level ($\text{pCa} = 6.3$), phenylephrine (PE) induces additional contractile force (Kitazawa et al, 1989). This Ca^{2+} -sensitization is accompanied by an increase in myosin LC_{20} (MLC_{20}) phosphorylation (Kitazawa and Somlyo, 1990), presumably resulting from inhibition of MLCP instead of activation of MLCK (Kitazawa et al, 1991a; Kubota et al, 1992). The pathway that leads to agonist-induced Ca^{2+} -sensitization of smooth muscle remains to be established. GTP-binding proteins and arachidonic acid (AA), produced during agonist stimulation, may be involved in the sensitization process of MLC_{20} phosphorylation to Ca^{2+} (Kitazawa et al, 1989; Kitazawa et al, 1991b, Alessi et al, 1992, Gong et al, 1992).

Myosin light chain phosphorylation can be decreased by Ca^{2+} itself (Kitazawa and Somlyo, 1990; Himpens et al, 1990; Stull et al, 1990). A putative mechanism for this desensitization involves activation of Ca^{2+} /calmodulin-dependent protein kinase II (CaM Kinase II), which catalyses phosphorylation of MLCK (Olson et al, 1990; Walsh 1994),

resulting in a decrease in the affinity of MLCK for Ca^{2+} /calmodulin complex and hence the decreased myosin light chain phosphorylation. (Tang et al, 1992; Tansey et al, 1992). In vivo MLCK phosphorylation catalyzed by CaM Kinase II may occur in high K^+ - and agonist-induced contractions, both of which result in an increase in $[\text{Ca}^{2+}]_i$ (Stull et al, 1990; Walsh 1994).

1.2.4. Ca^{2+} -independent contraction

Recently it has become clear that in some situations agonists can induce Ca^{2+} -independent contraction. It was shown that in vascular smooth muscles bathed in a Ca^{2+} -free medium stimulation by tumor-promoting phorbol ester, an activator of PKC, induces sustained contractions with a slow initial phase. Such contraction is neither accompanied by a rise in $[\text{Ca}^{2+}]_i$, nor MLC_{20} phosphorylation (Jiang and Morgan, 1987, 1989; Singer and Baker, 1987; Itoh and Lederis, 1987). On the other hand, in physiological solution (PSS) containing Ca^{2+} , the initial onset of tension induced by either extracellular high K^+ or PE stimulation is associated with a rapid increase in both $[\text{Ca}^{2+}]_i$ and MLC_{20} phosphorylation (Jiang and Morgan, 1989). Phenylephrine induces the same small tensions in permeabilized ferret aortic smooth muscle cells when $[\text{Ca}^{2+}]_i$ is lowered from pCa 7.0 to pCa 8.6. These contractions are reversed by a peptide inhibitor of PKC. It appears that these contractions are Ca^{2+} -independent rather than Ca^{2+} -sensitized (Collins et al, 1992).

The most likely mechanisms for Ca^{2+} -independent contraction involves Ca^{2+} -independent PKCs, or novel PKC (nPKC), a subfamily of PKC that are activated by DAG or phorbol ester in a Ca^{2+} independent manner (Walsh 1994). A putative signal

transduction pathway for this type of contraction was proposed as follows (Walsh et al, 1995): Ligand occupancy of receptors that communicate GTP-binding proteins activates phosphatidylcholine-specific phospholipase C or D, generating DAG without a Ca^{2+} transient. DAG then activates Ca^{2+} -independent PKC isoenzymes. PKC leads to phosphorylation of the actin-binding proteins caldesmon and calponin (Umekawa and Hidaka, 1985; Adam and Hathaway, 1993; Winder and Walsh, 1990; Winder et al, 1993). When unphosphorylated, both caldesmon and calponin inhibit the actin activation of the myosin Mg-ATPase activity (actomyosin ATPase or actomyosin-P, Fig. 3). Therefore, the phosphorylation of these two kinds of proteins reverses their inhibitory actions on actomyosin ATPase, producing a slow, sustained contraction even in the absence of a Ca^{2+} transient and myosin phosphorylation.

1.3. The common intracellular Ca^{2+} store

Lanthanum (La^{3+}) blocks Ca^{2+} flux across the cell membrane and to displace cell membrane bound Ca^{2+} . van Breemen et al. (1972) observed that La^{3+} blocks rabbit aortic smooth muscle contractions induced by high K^{+} , but not by some hormonal agonists such as NA, angiotension II, and histamine. However, following the stimulation by one of these agonists, tissues become inexcitable by any of the three agonists. Measurement of Ca^{2+} efflux in response to these chemicals in a Ca^{2+} -EGTA medium (Deth and van Breemen, 1977) produced similar results. These observations gave rise to the concept of a

common intracellular Ca^{2+} store that can be depleted by various agonists. This can be understood from the fact that they all activate the IP_3 production pathway, thereby open IP_3 receptors to release Ca^{2+} from SR. CAF, releasing Ca^{2+} through a distinct mechanism, also share with NA a common intracellular Ca^{2+} pool, referred to as the NA/CAF-sensitive store. More details on this store are given in the following section after a brief introduction of mechanisms by which CAF releases Ca^{2+} from the SR.

1.3.1. Caffeine-induced Ca^{2+} release from SR

As early as 1960s it was known that caffeine has a strong Ca^{2+} -releasing action on the SR of striated muscles. Caffeine readily penetrating into the cytoplasm (Bianchi, 1962), inducing contraction in skeletal muscle in the absence of extracellular Ca^{2+} (Frank, 1960). These data suggest that caffeine acts on intracellular organelles to do so. In striated muscle caffeine also releases Ca^{2+} from the SR of smooth muscle. Studies on intact and saponin-treated skinned smooth muscle obtained from guinea pig taenia caecum (Saida, 1982) showed that caffeine induces transient contractions of smooth muscles in Ca^{2+} -free medium, as confirmed in cultured skinned smooth muscle cells (Yammoto and van Breemen, 1986). Herrmann-Frank et al. (1991) using ryanodine receptors (RYA-R) purified from canine and porcine aortic SR and reconstructed into planar lipid bilayers, demonstrated that the Ca^{2+} channel in the RYA-R is activated by micromolar Ca^{2+} and caffeine, but inhibited by ruthenium red.

1.3.2. NA and CAF share a common Ca^{2+} store

In 1970s, it was realized that NA and CAF mobilize Ca^{2+} from a common intracellular store. Caffeine and NA stimulate Ca^{2+} efflux at similar rates, while the Ca^{2+} efflux rate induced by dinitrophenol (DNP), known to release Ca^{2+} from isolated mitochondria (Vasington and Murphy, 1962), is much slower (Deth and Casteels, 1977). The presence of EGTA in Ca^{2+} -free buffers decreases both NA- and CAF-induced Ca^{2+} effluxes of rabbit aortic tissues. EGTA does not affect DNP induced Ca^{2+} efflux (Deth and Lynch, 1981). These results indicate that Ca^{2+} mobilized by NA and CAF does not come from mitochondria but may be from the SR, a well-known intracellular Ca^{2+} source. After CAF (or NA)-induced Ca^{2+} efflux from rabbit aortic myocytes bathed in an EGTA- or La^{3+} - containing Ca^{2+} -free buffer, subsequent NA(or CAF)-stimulated efflux decreases. Furthermore, NA- and CAF-induced Ca^{2+} efflux is not additive in rabbit aortic tissue when stimulated by a simultaneous application of both NA and CAF, suggesting that NA and CAF mobilize a common cellular Ca^{2+} source (Deth and Lynch, 1981).

Consistent with these Ca^{2+} efflux results, many contractile studies on vascular smooth muscle (Itoh et al, 1983; Leijten and van Breemen, 1984; Saida and van Breemen, 1984) suggest that the NA-sensitive store and CAF-sensitive store overlaps. It is clear that these observations arise from the existence of two types of Ca^{2+} release channels on SR membrane: (1) the IP_3 receptor which is activated by NA via IP_3 production pathway, and (2) the RYA receptor which is directly activated by CAF that diffuses into the sarcoplasm. This common Ca^{2+} store can also be depleted by other agents. RYA,

activating RYA receptors on SR, induces a moderate increase in Ca^{2+} efflux, resulting in a decrease in the net cellular Ca^{2+} content (Hwang and van Breemen, 1987). Preincubating the isolated rabbit aorta with 10 μM ryanodine reduces both the NA- (10 μM) and CAF- (25 mM) induced tension and Ca^{2+} efflux in a Ca^{2+} -free medium (Hwang and van Breemen, 1987). These observations suggest that ryanodine depletes Ca^{2+} from the intracellular stores sensitive to both NA and CAF.

The degree of the overlap of the NA- and CAF-sensitive Ca^{2+} store seems to vary in different tissues. CAF induces a relative large contraction and a rise in $[\text{Ca}^{2+}]_i$ after the depletion of the NA-sensitive store in mesenteric arteries from rabbits and rats (Saida and van Breemen, 1984; Barö and Eisner, 1995), whereas the CAF-releasable Ca^{2+} store of rat aortic myocytes is smaller than NA-releasable one (Noguera and D'Ocon, 1992). Using both contraction and Ca^{2+} efflux measurements, Leijten and van Breemen (1984) demonstrated that the CAF- and NA- sensitive Ca^{2+} stores in rabbit aortic smooth muscle overlap even though the contraction induced by CAF is much smaller than that induced by NA. These authors suggested that the thickness of the tissue preparation may be one factor accounting for the small contractile response induced by CAF. In thinner aortic strips ($\sim 100 \mu\text{m}$) CAF-induced contraction is about 3 fold larger than that induced in thicker aortic strips ($\sim 250 \mu\text{m}$). Thus the limited contractile action of CAF can be explained by the fact that as CAF diffuses inwardly, different layers of smooth muscle would be separately activated and inhibited, preventing the summation of contraction by all of the smooth muscle cells.

1.4. Effects of cell membrane depolarization on vascular tone

In vascular smooth muscle, high K^+ -induced contraction is inhibited either by Ca^{2+} channel blockers or by removal of external Ca^{2+} . Therefore, such contraction is thought to be entirely dependent on Ca^{2+} influx (Kuriyama et al, 1982).

There are reports that in saponin-treated taenia caecum of guinea-pig (Saida, 1982) and porcine coronary artery strips (Itoh et al, 1982), depolarization induces contraction in the absence of extracellular Ca^{2+} . Using quin-2 microfluorometry, Kobayashi et al. (1985,1986) found that K^+ -depolarization of plasma membrane increases $[Ca^{2+}]_i$ in cultured rat aorta medial smooth muscle cells by releasing Ca^{2+} from the CAF-sensitive intracellular store, but the increase in $[Ca^{2+}]_i$ is not sufficiently high to induce contractile responses.

In rabbit mesenteric smooth muscle, $[Ca^{2+}]_i$ and tension induced by $10\mu M$ NA in Ca^{2+} -free solution (containing 2 mM EGTA) increase with KCl concentrations (Itoh et al, 1992). In that study high KCl concentrations did not change the resting IP_3 concentration. The absolute amount of IP_3 synthesized by $10\mu M$ NA is smaller in the presence of high K^+ (10 ~ 128 mM) than that in the presence of regular K^+ (5.9 mM). These investigators speculated that membrane depolarization may enhance the sensitivity of the IP_3 -induced Ca^{2+} release mechanism to IP_3 .

Ganitkevich and Isenberg (1993) reported that in vascular smooth muscle cells isolated from the coronary artery of guinea pig, depolarizing clamp steps increase and hyperpolarizing clamp steps decrease, respectively, the decaying phase of the $[Ca^{2+}]_i$ transient induced by $10\mu M$ acetylcholine (ACh) in nominally Ca^{2+} -free bathing solutions.

These authors suggested that membrane depolarization stimulates the formation of IP_3 . Using the same tissue preparation, they showed that the latency of the initial rise of a Ca^{2+} transient in response to low concentrations of ACh ($1 \sim 10\mu M$) stimulation in Ca^{2+} -free medium is dependent on the membrane potential demonstrated (Ganitkevich and Isenberg, 1996). In this study, depolarizing voltage steps shortened the latency of the initial rise of Ca^{2+} transient without changing its maximal amplitude. Increasing ACh concentration to $100 \mu M$, presumably increasing the IP_3 production, also reduced the latency independent of the membrane holding potentials. Therefore, the reduced latency was ascribed to the effect of depolarization on the rate of IP_3 accumulation.

Yanagisawa and Okada (1994) observed that the tensions generated by isolated canine coronary arteries in high K^+ and Ca^{2+} -containing physiological saline solution (PSS) are larger than those in low K^+ PSS at the same $[Ca^{2+}]_i$ level. Also at the same $[Ca^{2+}]_i$ level, the tissues which have been contracted with high K^+ Ca^{2+} -containing PSS relax faster in a low K^+ Ca^{2+} -containing PSS than in a high K^+ Ca^{2+} -free PSS. They attributed these observations to the effect that KCl depolarization increases Ca^{2+} sensitivity of the muscle contractile elements.

In summary, cell membrane depolarization has been found to regulate vascular tone through mechanisms more than activating VOCs. For instance, it enhances NA-induced tension and $[Ca^{2+}]_i$ without increasing the IP_3 production. It shortens the latency of the initial rise of Ca^{2+} transient in response to ACh and, at the same level of $[Ca^{2+}]_i$, it increases the tension. In addition, it was observed that cell membrane depolarization

releases a small amount of Ca^{2+} from the CAF-sensitive intracellular store. The underlying mechanisms for these observations remain unclear.

2. Purpose of study

The above studies indicate that cell membrane depolarization plays an important role in the coupling of agonist stimulation and vascular tone. The mechanism underlying the depolarization-induced enhancement of tension in response to an agonist can not be attributed to the activation of the cell membrane Ca^{2+} channels. The mechanisms proposed in the literature are not only varied but also inconsistent with the existing experimental observations. An alternative hypothesis is needed. This study presents data that suggests such an alternative mechanism underlying the KCl-enhanced contraction induced by NA. This mechanism is based on a concept that there are two intracellular Ca^{2+} stores in guinea pig aortic smooth muscle cells: one is the NA- and CAF- (NA/CAF-) sensitive one, the other is a voltage-dependent NA-sensitive (VDNAS) one. Ca^{2+} in the latter store can be released only by a combination of NA and depolarization. This proposed VDNAS Ca^{2+} store is able to account for the experimental observations on the depolarization-induced enhancement of NA-induced tension in guinea pig aortic smooth muscle.

3. Methods

3.1. Rationale for choosing guinea pig aortas as experimental tissues

Adrenergic receptors are classified into α - and β -receptors. These two receptor types have been further subdivided into α_1 -, α_2 - and β_1 -, β_2 -receptors respectively. The α_1 -receptors are located at postsynaptic sites on tissues innervated by adrenergic neurons. Most of the α_2 -adrenoceptors have presynaptic locations, but the evidence for any functional roles of the postjunctional α_2 -adrenoceptors is poor, especially in the arterial system. Activation of either β_1 - or β_2 -receptors of vascular smooth muscle stimulates adenylate cyclase which increases intracellular cyclic AMP to induce relaxation (Lincoln and Fisher-Simpson, 1984; Bulbring and Tomita, 1987; Stiles et al, 1984).

Postjunctional α_1 -adrenoceptors are found in arteries, arterioles and veins. The predominant adrenoceptors in aortas of dog, guinea pig and rabbit are the α_1 -type (Docherty et al, 1981; Griendling et al, 1984; Polonia et al, 1985; Ruffolo and Waddell, 1982; Ruffolo et al, 1982; Bulbring and Tomita, 1987), while rat aorta may contain both α_1 - and α_2 -receptors (Ruffolo and Waddell 1982; Ruffolo et al, 1982).

It was shown that adrenergic agonists, such as noradrenaline (high affinity to α_1 -, α_2 - and β_1 -receptors), phenylephrine (α_1 -agonist) and methoxamine (α_1 -agonist), increase IP_3 production to release intracellularly stored Ca^{2+} through activation of α_1 -receptors in

rat tail arteries (Fox et al, 1985), rabbit aortas (Villalobos-Molina et al, 1982) and rat aortas (Legan et al, 1985).

Therefore, the guinea pig aortas were chosen as the experimental tissues in this study because of: (1) the guinea pig aortas have α_1 -adrenoceptors which mediate noradrenaline-induced IP_3 synthesis; (2) guinea pigs are relatively economic animals for experiments, and (3) guinea pigs have been often used in cardiovascular research.

3.2. Tissue Preparation

Albino guinea pigs, weighting 300-400 g, were sacrificed by a blow to the back of the neck. The chest of guinea pig was opened, and thoracic aorta isolated and put in ice-cold Krebs solution (KS) (see section on “Composition of the solution used” below). Fat and connective tissue were removed from the aorta, and the aorta was cut into 3-4 mm wide rings. Endothelial cells in aortic rings were removed by gently rubbing the internal surface of the vessel with the teeth of a small forceps. (Absence of the endothelial cells was confirmed by the inability of acetylcholine to cause relaxation during noradrenaline (NA) induced contraction in regular Krebs solutions). Since a guinea pig aortic ring was too small to be mounted on a pair of stainless hooks without damaging the muscle, two rings were tied together in parallel with a surgical silk thread passing through the lumen of the two rings (Fig. 5). Then each ring was mounted on one of a pair of stainless steel hooks in a 20 ml double jacked organ bath containing Krebs solution at pH 7.4. Temperature of Krebs solutions was maintained at 25°C by circulating water through the double jacked baths. The experimental temperature was set at 25°C because caffeine-

Figure 5. Schematic diagram of the system used to record contractions of aortic smooth muscles of guinea pigs. Two aortic rings are tied with a surgical silk passing through their lumen. One ring is mounted on the upper stainless steel hook, the other on the lower hook. The upper hook is attached by a silk thread to the force transducer (FT03). Contractile activity is recorded via an amplifier onto a pen recorder (Grass 7PIA).

induced contraction in Ca^{2+} -free Krebs solution at 37°C was very small in pilot studies, as has been reported previously (Noguera and D'Ocon, 1992). One hook was connected to a Grass force transducer (FT03) and the other was fixed to the end of an aerator, through which the organ bath solution was bubbled continuously with 95% O_2 - 5% CO_2 . Signals generated during muscle contraction were sent via the force transducer and a pre-amplifier (Model 7P1A) and recorded on a Grass polygraph (Model 7). The rings were preloaded with 2g tension and equilibrated for 90 min. Afterwards, the rings were exposed 2 times to a 105 mM KCl Krebs solution (see section on “Composition of the solutions used” below) to test their ability to contract. The rings which developed peak tension less than 350 mg in response to 105 mM K^{+} stimulation were not studied, because the tension developed by these tissues in response to agonists in Ca^{2+} -free KS would be weak. Thus measurement errors would be enhanced. Therefore only rings that developed tensions equal to or greater than 350 mg during 105 mM K^{+} stimulation were studied. They were equilibrated in regular Krebs solution for 20 ~ 30 minutes for subsequent studies.

3.3. Contractility study

After incubation in regular Ca^{2+} -containing KS, the tissue was carried through a process termed wash/incubation to remove extracellular Ca^{2+} : the bath solution was drained and then the tissue was rinsed with Ca^{2+} -free KS (2 ~ 3 times over a 10 min. period). Afterwards NA (1 μM) or CAF (10 mM) was added to the Ca^{2+} -free KS. After peak tension had been reached for 5 ~ 10 min., NA or CAF was removed by using the

wash/incubation process described above. A subsequent NA or CAF stimulation in Ca^{2+} -free KS elicited a decreasing tension. In parallel experiments conducted in a regular Ca^{2+} -containing Krebs solution, repeated exposures of aortic rings to NA or CAF induced relatively constant responses. Therefore, after the first stimulation by NA or CAF in Ca^{2+} -free KS, the tissue was considered to have been depleted of most of its NA/CAF-sensitive Ca^{2+} store.

To stimulate an aortic ring with NA or CAF in a Ca^{2+} -free KS with high K^+ concentration, the bath solution was changed from a Ca^{2+} -free KS to a high K^+ Ca^{2+} -free KS, then the tissue was incubated in this solution for 10 min. prior to its exposure to NA or CAF. After reaching peak tension, the tissue was washed/incubated with a Ca^{2+} -free KS.

After depletion of the NA/CAF-sensitive store, tissues were incubated in a regular Ca^{2+} -containing Krebs solution for 20 minutes. This process is referred to as refilling of the intracellular Ca^{2+} stores. Its effectiveness was determined by a subsequent stimulation with NA(1 μM) or CAF(10 mM) in Ca^{2+} -free KS. If the resulting contractile responses to NA or CAF were similar to those induced in control state (i.e., NA or CAF-induced tension in Ca^{2+} -free KS before the depletion and refilling process of the NA/CAF-sensitive store), the NA/CAF-sensitive store was considered to have been fully refilled. Pilot experiments showed that a 20 min. incubation in regular Krebs solution was sufficient to fully accomplish this.

3.4. Composition of Solutions used

Regular Krebs solution: (mM) containing NaCl 115, KCl 4.6, NaH_2PO_4 1.2, MgCl_2 1.2, CaCl_2 2.5, NaHCO_3 21.9, and glucose 11.

High KCl (K^+) Krebs solution: prepared by replacing NaCl with equimolar KCl from the regular Krebs solution.

Ca^{2+} -free Krebs solution: prepared by omitting CaCl_2 from the regular Krebs solution.

High KCl (K^+) Ca^{2+} -free Krebs solution: prepared by replacing NaCl with equimolar KCl from the Ca^{2+} -free Krebs solution.

The pH of the solutions was adjusted to 7.4 by HCl (1N) or NaOH (1N), as needed.

3.5. Chemicals

Noradrenaline (NA) was dissolved in 0.01 N HCl to make a stock solution of 0.2 mM. Caffeine (CAF) was dissolved in distilled water to make a stock solution of 0.2 M. A 0.2 mM EGTA stock solution was prepared by dissolving it in distilled water and adding 1N NaOH. Angiotensin II (Ang II) was dissolved in distilled water and made into a stock of 20 and 200 μM . Cyclopiazonic acid (CPA) and pinacidil (PIN) were dissolved in dimethyl sulfoxide (DMSO) to make a stock solution of 20 mM and 40 mM, respectively. NA and EGTA stock solutions were kept at 4°C, CAF stock kept in a 37°C water bath, and Ang II, CPA and PIN stocks kept at 0°C.

Concentrations of the chemicals used were: 1 μM NA, 10 mM CAF, 2.0 mM EGTA, 0.1 μM and 1 μM Ang II, 0.05% DMSO, 10 μM CPA, 20 μM PIN. The majority

of these stock solutions were injected into the 20 ml bath chamber by a syringe or pipette. The stock solution of CPA was first added into the Krebs solution in a beaker which was shaken in order to prevent precipitation. Then this CPA containing Krebs solution was transferred to the bath chamber. All the chemicals were purchased from Sigma, St. Louis, MO, USA.

3.6. Statistical Analysis

Data are expressed as mean $\pm$ SEM of tension or the relative tension, which is the ratio of two peak tensions. The Student's t-test (paired or unpaired) was applied to determine the significance of differences between the mean values. $P \leq 0.05$ was considered as significant.

4. Results

4.1. CAF and NA share a common intracellular Ca^{2+} store.

Contractile responses and Ca^{2+} efflux measurements in rabbit (Leijten and van Breemen, 1984) and rat (Noguera and D'Ocon, 1992) aortas indicate that NA and CAF mobilize Ca^{2+} from a common intracellular store, termed the NA/CAF-sensitive Ca^{2+} store. The following contractile studies were carried out to confirm if this store exists in guinea pig aortas.

In the Ca^{2+} -free KS, NA (1 μM) or CAF (10 mM) induced a rapid rise in tension (Fig. 6(A) and 7(A)). After the tension reached its peak, the tissue gradually relaxed. After the ring was stimulated with CAF (Fig. 6(B)), the contractile response to NA became markedly reduced. This NA-induced peak tension was only 34% ($n = 4$) of that without a preceding CAF stimulation (Fig. 6(A)), suggesting that the NA-sensitive store is also releasable by the previous CAF-stimulation. Likewise, following a NA stimulation the subsequent CAF-induced contraction was also reduced (Fig. 7(B)) to 39% ($n = 4$) of its control size (Fig. 7(A)), indicating that the CAF-sensitive store is releasable by NA stimulation. These observations are in agreement with those published in the literature

Figure 6. Contractile response of guinea pig aortic rings to NA (●, 1 μ M) without (A) and following (B) exposure to CAF (◆, 10 mM) in the Ca^{2+} -free KS. In A, the aortic ring was washed/incubated with the Ca^{2+} -free KS for about 25 minutes, then stimulated with 1 μ M NA. In B, the tissue was washed/incubated with the Ca^{2+} -free KS for 10 minutes, then stimulated by 10 mM CAF. After inducing a contraction, CAF was removed before the tissue was stimulated with 1 μ M NA. W = wash/incubate with the Ca^{2+} -free KS.

Figure 7. Contractile responses of guinea pig aortic rings to CAF (◆, 10 mM) without (A) and following (B) exposure to NA (●, 1 μM) in the Ca^{2+} -free KS. W = wash/incubate with Ca^{2+} -free KS.

Note: following exposure to NA the response to CAF is markedly reduced.

(Leijten and van Breemen, 1984; Noguera and D'Ocon, 1992). Therefore, these results confirm the existence of the NA/CAF-sensitive store in guinea pig aortic smooth muscle.

4.2. Effects of K^+ on NA- and CAF-induced contractions in Ca^{2+} -free KS

It is generally accepted that high concentration of extracellular K^+ depolarizes cell membrane and activates the VOCs on the cell membrane, resulting in Ca^{2+} influx and concomitant increase in tension. This tension development is believed to be dependent on the presence of extracellular Ca^{2+} . In another words, high K^+ is unable to induce contraction in Ca^{2+} -free medium even though the cell membrane is depolarized (Karaki and Weiss, 1988). In the absence of extracellular Ca^{2+} , both NA and CAF can release intracellular Ca^{2+} from SR and induce contraction. The effects of high K^+ -induced depolarization on NA- and CAF- induced contraction in Ca^{2+} -free KS were studied in the following experiments.

4.2.1. Filled NA/CAF-sensitive Ca^{2+} store

Figure 8 shows the contractile responses of a guinea pig aorta to $1\mu M$ NA (A) or 10 mM CAF (B) in a regular (a) or a high K^+ (b) Ca^{2+} -free KS. After the tissue had been incubated in the regular KS, extracellular Ca^{2+} was removed by washing/incubating with a Ca^{2+} -free KS (see the Methods section). The bath solution was either kept in the bath chamber (a) or replaced with 105 mM K^+ Ca^{2+} -free KS (b). The results show that the NA-induced contraction in the regular Ca^{2+} -free KS (Fig. 8(Aa)) was smaller that in the presence (Fig. 8(Ab)) of high K^+ (583.3 ± 50.3 mg vs. 762.5 ± 48.3 mg, $n=6$, $P < 0.001$,

Figure 8. Contractile responses of guinea pig aortic rings to $1\mu\text{M}$ NA (A, ●) and 10 mM CAF (B, ◆) in the Ca^{2+} -free KS in absence (a) and presence (b) of 105 mM K^{+} . The aortic rings were washed/incubated with the Ca^{2+} -free KS for 10 min. and then either kept in the Ca^{2+} -free KS (a) or incubated with the 105 mM KCl Ca^{2+} -free KS (b) for another 10 minutes before addition of NA (A) or CAF(B). W = wash/incubate with the Ca^{2+} -free KS.

paired t-test). However, the CAF-induced contractions in the absence (Fig. 8(Ba)) and presence (Fig. 8(Bb)) of high K^+ did not show significant difference (215.0 ± 18.3 mg vs. 200.0 ± 22.4 mg, $n=5$, $P=0.24$, paired t-test). It should be noted that high K^+ alone fails to elicit any observable tension in Ca^{2+} -free KS.

4.2.2. Depleted NA/CAF-sensitive Ca^{2+} store

The above experimental results show that in the Ca^{2+} -free KS, high K^+ enhanced the NA-but not the CAF-induced contraction, probably due to Ca^{2+} release from a store which is sensitive to NA but not to CAF. To explore this possibility, experiments were conducted in such a way that the aortic rings were first depleted of the NA/CAF-sensitive store by stimulating with NA (Fig. 9(A)) or CAF (Fig. 9(B)) twice in Ca^{2+} -free KS, then the rings were incubated in high KCl Ca^{2+} -free KS for 10 minutes before subsequent NA or CAF stimulation.

The results showed that in the regular Ca^{2+} -free KS, the first NA (Aa) or CAF (Ba) stimulation induced a rapid phasic contraction, followed by a much smaller contraction in response to a second agonist stimulation (Fig. 9 (Ab, Bb)). However, the reduced tension induced by NA was recovered in the presence of 65 mM K^+ (Ac), but CAF-induced tension was not recovered even in the presence of 105 mM K^+ (Fig. 9(Bc)). As shown in the previous experiments, these results indicate that the high K^+ -induced enhancement is specific to NA, but not to CAF. The effect of various concentrations of KCl on NA-induced tension is depicted in Fig. 10. The data show that the enhancement increases with the extracellular K^+ concentrations ranging from 20 to 105 mM (Fig. 10).

Figure 9. Contractile response of guinea pig aortic rings to repeated stimulations by 1 μ M NA(A, ●) and 10 mM CAF (B, ◆) in the Ca²⁺-free KS in the absence and presence of high K⁺. In the Ca²⁺-free KS, the rings were stimulated by NA(A) or CAF (B) twice (a,b). Then the rings were bathed in a 65 (A) or 105 (B) mM KCl Ca²⁺-free KS for 10 minutes before exposed to the third NA or CAF stimulation (A(c), B(c)). W = wash/incubate with Ca²⁺-free KS.

Note: the response to NA but not to CAF was enhanced/increased in the presence of high K⁺.

A **B**

Figure 10. Effects of increasing the extracellular KCl concentration (A) and the cell membrane potential (B) on NA-induced contractions of guinea pig thoracic aorta in the Ca^{2+} -free KS. Relative tension is expressed as the ratio of the peak tension in the presence of high K^+ (Fig. 9A(c)) to its previous one in the absence of high K^+ (Fig. 9A(b)). Each data point is the mean $\pm$ SEM of six preparations (A). The cell membrane potentials (MP) were computed (see Appendix) with the Goldman-Hodgkin-Katz equation based on the measured MP-KCl relation of guinea pig abdominal aortic smooth muscle cells (Kajiwara 1982).

4.3. Effect of high K^+ on Ca^{2+} accumulation in the SR

It has been postulated that the superficial SR of smooth muscle may function as a buffer barrier to calcium entry (van Breemen et al, 1995). Instead of directly diffusing to the myofilaments and causing contractions, a portion of Ca^{2+} influx will accumulate in the superficial SR according to this postulate. In the previous experiments nominally Ca^{2+} -free KS was used. It could contain traces of Ca^{2+} which might enter the cell to refill the superficial SR. To investigate the possibility that the enhanced contractile responses to NA is a result of the high K^+ -induced Ca^{2+} accumulation into the superficial SR, the following experiments were conducted.

In a Ca^{2+} -free KS, the first NA stimulation in Fig. 11(a) induced a rapid rise in tension. After NA was removed, the tissue was either kept in the Ca^{2+} -free KS (Fig. 11(A)) or incubated in a 105 mM KCl Ca^{2+} -free KS for 10 min., followed by wash (Fig. 11(B)). After this treatment, the responses to subsequent NA stimulation in the both tissues (Fig. 11 A(b), B(b)) were not significantly different from each other. The ratios of their peak tensions of (b) to (a) were 0.41 ± 0.004 (Fig. 11(A)) and 0.44 ± 0.03 (Fig. 11(B)) ($p = 0.44$, $n=4$, paired t-test). In both cases the peak tensions decreased to about 40% of their previous ones ($n=4$). However, when 105 mM K^+ was present during NA stimulation, the contractile response was greatly enhanced (Fig. 11 C(b)) in comparison to those in (A(b)) and (B(b)). The results of four such experiments are summarized in Fig. 12.

Figure 11. Contractile response of guinea pig aortic rings to stimulations by NA (●, 1μM) in the Ca²⁺-free KS.

(A(b)): control responses to NA.

(B(b)): NA responses after incubation with the 105 mM K⁺ for 10 min. and washing with the Ca²⁺-free KS.

(C(b)): NA response in the presence of 105 mM K⁺.

The interval between two stimulations was 40 minutes. The 105 mM KCl Ca²⁺-free KS were used to incubate the tissue for a 10 min. period, followed by a 10 min. wash/incubation with the Ca²⁺-free KS (B(b)), or kept in the bath (C(b)) before the 2nd NA stimulation. W = wash/incubate with the Ca²⁺-free KS.

Note: the response to NA is only enhanced when high K⁺ is present during NA stimulation.

Figure 12. Statistic results of NA-induced contractions of guinea pig aortic rings treated with high K⁺. The experimental procedures are shown in figure 11.

“regular K⁺”, “high K⁺ + wash”, and “high K⁺” stand for different treatments with the 105 mM K⁺ Ca²⁺-free KS in figure 11 (A), (B), (C). The relative tension on the Y axis is calculated as the ratio of the peak tension induced by the 2nd to the 1st NA stimulation.

*: $p < 0.005$, $n = 4$, paired t-test, significant different from the other two groups

4.4. Characteristics of a voltage-dependent NA-sensitive(VDNAS) Ca^{2+} store

The previous experiments indicated that exposure to high K^+ followed by washing with a Ca^{2+} -free KS did not cause an enhancement of the contractile response to the NA stimulation. Enhancement was observed when NA and high K^+ were present together (Fig. 11(C), 12). As shown previously in Fig. 9, this enhancement was NA specific and occurred when the NA/CAF-sensitive store had been depleted. Therefore, the enhanced tension may be attributed to Ca^{2+} release from another unknown intracellular store. It is proposed that in smooth muscle cells of the guinea-pig aorta, there are two intracellular Ca^{2+} stores: one is the well-known NA/CAF-sensitive, the other one is NA-sensitive when cell membrane is depolarized. The latter store is termed the voltage-dependent NA-sensitive (VDNAS) store. High K^+ -induced enhancement of contraction might be due to Ca^{2+} -release from this proposed VDNAS store. Following experiments were conducted to further characterize this store.

4.4.1. Ca^{2+} recycling by the VDNAS store

In the regular Ca^{2+} -free KS repetitive NA stimulation elicited decreasing contractile responses in aortic rings (Fig. 13(A)) similar to those observed by Saida and van Breemen (1984) and Naugera and D'Ocon (1992). After the first NA-induced contraction in the regular Ca^{2+} -free KS, repetitive NA stimulation in the presence of 105 mM K^+ elicited contractions equal to or greater than the first one (Fig. 13(B)). These results are summarized in Fig. 13(C), which depicts the ratio of the peak tension induced by the n^{th} NA stimulation to that of the first contraction.

Figure 13. Contractile response of guinea pig aortic rings to repeated NA (●, 1μM) stimulations in the Ca²⁺-free KS in the absence (A) and presence (B) of 105 mM K⁺. The interval between two NA stimulations was 30 minutes. In B, the 105 mM KCl Ca²⁺-free KS was used to bath the tissue 10 minutes prior to the subsequent NA stimulation. W = wash/incubate with the Ca²⁺-free KS.

C: The relationship of the relative tension, expressed as the ratio of the peak tension induced by the Nth to the 1st NA stimulation in the regular (●) and the 105 mM K⁺ (○) Ca²⁺-free KS, with the number of NA exposure. The experimental procedures are shown in (A) and (B). Data are from six preparations.

4.4.2 Effect of CPA on Ca^{2+} recycling of the VDNAS store.

In the previous experiments the maintained contractile responses to repetitive NA stimulations in high K^+ Ca^{2+} -free KS might be explained by an effective Ca^{2+} recycling process by the intracellular stores. To test this hypothesis, CPA, a putative selective sarcoplasmic reticulum Ca^{2+} -ATPase inhibitor (Uyama et al, 1992), was used. It has been shown that CPA neither interferes with IP_3 or NA-induced Ca^{2+} -release in chemically skinned smooth muscle fibers, nor affects the contractile protein activities (Uyama et al, 1992; Gonzalez et al, 1995).

Two groups of aortic rings were stimulated with $1\mu\text{M}$ NA in the Ca^{2+} -free KS (Fig. 14 A(a), B(a)). Then the rings were incubated in the 105 mM K^+ Ca^{2+} -free KS without or with $10\mu\text{M}$ CPA prior to the 2nd NA stimulation. Following this, subsequent NA stimulation in the presence of 105 mM K^+ elicited near maximum tensions in the group of rings without CPA treatment (Fig. 14A(c)), but induced decreased tension in the CPA-treated rings even in the absence of CPA (Fig. 14B(c)). The ratio of peak tensions in (c) to (b) (Fig. 14A) was 0.89 ($n = 4$), but it decreased to 0.26 ($n = 7$) in Fig. 14B. Note that in the presence of CPA and 105 mM K^+ the ratio of peak tensions in (b) to (a) was 0.91 ± 0.04 (Fig. 14B(b)), which is significantly greater than ($p < 0.00001$, unpaired t-test) that in the absence of high K^+ (0.43 ± 0.03 , $n=6$) (Fig. 13(A(b))).

Figure 14. Contractile response of guinea pig aortic rings to repeated NA(●, 1μM) stimulations in the regular Ca^{2+} -free KS (a) and then in the high K^{+} Ca^{2+} -free KS in absence (A(b,c) and B(c)), and presence (B(b)) of 10 μM CPA. The interval between two NA stimulations was 30 minutes. The 105 mM KCl Ca^{2+} -free KS (b,c) was used to incubate the aortic rings for 10 minutes prior to the subsequent NA stimulation. 10μl DMSO (A(b), not indicated by arrow) or 10 μl CPA (10 μM) (B(b)) was added into the bath 5 minutes before the 2nd NA stimulation. W = wash/incubate with the Ca^{2+} -free KS.

4.4.3 Effect of high K^+ on the VDNAS store in the presence of EGTA

When studying the role of intracellular Ca^{2+} stores in vascular contraction, EGTA is often used to chelate the residual Ca^{2+} in bath medium. To assess the enhancement of high K^+ on the VDNAS store in presence of EGTA, aortic rings were first stimulated with $1\mu M$ NA in a Ca^{2+} -free KS to deplete the NA/CAF store. After a wash/incubation period followed by a 10 minute period either in a regular (Fig. 15A(b)) or a $105\text{ mM } K^+$ (Fig. 15B(b)) Ca^{2+} -free KS containing 2 mM EGTA, the rings were stimulated again with NA. The relative tensions (ratio of peak tensions of the 2nd contraction to that of the 1st contraction) in (A) and (B) were 0.13 ± 0.04 and 0.37 ± 0.04 respectively ($n=4$, $p < 0.05$, paired t-test), whereas in regular and in high K^+ Ca^{2+} free KS without EGTA the ratios were 0.43 ± 0.03 (Fig. 13A(a,b)) and 1.36 ± 0.08 (Fig. 13B(a,b)) ($p < 0.00001$, $n=6$, paired t-test). It should be noted that in the absence or presence of EGTA 105 mM KCl enhanced the NA-induced contractions attributed to the suggested VDNAS by similar ratio ($1.36/0.43 = 3.2$, $0.37/0.13 = 2.8$).

4.5. Effect of pinacidil on the K^+ -induced enhancement of NA response

Pinacidil is a peripheral vasodilator (Petersen et al, 1978; Eilertsen et al, 1982). Its antihypertensive action seems to be associated with the activation of the ATP-sensitive K^+ channel and resultant cell membrane hyperpolarization (Southerton et al, 1988). In addition, it has been reported that pinacidil inhibits the rise of $[Ca^{2+}]_i$ and tension in response to NA, associated with a decrease in IP_3 synthesis (Itoh et al, 1992). Pinacidil has been reported to have no effect on cGMP and cAMP levels (Southerton et al, 1988;

Figure 15. Contractile response of guinea pig aortic rings to NA($1\mu\text{M}$, $\bullet$) stimulation in the Ca^{2+} -free KS and in the 2 mM EGTA-containing Ca^{2+} -free KS in absence (A) and presence (B) of 105 mM K^+ . The interval between the two NA stimulations was 30 minutes. 2 mM EGTA and the 105 mM KCl Ca^{2+} -free KS (B) was applied 10 minutes before the subsequent NA stimulation. **W** = wash/incubate with the Ca^{2+} -free KS.

Kauffman et al, 1986), nor to act as a calcium entry blocking drug (Kærgaard Nielsen and Arrigoni-Martelli, 1981; Mikkelsen and Pedersen, 1982).

The previous experiments demonstrated that high K^+ potentiated the NA-induced tension, probably by releasing Ca^{2+} from the suggested VDNAS Ca^{2+} store. Since pinacidil hyperpolarizes the cell membranes, it was interesting to investigate the action of pinacidil on the NA-induced tension specifically attributed to the VDNAS store.

The effect of pinacidil is shown in Fig. 16. After the NA/CAF-sensitive store had been depleted by repeated NA ($1\ \mu M$) stimulation in the Ca^{2+} -free KS (Fig. 16A(a,b)), the enhancement of the NA-induced contraction by high K^+ in the presence of $20\ \mu M$ pinacidil is reduced (Fig. 16 (A(c))). The effects of various concentrations of KCl on NA-induced increase in relative tension in the presence and absence of $20\ \mu M$ pinacidil are depicted in Fig. 16(B). The data show that in the presence of pinacidil the curve of the relative tension versus KCl concentration is shifted to the right of that in the absence of pinacidil, indicating an inhibitory effect of pinacidil on the contraction attributed to the Ca^{2+} released from the VDNAS store.

4.6. Effect of K^+ on Angiotension II (Ang II)- induced contraction in Ca^{2+} -free KS

Angiotension II (Ang II), circulating in the blood stream, is one of the most potent vasoconstrictors. Ang II, acting through AT_1 receptors, induces smooth muscle contraction by inducing Ca^{2+} influx, and mobilizing intracellular Ca^{2+} stores (Zhang et al,

B

Figure 16. Contractile response of guinea pig aortic rings to NA (●, 1 μ M) in the regular Ca^{2+} -free KS and in the high K^+ Ca^{2+} -free KS in presence of 20 μ M pinacidil.

A: The experimental details are similar to those in figure 9 (A). 10 μ l DMSO (not shown), serving as control, or 10 μ l pinacidil (20 μ M, ■) was added 5 minutes before the third NA stimulation (c).

B. Effect of increasing concentrations of KCl on 1 μ M NA-induced tension in the Ca^{2+} -free KS in the absence (●) and presence (■) of 20 μ M pinacidil. Experimental procedures are shown in (A). The relative tension on the Y-axis is expressed as the ratio of the peak tensions induced by the 3rd to the 2nd NA stimulations.

*: $p < 0.05$, unpaired t-test.

**: $p < 0.01$, unpaired t-test.

***: $p < 0.005$, unpaired t-test.

****: $p < 0.0005$, unpaired t-test.

1994) as well. Ang II induces a transient contraction when extracellular Ca^{2+} is removed, suggesting that it may mobilize intracellular Ca^{2+} stores (Zhang et al, 1994). This action is associated with the activation of phospholipase C, which produces DAG and IP_3 (Alexander et al, 1985; Brock et al, 1985). Ang II-induced IP_3 formation is rapid, correlating temporally with a rapid increase in $[\text{Ca}^{2+}]_i$ and an initial contraction. In contrast, the rise of DAG production was much slower and sustained (Griendling et al, 1987). Therefore, the mechanisms of Ca^{2+} release from intracellular stores in response to Ang II and NA seem to be similar. As the action of Ang II on vascular smooth muscle cells with depleted NA/CAF sensitive stores has not been reported, experiments were conducted to delineate the effect of KCl on Ang II induced contraction in the Ca^{2+} -free KS.

4.6.1. Tachyphylaxis of Ang II-induced contractions

In Ca^{2+} -containing KS, guinea pig aortic rings show tachyphylaxis in response to a repeated Ang II stimulation (Fig. 17). As shown, the first stimulation by $1\mu\text{M}$ Ang II induced a transient contraction, but the second Ang-II stimulation failed to produce any response.

4.6.2. Effect of K^+ on Ang II-induced contraction in Ca^{2+} -free KS

The experimental procedure used in this study (Fig. 18A) was similar to that in Fig. 9(A) except that Ang II was studied instead of NA. It is briefly described as follows: aortic rings were stimulated twice with $1\mu\text{M}$ NA in regular Ca^{2+} -free KS to deplete the

Figure 17. Contractile responses of guinea pig aortic rings to repeated Ang II (■, 1 μ M) stimulation in a Ca²⁺-containing regular KS. W = wash/incubation with the regular KS for 20 minutes.

B **C**

Figure 18. Contractile response of guinea pig aortic rings to NA (●, 1 μ M) in the regular Ca^{2+} -free KS and to Ang II (0.1 μ M, ■) in the high K^+ Ca^{2+} -free KS.

A: The experimental details are similar to those in figure 9(A). Instead of NA, Ang II was added in the presence of high K^+ .

B: KCl dose effect on NA (●, 1 μ M) and Ang II (■, 0.1 μ M) induced contraction in the Ca^{2+} -free KS. The experimental procedures are shown in Fig. 9(A) and 18(A). The relative tension is expressed as the ratio of the peak tension induced by NA (Fig. 9(Ac)) or Ang-II (Fig. 18(Ac)) in the presence of high K^+ to its previous NA response in the absence of high K^+ (Fig. 9(Ab), 18(Ab)).

C: Effects of membrane potentials of the aortic smooth muscle cells of guinea pig aorta on NA-(●, 1 μ M) and Ang II (■, 0.1 μ M)-induced relative tensions. The cell membrane potentials (MP) were computed with the Goldman-Hodgkin-Katz equation based on the measured MP-KCl relation of guinea pig abdominal aortic smooth muscle cells (Kajiwara 1982).

NA/CAF-sensitive store. After washing/incubating in the regular Ca^{2+} -free KS for 10 min. and then in the high K^+ Ca^{2+} -free KS for another 10 min., the rings were stimulated with $0.1\mu\text{M}$ Ang II in the same high K^+ solution. The K^+ -induced enhancement of Ang II response is expressed as relative tension, i.e., the ratio of peak tension induced by Ang II to that of the second NA-induced contraction.

The relation of Ang II-induced relative tension and extracellular K^+ concentration is shown in Fig. 18 (B). For comparison the effect of various KCl concentrations on NA-induced tensions, extracted from Fig. 10, is included. The results show that Ang II-induced tension is enhanced by KCl at moderate concentrations (5 ~ 45 mM). Ang II responses are not further affected by higher KCl concentrations (50 ~ 105 mM). They are less than the NA-induced ones.

5. Discussion

The experiments described herein showed that NA-induced contraction in the Ca^{2+} -free KS was enhanced in presence of high K^+ . In contrast, the contractile response to CAF was not affected by high K^+ (Fig. 8, 9). These results are consistent with those reported (Itoh et al, 1992, Ganitkevich and Isenberg, 1993) and suggest that K^+ -induced enhancement of contraction is specific to NA, but not CAF. Our results further indicated that the K^+ -induced enhancement occurred after the NA/CAF-sensitive store had been depleted (Fig. 9(A)), and this enhancement could be demonstrated by repetitive NA stimulation in the presence of 105 mM K^+ (Fig. 13). Underlying mechanisms are discussed in the following.

5.1. Possible Mechanisms for high K^+ -induced enhancement

The above experimental observations may be attributed to the following possible mechanisms: (1) an increase in intracellular Na^+ concentration ($[\text{Na}^+]_i$) promotes Ca^{2+} influx, or a reduction of transmembrane Na^+ gradient increases Ca^{2+} influx and decreases Ca^{2+} efflux via $\text{Na}^+/\text{Ca}^{2+}$ exchange (Wong and Bassingthwaighe, 1981); (2) depolarization stimulates formation of IP_3 (Ganitkevich and Isenberg, 1993); (3) membrane depolarization enhances sensitivity of the IP_3 -induced Ca^{2+} release mechanism

to IP_3 (Itoh et al, 1992); or increases Ca^{2+} sensitivity of contractile elements (Yanagisawa and Okada, 1994). (4) Depolarization causes Ca^{2+} accumulation into SR. These mechanisms are discussed in the following section.

5.1.1. Role of $\text{Na}^+/\text{Ca}^{2+}$ exchange

The experiments were conducted in Ca^{2+} -free medium, therefore eliminating or minimizing Ca^{2+} influx via $\text{Na}^+/\text{Ca}^{2+}$ exchange or ROCs and VOCs. Reduction of external Na^+ (Na^+_o) by KCl on an equimolar basis tends to reduce resting $[\text{Na}^+]_i$, which was estimated to be 11 ~ 13 mM (Gillespie et al, 1992a). It is most unlikely that the enhanced contraction induced by NA in the low Na^+ and high K^+ Ca^{2+} -free KS is attributed to Ca^{2+} influx via the exchange.

A number of studies support the idea that the $\text{Na}^+/\text{Ca}^{2+}$ exchange plays little role in contraction of smooth muscle cells in Ca^{2+} -containing solutions in the presence and absence of Na^+_o . Smith et al (1989) reported that removing Na^+_o or replacing Na^+_o with K^+ caused no detectable change in $[\text{Ca}^{2+}]_i$ in rat aortic muscle. Gillespie et al (1992a) observed that in cultured rat aortic cells line (A10), presence or absence of Na^+_o caused no change in either $[\text{Ca}^{2+}]_i$ transient induced by arginine vasopressin (AVP) or the recovery phase of $[\text{Ca}^{2+}]_i$ transient following a brief stimulation with AVP. Gillespie et al (1992a) also demonstrated that in the absence of external Na^+ , in cells initially exposed to nominally Ca^{2+} -free solution and stimulated with AVP to deplete any internal Ca^{2+} stores, subsequent brief or prolonged incubation in a Ca^{2+} -containing solution did not activate a large Ca^{2+} influx. These observations strongly suggest that in the cells with depleted

internal store, Ca^{2+} influx/efflux via $\text{Na}^+/\text{Ca}^{2+}$ exchange is insignificant even in the Ca^{2+} -containing solution. Removal of Na^+ from a Ca^{2+} -containing solution caused insignificant Ca^{2+} influx in A7r5 cells (Gillespie et al, 1992b). In the same cell line $\text{Na}^+/\text{Ca}^{2+}$ exchange appears to play very little role in the AVP-induced Ca^{2+} transients (Vigne et al, 1988). In the study of Yanagisawa and Okada (1994) on canine coronary arterial rings in a Ca^{2+} -containing medium, change of $[\text{Na}^+]_o$ did not affect the relaxation phase of the $[\text{Ca}^{2+}]_i$ -force relationship. Reduction in $[\text{Na}^+]_o$ had no effect on the agonist-induced contractions of vascular smooth muscle cells in a Ca^{2+} -free medium (Bova et al, 1990).

In principle, reduction of $[\text{Na}^+]_o$ suppresses Na^+ -activated Ca^{2+} efflux. In view of the above cited studies, it appears that the KCl enhancement of NA-induced tension is not likely caused by the reduction of Ca^{2+} efflux for the following reasons: (1) The CAF-induced contractions in high Na^+_o and low K^+ , or low Na^+_o and high K^+ Ca^{2+} -free solutions are equal in magnitude and relaxation rate (Fig. 8(B)). If the $\text{Na}^+/\text{Ca}^{2+}$ exchange was a dominant determinant in Ca^{2+} homeostasis the contractions in the low Na_o and high K^+ solutions would be much higher and the relaxation rate would be much slower. The tension developed by the CPA-treated rings (Fig. 14(Bb)) would be tonic with very slow relaxation rate because Ca^{2+} recycling by the Ca^{2+} store is inhibited by CPA. However, phasic contraction with fast relaxation rate was observed. Furthermore, low Na^+ and high K^+ failed to enhance the CAF-induced contractions either before (Fig. 8(Bb)) or after (Fig. 9(Bc)) the NA/CAF-sensitive store had been depleted. (2) The contractions induced by repetitive NA-stimulations in the low Na_o and high K^+ (the 2nd ~ 5th NA stimulation in Fig. 13(B)) did not relax slower than that of the control contraction (the 1st NA

stimulation in Fig. 13(B)). (3) Intracellular Ca^{2+} concentration is reduced by the following processes: (a) Ca^{2+} uptake by Ca^{2+} stores, (b) Na^{+} -activated Ca^{2+} efflux, (c) $[\text{Ca}^{2+}]_i$ -activated Ca^{2+} efflux via $\text{Na}^{+}/\text{Ca}^{2+}$ exchange, (d) sarcolemmal Ca^{2+} pump, and (e) Ca^{2+} diffusion out of the cell. The reduction in Na^{+} -activated Ca^{2+} efflux would have to be so much larger than the combined Ca^{2+} removal processes (a, c, d, e) to account for a four-fold increase in contraction (Fig. 10, $[\text{K}^{+}] = 105 \text{ mM}$).

In the studies of Genitkevich and Isenberg (1993, 1996) on isolated myocytes from guinea pig coronary artery depolarizing clamp steps increased the decaying phase of Ca^{2+} transient induced by acetylcholine in nominally Ca^{2+} -free solution in which $[\text{Na}^{+}]_o$ was kept constant. The increase in $[\text{Ca}^{2+}]_i$ cannot be attributed to a reduction of Ca^{2+} efflux via the $\text{Na}^{+}/\text{Ca}^{2+}$ exchanges. Since the exchanges are electrogenic, depolarization tends to reduce Na^{+} -activated Ca^{2+} efflux (Wong and Bassingthwaite, 1981). Information on effect of membrane potential on the Na^{+} -activated Ca^{2+} efflux from smooth muscle is lacking. However, in squid axon, in the absence of both extracellular Ca^{2+} and Na^{+} , the Ca^{2+} efflux was independent on membrane potential, and the maximal efflux was only $1 \sim 2 \text{ p mole/cm}^2 \text{ sec}$ (Mullins and Brinley, 1975). The independence of Na^{+} -activated Ca^{2+} efflux on membrane potential was also observed in guinea-pig auricles (Jundt et al, 1975). Therefore, the high KCl enhanced contractions induced by NA cannot be possibly attributed to the $\text{Na}^{+}/\text{Ca}^{2+}$ exchanges.

5.1.2. Does IP₃ production or sensitivity of contractile elements increase?

The idea that depolarization stimulates IP₃ formation is not supported by observations of Itoh et al. (1992). They found that high K⁺ (20 ~ 128 mM) decreased the absolute amount of IP₃ by half. The reduction in IP₃ synthesis by NA may be associated with parallel decreases in diacylglycerol, protein kinase C, and arachidonic acid, the suggested Ca²⁺-sensitization agents (Somlyo and Somlyo, 1994). As at least three IP₃ molecules bind to one IP₃ receptor, a 50% reduction in IP₃ could result in a 8-fold reduction in the releasing capability of the Ca²⁺ store, and thus the amount of Ca²⁺ released. With a possible 8-fold reduction in released Ca²⁺, and the reduced production of the above mentioned Ca²⁺-sensitization agents, a 4-fold increase in NA-induced tension by 105 mM KCl (Fig. 10) would require an increase in the Ca²⁺ sensitivity of contractile elements more than 32 times. Such a large increase in sensitivity may not be possible. In addition, tension development by vascular smooth muscle, besides being dependent on [Ca²⁺]_i and the Ca²⁺ sensitivity of contractile elements, may be affected by the rate of increase in [Ca²⁺]_i. A higher rate may elicit a greater tension than a lower one even though the resultant [Ca²⁺]_i level and the Ca²⁺ sensitivity of contractile elements are the same. Therefore, at the same [Ca²⁺]_i level, higher tension may not be ascribed to the increase in Ca²⁺ sensitivity of contractile elements as suggested by Yanagisawa and Okada (1994). Furthermore, the CAF response was not affected by the presence of high K⁺ (Fig. 8(B), 9(B)). This result does not support the idea of increased sensitivity of contractile elements to Ca²⁺.

5.1.3. Is the sensitivity of IP₃ receptor to IP₃ greatly increased?

Itoh et al. (1992) showed that in the presence of 128 mM KCl, $[Ca^{2+}]_i$ and tension induced by 10 μ M NA were two fold, and IP₃ production was half of those in 5.9 mM KCl. They suggested that membrane depolarization enhances sensitivity of the IP₃-induced Ca^{2+} release mechanism to IP₃ (IICR). Based on the similar argument against an increase in sensitivity of contractile elements to Ca^{2+} , a two fold increase in $[Ca^{2+}]_i$ would require an increase in the sensitivity of IICR 16 times. While such a large increase may not be possible, Itoh et al's observations can be accounted for with our proposed VDNAS store, which is detailed in section 5.2. As Itoh et al. did not deplete the NA/CAF-sensitive store prior to KCl depolarization, the increase in $[Ca^{2+}]_i$ could be attributed to Ca^{2+} released from both the NA/CAF-sensitive and the VDNAS stores. It is worth pointing out that the NA-induced contraction attributed to the VDNAS store was equal to or greater than that due to the NA/CAF-sensitive store (Fig. 9(A), 13(B)) despite the possibility that KCl may depress the NA-induced synthesis of IP₃ or other messengers.

5.1.4. Does high K⁺ cause Ca²⁺ accumulation into SR?

According to the Ca^{2+} -buffer barrier postulate (van Breemen et al, 1995), the Ca^{2+} influx through cell membrane may refill the depleted superficial SR. However, in this study incubation with high KCl alone did not affect the subsequent NA-induced tension in Ca^{2+} -free KS (Fig. 11(B), 12). These results suggest that the amount of Ca^{2+} accumulated into SR due to high K⁺-induced Ca^{2+} influx, if any, is not sufficient to account for the enhanced contractile response to NA in the presence of high K⁺. Only the

combination of high K^+ and NA together can induce a significantly enhanced response (Fig. 11(C), 12).

5.2. Proposed model of two intracellular Ca^{2+} store in guinea pig aorta

Based on our data we propose that there are two intracellular Ca^{2+} stores in guinea-pig aortic smooth muscle cells: a NA/CAF-sensitive one, and a voltage-dependent NA-sensitive (VDNAS) one (Fig. 19). In the VDNAS store, voltage-sensitive 'gates' (VSG) cover or mask the Ca^{2+} release sites or IP_3 receptors. These 'gates' are voltage- and time-dependent. Depolarization activates or opens these 'gates', exposing the IP_3 receptors with high sensitivity to IP_3 or exposing Ca^{2+} releasing sites highly sensitive to other metabolites of the phosphatidylinositol cascade generated by agonist. In the absence of extracellular Ca^{2+} and agonist, the opening of the 'gates' by depolarization may cause a leakage of Ca^{2+} from the store, resulting in an increase in $[Ca^{2+}]_i$ insufficient to elicit contraction (Kobayashi et al, 1985, 1986). The long delay between depolarization and the rise in $[Ca^{2+}]_i$ (Ganitkevich and Isenberg, 1993, 1996) may not be attributed to the IP_3 formation process containing a voltage-sensitive step (Vergara et al, 1985), but to the time-dependent activation of the 'gates'.

Electron microscopy studies (Somlyo and Franzini-Armstrong, 1985) reveal that the sarcoplasmic reticulum (SR) tubules at the periphery of a vascular smooth muscle cell frequently establish close couplings with the surface membrane. The junctional gap between the two membranes at close coupling sites is approximately 10 ~ 20 nm with periodic 'bridging structures' crossing the gap resembling the surface couplings of

Figure 19. A proposed two intracellular Ca^{2+} stores model for smooth muscle cells of the guinea-pig aorta. Noradrenaline (NA) or caffeine (CAF) releases Ca^{2+} from the intracellular NA/CAF-sensitive Ca^{2+} store. Membrane depolarization activates or opens the voltage-sensitive 'gates' (VSG) of the voltage-dependent NA-sensitive (VDNAS) Ca^{2+} store, exposing the Ca^{2+} releasing sites (RS) for IP_3 or other phosphatidylinositol metabolites generated by NA. R = receptor, VOC = voltage-operated Ca^{2+} channel, ROC = receptor-operated Ca^{2+} channel, G=GTP-binding protein, PLC = phospholipase C, RYA = ryanodine receptor, ECS = extracellular space, M = membrane.

cardiac and the triads of skeletal muscle. Therefore, depolarization of the plasma membrane by high K^+ may be conducted to the plasma membrane junction and thus lead to depolarization of the SR. This in turn would activate the voltage-sensitive 'gates' of the VDNAS store.

5.3. Ca^{2+} recycling into SR or Ca^{2+} accumulating at cell membrane?

In the absence of high K^+ , the attenuated contractions in response to repeated NA stimulations in Ca^{2+} -free KS (Fig. 13(A)) may be caused by NA-stimulated Ca^{2+} efflux (Deth and Casteels, 1977; Leijten and van Breemen, 1986) and an ineffective Ca^{2+} recycling mechanism. In the presence of high K^+ (105 mM) (Fig. 13(B)) the maintained responses to NA suggest that high K^+ depolarization not only potentiates contraction but also facilitates Ca^{2+} recycling into the VDNAS store. This idea is supported by the fact that after the tissue had been treated with CPA, high K^+ and NA together induced response was greatly reduced (Fig. 14B(c)). This depressed tension is likely attributable to the failure of the VDNAS store to recycle the released Ca^{2+} induced by the preceding NA stimulation (b). These results imply that the maintained contractions attributed to the VDNAS store (Fig. 13) are due to recycling of the released Ca^{2+} by the SR Ca^{2+} -ATPase.

It can be argued that without the Ca^{2+} chelator EGTA in the bathing solution, Ca^{2+} efflux could have accumulated and elevated $[Ca^{2+}]$ in the extracellular space (ECS); the maintained contractions could be mainly attributed to Ca^{2+} influx from the ECS via the VOCs and the ROCs. Therefore, in the presence of EGTA the Ca^{2+} influx could be eliminated resulting in the depressed responses to the subsequent NA stimulations (Fig.

15). This concept is not supported by the following observations: (1) prior to each NA stimulation, no contraction was observed during the wash/incubation period in high KCl Ca^{2+} -free KS without EGTA, indicating no or insignificant Ca^{2+} influx via VOC; (2) as CPA inhibits Ca^{2+} recycling by the store, all released Ca^{2+} induced by NA would be extruded to, and accumulated in the ECS. During wash/incubation period in high KCl Ca^{2+} -free KS prior to the subsequent NA stimulation (Fig. 14B(c)), significant contraction due to Ca^{2+} influx via VOC should be observed. No such contraction was observed.

It is possible that K^+ -depolarization could release membrane bound Ca^{2+} into the cytosol via the VOC. This Ca^{2+} influx, insufficient to elicit contraction during wash/incubation period in high KCl Ca^{2+} -free KS, together with Ca^{2+} released by NA could trigger a Ca^{2+} -induced- Ca^{2+} -release (CICR) mechanism to release Ca^{2+} from a Ca^{2+} sensitive compartment (CaSC) of the NA/CAF-sensitive store. However, such a possibility may be ruled out for the following reasons:

(1) As high KCl reduces IP_3 production (Itoh et al, 1992), and the NA/CAF-sensitive store is apparently depleted, the Ca^{2+} content of the CaSC has to be exceedingly large to account for a 4-fold increase in NA-induced contraction in 105 mM KCl Ca^{2+} -free KS (Fig. 10).

(2) CICR channels can be activated by CAF (Liu et al, 1989). If the Ca^{2+} content of the CaSC is large, CAF should release sufficient Ca^{2+} from the store and elicit significant tension even though the NA-sensitive portion of the NA/CAF-sensitive store is depleted. However, it was observed that after a NA-induced contraction in the Ca^{2+} -

free KS subsequent CAF stimulation failed to elicit significant tension (Fig. 7B). In addition, CAF failed to induce tension in the 105 mM KCl Ca^{2+} -free KS after the NA/CAF-sensitive store had been depleted by CAF (Fig. 9(B)). Therefore, the membrane bound Ca^{2+} is unlikely the factor that enhances the NA-induced contraction.

5.4. Suppressed responses in the presence of EGTA

High K^+ enhanced NA-induced contractions in Ca^{2+} -free KS in both absence (Fig. 13) and presence (Fig. 15) of EGTA. However, contractile responses in the presence of EGTA were smaller. The underlying mechanisms for this suppression remain unclear. EGTA is relatively impermeable, and should have had no effect on either $[\text{Ca}^{2+}]_i$ or the Ca^{2+} store. However, studies on vascular smooth muscle cells have shown that EGTA lowers the basal cytosolic Ca^{2+} level (Kobayashi et al, 1985), and induces transient Ca^{2+} efflux (Deth and Lynch, 1981). Long duration incubation with EGTA could destabilize the cell membrane and deplete the intracellular Ca^{2+} stores, resulting in attenuated contractile responses to an agonist (Guan et al, 1988). In the experiments described herein EGTA could have accelerated the depletion of the NA/CAF-sensitive store prior to the second NA stimulation. High KCl could activate or open the voltage-sensitive 'gates' to expose the Ca^{2+} -releasing sites of the VDNAS store. Consequently EGTA could chelate the Ca^{2+} leaking through these releasing sites and therefore deplete the Ca^{2+} content of the store. Recent studies showed that in human duodenum Ca^{2+} -EGTA complex inhibits adenylate cyclase activity (Smith et al, 1993), and in airway epithelial cells, unbound form of EGTA may interfere with calcium signaling through inhibition of Ca^{2+} release

from the intracellular store (Harris and Hanrahan, 1994). These mechanisms may be possibly involved in the reduced contractile responses of EGTA-treated rings to NA.

5.5. Effect of pinacidil

The curve of NA-induced relative tension vs. [KCl] in the presence of 20 μ M pinacidil shifts to the right of that in the absence of pinacidil (Fig. 16B), indicating that pinacidil inhibits NA responses. At low [KCl] (4.6 mM) the reduced NA contraction may be attributed to an inhibitory effect of pinacidil on IP_3 production (Itoh et al, 1992), whereas at moderate to high [KCl] (45 ~ 85 mM), the depressed relative tension are unlikely to be due to the reduction in IP_3 production or decreased sensitivity of contractile protein to Ca^{2+} because of the following observations in the study of Itoh et al. (1992):

(1) At [KCl] of 40 ~ 128 mM the [IP_3] produced by 10 μ M NA in the presence or absence of 1 μ M pinacidil was unchanged;

(2) Caffeine-induced tension and [Ca^{2+}]_i were not affected by pinacidil, suggesting that pinacidil has no effect on the sensitivity of contractile protein to Ca^{2+} ;

(3) Pinacidil of 0.1 ~ 10 μ M has no effect on the concentrations of cyclic AMP and cyclic GMP which are reputed to inhibit the synthesis of IP_3 in vascular tissues (Kajikuri and Kuriyama, 1990).

Inhibitory effect of pinacidil on the NA-induced tensions may have been due to hyperpolarization of the VDNAS store. It is known that pinacidil hyperpolarizes cell membrane by activation of ATP-sensitive and [Ca^{2+}]_i-insensitive K^+ channels in smooth muscle cells of rabbit mesenteric artery (Quast and Cook, 1989; Standen et al, 1989). The

hyperpolarizing action of pinacidil persists in low Na^+ Krebs solution in this tissue (Nakashima et al, 1990). In the VDNAS store the activation of the proposed 'voltage sensitive gate' depends on membrane depolarization. The hyperpolarizing action of pinacidil tends to reduce the depolarizing effect of KCl, thus decreasing the activation of the 'voltage sensitive gate'. Consequently a smaller number of Ca^{2+} releasing sites to IP_3 or other NA-generated second messengers are exposed or unmasked, resulting in a reduction of Ca^{2+} release from the VDNAS store and thus a decrease in tension.

It is also plausible that any membrane hyperpolarization induced by pinacidil activates the Ca^{2+} extrusion mechanism mediated by $\text{Na}^+/\text{Ca}^{2+}$ exchange through activation of K^+ efflux, and thus causes vasodilation. However, since pinacidil inhibits the NA-induced contraction in low Na^+ solution more potently than in Krebs solution (Itoh et al, 1992), this mechanism can not play a major role.

5.6. Effect of K^+ on Ang II-induced contraction

Ang II induces contractions in guinea pig aortic rings that exhibit tachyphylaxis (Fig. 17), which has been reported by some (St-Louis et al, 1977; Oshiro, 1984), but not all (Lalanne et al, 1984) authors. The mechanisms underlying this tachyphylaxis are not clear. However, rapid ligand-receptor internalization has been proposed as one possible explanation (Griendling et al, 1987; Auguet et al, 1991).

After the NA/CAF-sensitive store has been depleted by NA, Ang II-induced contractions were enhanced by KCl. The Ang II-induced contractions are not as large as the NA-induced ones in the presence of $[\text{KCl}]_0$ that is higher than 50 mM. Such

enhancement reached maximal level at about 50 mM KCl. According to the proposed VDNAS store model, high KCl should activate the voltage sensitive gates (VSG) and expose the Ca^{2+} releasing sites to IP_3 or other second messengers generated by Ang II. The mechanism underlying the reduced contractions (relative to the NA-induced ones) attributed to VDNAS store remains unknown. The follows are possible explanations for this:

(a) The suggested rapid ligand-receptor complex internalization may limit the amount of IP_3 generated by Ang II, thus limiting the amount of Ca^{2+} released from the VDNAS store;

(b) Besides IP_3 , Ang II also generates a metabolite which modulates or partially inhibits the activation of the VSG. Consequently less number of Ca^{2+} releasing sites are exposed;

(c) The Ca^{2+} releasing sites are not only sensitive to IP_3 but also sensitive to certain second messenger generated by NA but not Ang II.

Based on the results of this study, NA appears to be a more potent vasoconstrictor of guinea pig aortic smooth muscle than Ang II under the conditions of the experimental methods described above.

6. Summary

This study investigated the effects of high K^+ -induced depolarization on the contractile responses of guinea pig aortic smooth muscle to noradrenaline (NA) and caffeine (CAF) in Ca^{2+} -free Krebs solution. NA (1 μ M) or CAF (10 mM) in regular Ca^{2+} -free KS induced a (termed 1st) phasic contraction, but responses to a second NA or CAF stimulation were significantly reduced, suggesting the depletion of a common intracellular (NA/CAF-sensitive) store previously reported by investigators. After the depletion of this store: (1) neither 105 mM KCl Ca^{2+} free KS nor 10 mM CAF in a high K^+ solution induced contraction; (2) NA-induced contractions were enhanced by KCl, increasing in a dose-dependent manner ([KCl] ranging from 25 to 105 mM); (3) in 105 mM KCl Ca^{2+} -free Krebs solution repetitive NA stimulations elicited enhanced and non-decreasing responses equal to or greater than the 1st contraction, suggesting an efficient recycling of intracellular Ca^{2+} . In the presence of 10 μ M cyclopiazonic acid (CPA) NA-induced contraction was enhanced. However the response to a subsequent NA stimulation in the absence of CPA was greatly reduced, implying an inhibition of the Ca^{2+} recycling function; (4) 2 mM EGTA attenuated the NA-induced contractions in regular and 105 mM KCl Ca^{2+} free KS; (5) 20 μ M pinacidil inhibited 1 μ M NA-induced tension in Ca^{2+} -free KS with KCl ranging from 5 ~ 85 mM; (6) as NA, 10^{-7} M Ang II-induced tension in

Ca^{2+} -free KS was enhanced by KCl (5 ~ 105 mM), but the enhancement was less than those of NA-induced tension at relative high concentrations of K^+ (85 ~ 105 mM). Based on the above observations it is proposed that there are two intracellular Ca^{2+} stores in smooth muscle cells of guinea-pig aorta: (1) a NA/CAF-sensitive store and (2) a voltage-dependent NA-sensitive store which has effective Ca^{2+} recycling capabilities.

7. Future Directions

In order to characterize the existence of a VDNAS Ca^{2+} store in guinea pig aortic smooth muscle cells, experiments should be conducted on isolated myocytes. In such experiments, cytosolic Ca^{2+} concentration would have to be measured with the aid of a Ca^{2+} -sensitive dye such as fura-2. Depolarization of membrane potential would have to be controlled by the patch-clamp technique (Ganitkevich and Isenberg, 1993, 1996) and IP_3 production should be measured (Itoh et al, 1992). To test whether the Ca^{2+} releasing sites of the VDNAS stores are sensitive to IP_3 or other second messengers, caged IP_3 should be used. In addition, the existence of a VDNAS store should be explored in the vascular smooth muscle cells of different species.

8. Appendix

The permeability of smooth muscle cells to Na^+ is relatively large, therefore the cell membrane potentials (MP) of guinea pig aortic smooth muscles were computed with the Goldman-Hodgkin-Katz equation instead of the Nernst equation, which is simplified as:

$$\text{MP} = -61 \cdot \log \frac{K_i + (P_{\text{Na}} / P_K) \cdot \text{Na}_i}{K_o + (P_{\text{Na}} / P_K) \cdot \text{Na}_o}$$

where MP is the membrane potential, K_i and K_o are intracellular and extracellular K^+ concentrations, Na_i and Na_o are intracellular and extracellular Na^+ concentrations, and (P_{Na} / P_K) is the ratio of Na^+ permeability to K^+ permeability.

The measured MP- K_o relationship of guinea pig abdominal aortic smooth muscle cells (Kajiwara 1982) was curve fitted by a polynomial. After the coefficients of this polynomial were determined by the Least Square method, the values of $[K_i + (P_{\text{Na}} / P_K) \cdot \text{Na}_i]$ and (P_{Na} / P_K) were estimated. The MPs of the guinea pig thoracic aortas in varying concentrations of K_o and Na_o were calculated.

9. References

- Adam LP and Hathaway DR (1993). Identification of mitogen-activated protein kinase phosphorylation sequences in mammalian h-caldesmon. *FEBS Lett.*, 322: 56-60.
- Alessi D, MacDougall LK, Sola MM, Ikebe M and Cohen P (1992). The control of protein phosphatase-1 by targeting subunits. The major myosin phosphatase in avian smooth muscle is a novel form of protein phosphatase-1. *Eur. J. Biochem.*, 210: 1023-1035.
- Alexander RW, Brock TA, Gimbrone MA Jr and Rittenhouse SE (1985). Angiotensin increases inositol trisphosphate and calcium in vascular smooth muscle. *Hypertension*, 7: 447-451.
- Auguet M, Delaflotte S, Guillon JM, Chabrier PE and Braquet P (1991). Different regulation of vascular tone by angiotensin II and endothelin-1 in rat aorta. *Eur. J. Pharmacol.*, 196: 21-27.
- Barö I and Eisner DA (1995). Factors controlling changes in intracellular Ca^{2+} concentration produced by noradrenaline in rat mesenteric artery smooth muscle cells. *J. Physiol.*, 482: 247-258.
- Berridge MJ (1993). Inositol trisphosphate and calcium signaling. *Nature*, 361: 315-325.
- Berridge MJ and Irvine RF (1984). Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature*, 312: 315-321.
- Bianchi CP (1962). Kinetics of radiocaffeine uptake and release in frog sartorius. *J Pharmacol. Exp. Ther.*, 138: 41- 47.
- Bolton TB (1979). Mechanisms of action of transmitters and other substances on smooth muscle. *Physiol. Rev.*, 59: 606-718.
- Bond M, Kitazawa T, Somlyo AP and Somlyo AV (1984). Release and recycling of calcium by the sarcoplasmic reticulum in guinea-pig portal vein smooth muscle. *J. Physiol.*, 355: 677-695.

- Bova S, Goldman WF, Yuan XJ and Blaustein MP (1990). Influence of Na^+ gradient on Ca^{2+} transients and contraction in vascular smooth muscle. *Am. J. Physiol.*, 259: H409- H423.
- Brock TA, Alexander RW, Ekstein LS, Atkinson WJ and Gimbrone MA Jr (1985). Angiotensin increases cytosolic free calcium in cultured vascular smooth muscle cells. *Hypertension*, 7 (suppl. I): I105-I109.
- Bulbring E and Tomita T (1987). Catecholamine action on smooth muscle. *Pharmacol. Rev.*, 39: 49-96.
- Carsten ME (1969). Role of calcium binding by sarcoplasmic reticulum in the contraction and relaxation of uterine smooth muscle. *J. Gen. Physiol.*, 53: 414-426.
- Cauvin C, Loutzenhiser R and van Breemen C (1983). Mechanisms of calcium antagonist-induced vasodilatation. *Annu. Rev. Pharmacol. Toxicol.*, 23: 373-396.
- Chamley-Campbell J, Campbell GR and Ross R (1979). The smooth muscle cell in culture. *Physiol. Rev.*, 59: 1-61.
- Collins EM, Walsh MP and Morgan KG (1992). Contraction of single vascular smooth muscle cells by phenylephrine at constant $[\text{Ca}^{2+}]_i$. *Am. J. Physiol.*, 262: H754-H762.
- Dabrowska R, Sherry JM, Aromatorio DK and Hartshorne DJ (1978). Modulator protein as a component of the myosin light chain kinase from chicken gizzard. *Biochemistry*, 17: 253-258.
- Defeo TT and Morgan KG (1985). Calcium-force relationships as detected with aequorin in two different vascular smooth muscles of the ferret. *J. Physiol.*, 369:269-282.
- Deth R and Casteels R (1977). A study of releasable Ca fractions in smooth muscle cells of the rabbit aorta. *J. Gen. Physiol.*, 69: 401-416.
- Deth R and van Breemen C (1977). Agonist induced release of intracellular Ca^{2+} in the rabbit aorta. *J. Memb. Biol.*, 30: 363-380.
- Deth RC and Lynch CJ (1981). Mobilization of a common source of smooth muscle Ca^{2+} by norepinephrine and methylxanthines. *Am. J. Physiol.*, 240: C239-C247.
- Devine CE, Somlyo AV and Somlyo AP (1972). Sarcoplasmic reticulum and excitation-contraction coupling in mammalian smooth muscles. *J. Cell Biol.*, 52: 690-718.

- Docherty JR, Constantine JW and Starke K (1981). Smooth muscle of rabbit aorta contains α_1 - but not α_2 -adrenoceptors. *Arch. Pharmacol.*, 317: 5 - 7.
- Ehrlich BE and Watras J (1988). Inositol 1,4,5-trisphosphate activates a channel from smooth muscle sarcoplasmic reticulum. *Nature* 336: 583-586.
- Ehrlich BE, Kaftan E, Bezprozvannaya S and Bezprozvanny I (1994). The pharmacology of intracellular Ca^{2+} -release channels. *Trends Pharmacol. Sci.*, 15: 145-149.
- Eilertsen E, Hart JW, Magnussen MP, Sorensen H and Arrigoni-Mortelli E (1982). Pharmacokinetics and distribution of the new antihypertensive agent pinacidil in rat, dog and man. *Xenobiotica*, 12: 177-185.
- Filo RS, Bohr DF and Ruegg JC (1965). Glycerinated skeletal and smooth muscle: calcium and magnesium dependence. *Science*, 147:1581-1583.
- Fitzpatrick DF, Landon EJ, Debbas G and Hurwitz L (1972). A calcium pump in vascular smooth muscle. *Science*, 176: 305-306.
- Forssmann WG and Girardier L (1970). A study of the T system in rat heart. *J. Cell Biol.*, 44: 1-19.
- Fox AW, Abel PW and Minneman KP (1985). Activation of α_1 -adrenoceptors increases ^3H -inositol metabolism in rat vas deferens and caudal artery. *Eur. J. Pharmacol.*, 116: 145-152.
- Frank GB (1960). Effects of changes in extracellular calcium concentration on the potassium-induced contracture of frog's skeletal muscle. *J. Physiol.*, 151: 518-538.
- Ganitkevich VYa and Isenberg G (1993). Membrane potential modulates inositol 1,4,5-trisphosphate-mediated Ca^{2+} -transients in guinea-pig coronary myocytes. *J. Physiol.*, 470: 35-44.
- Ganitkevich VY and Isenberg G (1996). Effect of membrane potential on the initiation of acetylcholine-induced Ca^{2+} transients in isolated guinea pig coronary myocytes. *Circ Res.*, 78: 717-723.
- Gillespie JI, Otun H, Greenwell JR and Dunlop W (1992a) Evidence for Na^+ - Ca^{2+} exchange and Ca^{2+} -induced Ca^{2+} release in a cultured vascular smooth muscle cell line from the rat. *Exp. Physiol.*, 77: 141-152.
- Gillespie JI, Otun H, Greenwell JR and Dunlop W (1992b). The effect of $[\text{Na}^+]_i$ and $[\text{Na}^+]_o$ on Ca^{2+} mobilization in the rat aortic smooth muscle cell line A7r5. *Exp. Physiol.*, 77: 627-635.

- Gonzalez De-La-Fuente-P, Savineau JP and Marthan R (1995). Control of pulmonary vascular smooth muscle tone by sarcoplasmic reticulum Ca^{2+} pump blockers: thapsigargin and cyclopiazonic acid. *Pflügers Arch.*, 429:617-624.
- Gong MC, Fuglsang A, Alessi D, Kobayashi S, Cohen P, Somlyo AV and Somlyo AP (1992). Arachidonic acid inhibits myosin light chain phosphatase and sensitizes smooth muscle to calcium. *J. Biol. Chem.*, 267: 21492-21498.
- Griendling KK, Delafontaine P, Rittenhouse SE, Gimbrone MA Jr and Alexander RW (1987). Correlation of receptor sequestration with sustained diacylglycerol accumulation in angiotensin II-stimulated cultured vascular smooth muscle cells. *J. Biol. Chem.*, 262: 14555-14562.
- Griendling KK, Sastre A and Milnor WR (1984). Regional differences in α_1 -adrenoceptor numbers and responses in canine aorta. *Am. J. Physiol.*, 247: H928-H935.
- Guan YY, Kwan CY and Daniel EE (1988) The effects of EGTA on vascular smooth muscle contractility in calcium-free medium. *Can. J. Physiol. Pharmacol.*, 66: 1053-1056
- Guyton AC (1991) *Textbook of Medical Physiology*. Eighth Edition, Philadelphia, PA. W.B.Saunders Company. Page 88.
- Harris RA and Hanrahan JW (1994). Effects of EGTA on calcium signaling in airway epithelial cells. *Am. J. Physiol.*, 267: C1426-1434.
- Hathaway DR, March KL, Lash JA, Adam LP and Wilensky RL (1991). Vascular smooth muscle. A review of the molecular basis of contractility. *Circulation*, 83: 382-390.
- Herrmann-Frank A, Darling E and Meissner G (1991). Functional characterization of the Ca^{2+} -gated Ca^{2+} release channel of vascular smooth muscle sarcoplasmic reticulum. *Pflügers Arch.*, 418:353-359.
- Himpens B, Kitazawa T and Somlyo AP. (1990). Agonist-dependent modulation of Ca^{2+} sensitivity in rabbit pulmonary artery smooth muscle. *Pflügers Arch.*, 417: 21-28.
- Hurwitz L (1986). Pharmacology of calcium channels and smooth muscle. *Annu. Rev. Pharmacol. Toxicol.* 26: 225-258.
- Hwang KS and van Breemen C (1987). Ryanodine modulation of ^{45}Ca efflux and tension in rabbit aortic smooth muscle. *Pflügers Arch.*, 408: 343-350.
- Itoh H and Lederis K (1987). Contraction of rat thoracic aorta strips induced by phorbol 12-myristate 13-acetate. *Am. J. Physiol.* 252: C244-C247.

- Itoh T, Kajiwarara M, Kitamura K and Kuriyama H (1982). Roles of stored calcium on the mechanical response evoked in smooth muscle cells of the porcine coronary artery. *J. Physiol.*, 322: 107-125.
- Itoh T, Kuriyama H and Suzuki H (1983). Differences and similarities in the noradrenaline- and caffeine- induced mechanical responses in the rabbit mesenteric artery. *J. Physiol.*, 337: 609-629.
- Itoh T, Seki N, Suzuki S, Ito S, Kajikuri J and Kuriyama H (1992). Membrane hyperpolarization inhibits agonist-induced synthesis of inositol 1,4,5-trisphosphate in rabbit mesenteric artery. *J. Physiol.*, 451: 307-328.
- Jiang MJ and Morgan KG (1989). Agonist-specific myosin phosphorylation and intracellular calcium during isometric contractions of arterial smooth muscle. *Pflügers Arch.*, 413: 637-643.
- Jiang MJ and Morgan KG (1987). Intracellular calcium levels in phorbol ester-induced contractions of vascular muscle. *Am. J. Physiol.*, 253: H1365-H1371.
- Jundt H, Porzig H, Reuter H and Stucki JW (1975). The effect of substances releasing intracellular calcium ions on sodium-dependent calcium efflux from guinea-pig auricles. *J. Physiol.*, 246: 229-253.
- Kærgaard Nielsen Chr and Arrigoni-Martelli E (1981). Effect of a new vasodilator, pinacidil (P1134), on potassium, noradrenaline and serotonin induced contractions in rabbit vascular tissues. *Acta Pharmacol Toxicol.*, 49: 427-431.
- Kajikuri J and Kuriyama H (1990). Inhibitory action of α -human atrial natriuretic peptide on noradrenaline-induced synthesis of myo-inositol 1,4,5-trisphosphate in the smooth muscle cells of rabbit aorta. *Br. J. Pharmacol.*, 99: 536-540.
- Kajiwarara M (1982). General features of electrical and mechanical properties of smooth muscle cells in the guinea-pig abdominal aorta. *Pflügers Arch.*, 393: 109-117.
- Karaki H and Weiss GB (1988). Calcium release in smooth muscle. *Life Sciences*, 42: 111-122.
- Kauffman RF, Schenck KW, Conery BG and Cohen ML (1986). Effects of pinacidil on serotonin-induced contractions and cyclic nucleotide levels in isolated rat aortae: comparison with nitroglycerin, minoxidil and hydralazin. *J. Cardiovasc. Pharmacol.*, 8: 1195-1200.

- Kitazawa T and Somlyo AP (1990). Desensitization and muscarinic re-sensitization of force and myosin light chain phosphorylation to cytoplasmic Ca^{2+} in smooth muscle. *Biochem. Biophys. Res. Commun.*, 172: 1291-1297.
- Kitazawa T, Gaylinn BD, Denney GH and Somlyo AP (1991a). G-protein-mediated Ca^{2+} sensitization of smooth muscle contraction through myosin light chain phosphorylation. *J. Biol. Chem.*, 266: 1708-1715.
- Kitazawa T, Kobayashi S, Horiuti K, Somlyo AV and Somlyo AP (1989). Receptor-coupled, permeabilized smooth muscle. Role of the phosphatidylinositol cascade, G-proteins, and modulation of the contractile response to Ca^{2+} . *J. Biol. Chem.*, 264: 5339-5342.
- Kitazawa T, Masuo M and Somlyo AP (1991b). G protein-mediated inhibition of myosin light-chain phosphatase in vascular smooth muscle. *Proc. Natl. Acad. Sci.* 88: 9307-9310.
- Kobayashi S, Kanaida H and Nakamura M (1985). K^{+} -depolarization induces a direct release of Ca^{2+} from intracellular storage sites in cultured vascular smooth muscle cells from rat aorta. *Biochem. Biophys. Res. Commun.*, 129: 877-884.
- Kobayashi S, Kanaida H and Nakamura M (1986). Complete overlap of caffeine- and K^{+} depolarization-sensitive intracellular calcium storage sites in cultured rat arterial smooth muscle cells. *J. Biol. Chem.*, 261: 15709-15713.
- Kowarski D, Shuman H, Somlyo AP and Somlyo AV (1985). Calcium release by noradrenaline from central sarcoplasmic reticulum in rabbit main pulmonary artery smooth muscle. *J. Physiol.*, 366: 153-175.
- Kubota Y, Nomura M, Kamm KE, Mumby MC and Stull JT (1992). GTP- γ S-dependent regulation of smooth muscle contractile elements. *Am. J. Physiol.*, 262: C405-C410.
- Kuriyama H, Ito Y, Suzuki H, Kitamura K and Itoh T (1982). Factors modifying contraction-relaxation cycle in vascular smooth muscles. *Am. J. Physiol.* 243: H641-H662.
- Lalanne C, Mironneau C, Mironneau J and Savineau JP (1984). Contractions of rat uterine smooth muscle induced by acetylcholine and angiotensin II in Ca^{2+} -free medium. *Br. J. Pharmacol.*, 81: 317-326.
- Legan E, Chernow B, Parrillo J and Roth BL (1985). Activation of phosphatidylinositol turnover in rat aorta by α_1 -adrenergic receptors stimulation. *Eur. J. Pharmacol.*, 110: 389-390.

- Leijten PAA and van Breemen C (1984). The effects of caffeine on the noradrenaline sensitive calcium store in rabbit aorta. *J. Physiol.*, 357: 327-339.
- Lincoln TM and Fisher-Simpson V (1984). A comparison of the effects of forskolin and nitroprusside on cyclic nucleotides and relaxation in the rat aorta. *Eur. J. Pharmacol.*, 101: 17-27.
- Liu QY, Lai A, Rousseau E, Jones RV and Meissner G (1989). Multiple conductance states of the purified calcium release channel complex from skeletal sarcoplasmic reticulum. *Biophys. J.*, 55: 415-424.
- Michell RH (1975). Inositol phospholipids and cell surface receptor function. *Biochim. Biophys. Acta.*, 415: 81-147.
- Mikkelsen E and Pedersen OL (1982). Comparison of the effects of a new vasodilator pinacidil and nifedipine on isolated blood vessels. *Acta Pharmacol. Toxicol.*, 51: 407-412.
- Missiaen L, De Smedt H, Droogmans G, Himpens B and Casteels R (1992). Calcium ion homeostasis in smooth muscle. *Pharmacol. Ther.*, 56: 191-231.
- Mueller E and van Breemen C (1979). Role of intracellular Ca^{2+} sequestration in β -adrenergic relaxation of a smooth muscle. *Nature*, 281: 682-683.
- Mullins LJ and Brinley FJ Jr (1975). Sensitivity of calcium efflux from squid axon to changes in membrane potential. *J. Gen. Physiol.*, 65: 135-152.
- Nakashima M, Li Y, Seki N and Kuriyama H (1990). Pinacidil inhibits neuromuscular transmission indirectly in the guinea-pig and rabbit mesenteric arteries. *Br. J. Pharmacol.*, 101: 581-586.
- Nelson MT, Patlak JB, Worley JF and Standen NB (1990). Calcium channels, potassium channels and voltage dependence of arterial smooth muscle tone. *Am. J. Physiol.*, 259: C3-C18.
- Nishimura J, Kolber M and van Breemen C (1988). Norepinephrine and GTP- γ -S increase myofilament Ca^{2+} sensitivity in α -toxin permeabilized arterial smooth muscle. *Biochem. Biophys. Res. Commun.*, 157: 677-683.
- Noguera MA and D'Ocon MP (1992). Different and common intracellular calcium-stores mobilized by noradrenaline and caffeine in vascular smooth muscle. *Naunyn-Schmiedeberg's Arch Pharmacol.*, 345: 333-341.

- Olson NJ, Pearson RB, Needleman DS, Hurwitz MY, Kemp BE and Means AR (1990). Regulatory and structural motifs of chicken gizzard myosin light chain kinase. *Proc. Natl. Acad. Sci.*, 87: 2284-2288.
- Oshiro ME, Miasiro N, Paiva TB and Paiva AC (1984). Angiotensin tachyphylaxis in the isolated rabbit aorta. *Blood vessels*, 21: 72-79.
- Petersen HJ, Nielsen CK, Arrigoni-Martelli E (1978). Synthesis and hypotensive activity of N-alkyl-N''-cyano-N'-pyridylguanidines. *J. Med. Chem.*, 21: 773-781.
- Polonia JJ, Paiva MQ and Guimaraes S (1985). Pharmacological characterization of postsynaptic 4-adrenoceptor subtypes in five different dog arteries in-vitro. *J. Pharm. Pharmacol.*, 37:205-208.
- Popescu LM, Panoiu C, Hinescu M and Nutu O (1985). The mechanism of cGMP-induced relaxation in vascular smooth muscle. *Eur. J. Pharmacol.*, 107: 393-394.
- Quast U and Cook NS (1989). Moving together: K⁺ channel openers and ATP-sensitive K⁺ channels. *Trends Pharmacol. Sci.*, 10: 431-435.
- Ruffolo RR and Waddell JE (1982). Receptor interactions of imidazolines: α -adrenoceptors of rat and rabbit aortae differentiated by relative potencies, affinities, and efficacies of imidazole agonists. *Br. J. Pharmacol.*, 77: 169-176.
- Ruffolo RR, Waddell JE and Yaden EL (1982). Heterogeneity of postsynaptic α adrenergic receptors in mammalian aortas. *J. Pharmacol. Exp. Ther.* 221: 309-314.
- Saida K (1982). Intracellular Ca release in skinned smooth muscle. *J. Gen. Physiol.*, 80: 191-202.
- Saida K and van Breemen C (1984). Characteristics of the norepinephrine-sensitive Ca store in vascular smooth muscle., *Blood vessels*, 21: 43-52.
- Shimizu H, Ito M, Miyahara M, Ichikawa K, Okubo S, Konishi T, Naka M, Tanaka T, Hirano K, Hartshorne DJ and Nakano T. (1994). Characterization of the myosin-binding subunit of smooth muscle myosin phosphatase. *J. Biol. Chem.*, 269: 30407-30411.
- Shirazi A, Iizuka K, Fadden P, Mosse C, Somlyo AP, Somlyo AV and Haystead TAJ (1994). Purification and characterization of the mammalian myosin light chain phosphatase holoenzyme. The differential effects of the holoenzyme and its subunits on smooth muscle. *J. Biol. Chem.*, 269: 31598-31606.

- Singer HA and Baker KM (1987). Calcium dependence of phorbol 12, 13-dibutyrate-induced force and myosin light chain phosphorylation in arterial smooth muscle. *J. Pharmacol. Exp. Ther.*, 243: 814-821.
- Smith JB, Zheng T and Smith L (1989). Relationship between cytosolic free Ca^{2+} and Na^{+} - Ca^{2+} exchange in aortic muscle cells. *Am. J. Physiol.*, 256: C147-C154.
- Somlyo AP and Himpens B (1989). Cell calcium and its regulation in smooth muscle. *FASEB J.*, 3: 2266-2276.
- Somlyo AP and Somlyo AV (1994). Signal transduction and regulation in smooth muscle. *Nature*, 372: 231-236
- Somlyo AP, Devine CE, Somlyo AV and North SR (1971). Sarcoplasmic reticulum and the temperature-dependent contraction of smooth muscle in calcium-free solutions. *J. Cell Biol.*, 51: 722-741.
- Somlyo AV and Franzini-Armstrong C (1985). New views of smooth muscle structure using freezing, deep-etching, and rotary shadowing. *Experientia*, 41: 841-856.
- Southerton JS, Weston AH, Bray KM, Newgreen DT and Taylor SG (1988). The potassium channel opening action of pinacidil; studies using biochemical, ion flux and microelectrode techniques. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, 338: 310-318.
- St-Louis J, Regoli D, Barabe J, Park WK (1977). Myotropic actions of angiotensin and noradrenaline in strips of rabbit aortae. *Can. J. Physiol. Pharmacol.*, 55: 1056-1069.
- Standen NB, Quayle JM, Davies NW, Brayden JE, Huang Y and Nelson MT (1989). Hyperpolarizing vasodilators activate ATP-sensitive K^{+} channels in arterial smooth muscle. *Science*, 245: 177-180.
- Stiles GL, Caron MG and Lefkowitz RJ (1984). β -adrenergic receptors: biochemical mechanisms of physiological regulation. *Physiol. Rev.*, 64: 661-743.
- Stull JT, Hsu LC, Tansey MG and Kamm KE (1990). Myosin light chain kinase phosphorylation in tracheal smooth muscle. *J. Biol. Chem.*, 265: 16683-16690.
- Suematsu E, Hirata M, Hashimoto T and Kuriyama H (1984). Inositol 1,4,5-trisphosphate releases Ca^{2+} from intracellular store sites in skinned single cells of porcine coronary artery. *Biochem. Biophys. Res. Commun.* 120: 481-485.

- Sumida M, Okuda H and Hamada M (1984) Ca^{2+} , Mg^{2+} -ATPase of microsomal membranes from bovine aortic smooth muscle. Identification and characterization of an acid-stable phosphorylated intermediate of the Ca^{2+} , Mg^{2+} -ATPase. *J. Biochem.*, 96:1365-1374.
- Tang DC, Stull JT, Kubota Y and Kamm KE (1992). Regulation of the Ca^{2+} dependence of smooth muscle contraction. *J. Biol. Chem.*, 267: 11839-11845.
- Tansey MG, Word RA, Hidaka H, Singer HA, Schworer CM, Kamm KE and Stull JT (1992). Phosphorylation of myosin light chain kinase by the multifunctional calmodulin-dependent protein kinase II in smooth muscle cells. *J. Biol. Chem.*, 267: 12511-12516.
- Taylor DA, Bowman BF and Stull JT (1989). Cytoplasmic Ca^{2+} is a primary determinant for myosin phosphorylation in smooth muscle cells. *J. Biol. Chem.*, 264: 6207-6213.
- Umekawa H and Hidaka H (1985). Phosphorylation of caldesmon by protein kinase C. *Biochem. Biophys. Res. Commun.*, 132: 56-62.
- Uyama Y, Imaizumi Y and Watanabe M (1992). Effects of cyclopiazonic acid, a novel Ca^{2+} -ATPase inhibitor, on contractile responses in skinned ileal smooth muscle. *Br. J. Pharmacol.*, 106:208-214.
- van Breemen C and Saida K (1989). Cellular mechanisms regulating $[\text{Ca}^{2+}]_i$ smooth muscle. *Annu. Rev. Physiol.*, 51: 315-329.
- van Breemen C, Aaronson P, Loutzenhiser R and Meisner K (1980). Ca^{2+} movements in smooth muscle. *Chest*, 78: 157-165.
- van Breemen C, Chen Q and Laher I (1995). Superficial buffer barrier function of smooth muscle sarcoplasmic reticulum. *Trends Pharmacol. Sci.*, 16: 98-105.
- van Breemen C, Farinas BR, Gerba P and McNaughton ED (1972). Excitation-contraction coupling in rabbit aorta studied by the lanthanum method for measuring cellular calcium influx. *Circ. Res.*, 30: 44-54.
- Vasington FD and Murphy JV (1962). Ca^{2+} uptake by rat kidney mitochondria and its dependence on respiration and phosphorylation. *J. Biol. Chem.*, 237: 2670-2677.
- Vergara J, Tsien RY and Delay M (1985). Inositol 1,4,5-trisphosphate: a possible chemical link in excitation-contraction coupling in muscle. *Proc. Natl. Acad. Sci.*, 82: 6352-6356.

- Vigne P, Breittmayer JP, Duval D, Frelin C and Lazdunski M (1988). The $\text{Na}^+/\text{Ca}^{2+}$ antiporter in aortic smooth muscle cells. Characterization and demonstration of an activation by phorbol esters. *J. Biol. Chem.*, 263: 8078-8083.
- Villalobos-Molina RUcM, Hong E and Garcia-Sainz (1982). A correlation between phosphatidylinositol labeling and contraction in rabbit aorta-effect of α -1 adrenergic activation. *J. Pharmacol. Exp. Ther.*, 222: 258-261.
- Walsh MP (1994). Regulation of vascular smooth muscle tone. *Can. J. Physiol. Pharmacol.*, 72: 919-936.
- Walsh MP, Kargacin GJ, Kendrick-Jones J and Lincoln TM (1995). Intracellular mechanisms involved in the regulation of vascular smooth muscle tone. *Can. J. Physiol. Pharmacol.*, 73: 565-573.
- Williams DA and Fay FS (1986). Calcium transients and resting levels in isolated smooth muscle cells as monitored with quin-2. *Am. J. Physiol.*, 250: C779-C791.
- Winder SJ and Walsh MP (1990). Smooth muscle calponin. Inhibition of actomyosin MgATPase and regulation by phosphorylation. *J. Biol. Chem.*, 265: 10148-10155.
- Winder SJ, Allen BG, Fraser ED, Kang HM, Kargacin GJ and Walsh MP (1993). Calponin phosphorylation in vitro and in intact muscle. *Biochem. J.*, 296: 827-836.
- Wong AYK and Bassingthwaite JB (1981). The kinetics of $\text{Na}^+/\text{Ca}^{2+}$ exchange in excitable tissues. *Math Biosci.*, 53: 275-310.
- Yamamoto H and van Breemen C (1986). Ca^{2+} compartments in saponin-skinned cultured vascular smooth muscle cells. *J. Gen. Physiol.*, 87: 367-389.
- Yanagisawa T and Okada Y (1994). KCl depolarization increases Ca^{2+} sensitivity of contractile elements in coronary arterial smooth muscle. *Am. J. Physiol.*, 267: H614-H621.
- Zhang JS, Van Meel JC, Pfaffendorf M, Zhang J and Van Zwieten PA (1994). Calcium dependency of the AT_1 -receptor mediated effects in the rat portal vein: influence of calcium antagonists. *Naunyn-Schmiedeberg's Arch Pharmacol*, 349: 437-442.
- Zschauer A, Scott-Burden T, Buhler FR and van Breemen C (1987). Vasopressor peptides and depolarization stimulated Ca^{2+} -entry into cultured vascular smooth muscle. *Biochem. Biophys. Res. Commun.*, 148: 225-231.

IMAGE EVALUATION TEST TARGET (QA-3)

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