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**The Caffeine-sensitive Ca^{2+} Store
in Vascular Smooth Muscle**

by

Peter Bernard Alexander

Department of Pharmacology
Faculty of Medicine
University of Ottawa
Ottawa, Ontario, CANADA

A thesis submitted in conformity with the
requirements of the degree of Master of Science
at the University of Ottawa



Peter Bernard Alexander, Ottawa, Canada, 1993



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to my parents

ABSTRACT

The objectives of this study were as follows: 1) to establish a technique to determine $[Ca^{2+}]_i$ in single enzymatically isolated vascular smooth muscle cells (VSMCs) from the rat tail artery by means of the fura-2 fluorescent dye, 2) to study the relationship between VSMC shortening and Ca^{2+} mobilization by caffeine, 3) to determine the mechanism of caffeine-induced Ca^{2+} mobilization by studying the effects of removal of extracellular Ca^{2+} and agents that modulate Ca^{2+} mobilization (ryanodine, TMB-8, nifedipine, thapsigargin), 4) to determine the possible physiological role of the caffeine-sensitive Ca^{2+} store in VSMCs by its interaction with noradrenaline.

The mean resting $[Ca^{2+}]_i$ level in VSMCs used in this study was 44 nM. The mean resting VSMC length was 134 μ m. Cells responded to increasing concentrations of caffeine by both shortening and exhibiting increases in $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ response consisted of two phases: A transient (rapid rise and decay) $[Ca^{2+}]_i$ response increasing in magnitude over the full range of caffeine concentrations, and a tonic (sustained) increase in $[Ca^{2+}]_i$ following the transient $[Ca^{2+}]_i$ response, but observed only at higher caffeine concentrations. Washing enabled $[Ca^{2+}]_i$ to return to pre-challenge levels. Mean transient and tonic $[Ca^{2+}]_i$ increase in response to maximal (10 mM) caffeine was 392 nM and 85 nM respectively. There was a significant correlation between transient and tonic $[Ca^{2+}]_i$ increases in response to caffeine. The threshold $[Ca^{2+}]_i$ level required to effect cell shortening was 53 nM. Half-maximal shortening occurred at 189 nM, with maximal VSMC shortening occurring at a $[Ca^{2+}]_i$ of 326 nM. Single VSMCs did not relax after contraction. However the $[Ca^{2+}]_i$ response to caffeine

was found to be independent of cell length. Repeated application of caffeine in contracted cells effected similar transient and tonic $[Ca^{2+}]_i$ responses to that seen in naive cells.

Following removal of extracellular Ca^{2+} , the resting $[Ca^{2+}]_i$ level dropped to 20 nM where it remained until Ca^{2+} was added back. In addition, VSMC tonic $[Ca^{2+}]_i$ response to caffeine was abolished. The transient $[Ca^{2+}]_i$ response was not significantly affected in the first caffeine challenge but was eliminated following the second and subsequent challenges in Ca^{2+} -free conditions. Ryanodine significantly reduced both transient and tonic increases in $[Ca^{2+}]_i$ in response to caffeine. This was consistent with a significant attenuation in VSMC shortening response. Ryanodine did not significantly alter resting $[Ca^{2+}]_i$ levels. Both TMB-8 and nifedipine did not significantly effect resting $[Ca^{2+}]_i$, caffeine-induced increases in transient and tonic $[Ca^{2+}]_i$, or decreases in VSMC length in response to caffeine. Thapsigargin induced a gradual but variable rise in $[Ca^{2+}]_i$ during the first 15 minutes but subsequently stabilized to 67 nM. Thapsigargin decreased cell lengths to 89 % of their original length. Pretreatment with thapsigargin had no significant effect on either transient or tonic $[Ca^{2+}]_i$ changes in response to caffeine. The noradrenaline-releasable and the caffeine-releasable Ca^{2+} store were found to overlap. Maximal concentrations of each applied to VSMCs deplete the releasable Ca^{2+} available to the other.

In conclusion, the fura-2 technique may be successfully employed to measure $[Ca^{2+}]_i$ in single vascular smooth muscle cells from the rat tail artery. The $[Ca^{2+}]_i$ response was found to be reproducible irrespective of cell length. This is the first study to show this, although others have used single cells to study $[Ca^{2+}]_i$ changes, the

question of whether cell morphology affects $[Ca^{2+}]_i$ has never been addressed. This allows comparative $[Ca^{2+}]_i$ studies to be performed on the same single vascular smooth muscle cell regardless of its contractile state. This is important as there can be great heterogeneity in the Ca^{2+} signal response between different cells in the same population.

On this basis, we have shown that ryanodine reduces the caffeine-induced Ca^{2+} response; whereas other agents known to affect Ca^{2+} regulation such as thapsigargin, TMB-8, and nifedipine had no effect on the caffeine-induced Ca^{2+} response. Extracellular Ca^{2+} was found to play an important role in the maintenance of resting and tonic $[Ca^{2+}]_i$ levels, as well as being essential to replenishment of the caffeine-sensitive Ca^{2+} store. We have also shown that caffeine and noradrenaline are equally effective in releasing intracellular Ca^{2+} and inducing a tonic increase in $[Ca^{2+}]_i$.

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LIST OF ABBREVIATIONS

AM	acetoxymethyl ester
ATP	adenosine triphosphate
BAPTA	1,2- <i>bis</i> (o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
Ca ²⁺	free calcium (II) ion
[Ca ²⁺] _i	intracellular Ca ²⁺ concentration
cAMP	adenosine 3':5'-cyclic monophosphate
cGMP	guanosine 3':5'-cyclic monophosphate
CICR	Ca ²⁺ -induced Ca ²⁺ release
DTT	dithiothreitol
EGTA	ethylene glycol-O,O'- <i>bis</i> (2-aminoethyl)-N,N,N',N'-tetraacetic acid
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid
InsP ₃	inositol-1,4,5-trisphosphate
IICR	InsP ₃ -induced Ca ²⁺ release
MLCK	myosin light chain kinase
MOPS	3-[N-morpholino]propanesulphonic acid
PIP ₂	phosphatidylinositol-4,5-diphosphate
PKA	protein kinase A
PKC	protein kinase C
SR	sarcoplasmic reticulum
TMB-8	3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester
VSMC	vascular smooth muscle cell

INTRODUCTION

Part I. Ca^{2+} and Smooth Muscle Contraction

The importance of Ca^{2+} as a factor in muscle contraction was discovered by the English physiologist Sidney Ringer in the year 1883. On replacing London city tap water with distilled water in his medium, he noticed that frog muscles failed to contract. Since then, a great deal of information has been gathered on the role of Ca^{2+} in muscle contraction, aided by increasingly elegant techniques for detecting $[\text{Ca}^{2+}]_i$ in muscle cells. Smooth muscle contraction has historically been based on comparisons with skeletal muscle.

(A) Excitation-Contraction Coupling

The divergence between smooth and skeletal muscle is evident in their capacity to respond to external stimuli. Skeletal muscle relies on stimulation of nicotinic receptors to depolarize the cell to trigger contraction. This is referred to as electromechanical coupling. Smooth muscles may contract in the absence of membrane depolarization in response to a variety of neurotransmitters and hormones. Contraction independent of membrane depolarization is referred to as pharmacomechanical coupling. Smooth muscle receptors are coupled to a multiplicity of ion channels as well as membrane phospholipases capable of generating the second messenger inositol 1,4,5-trisphosphate (InsP_3) (Warshaw *et al.*, 1986). InsP_3 acts on the internal stores to release Ca^{2+} into the cytoplasm (Berridge, 1986, see Part II).

(B) Myosin, Actin, and Cross-bridge Formation

The sliding filament mechanism is the generally accepted theory to explain the contraction of both smooth and skeletal muscle (Hartshorne & K2wamura, 1992). The fundamental contractile unit of muscle, the sarcomere, is present in smooth muscle although in a more loosely associated manner than is found in skeletal muscle. Tension is transmitted throughout the smooth muscle by thin filaments attached to dense bodies, structures resembling the Z-line common to skeletal muscle. Generation of tension occurs through the cyclic formation of cross-bridges between actin and myosin, fuelled by the hydrolysis of ATP. The rate of cross-bridge cycling is substantially slower in smooth muscle compared to skeletal muscle: However, the mechanics are fundamentally the same. The velocity of muscle contraction (under zero load) is proportional to the rate of cross-bridge cycling. Force developed is a summation of the cross-bridges acting (in parallel) at any given moment.

(C) Ca^{2+} , Calmodulin, Myosin Light Chain Kinase, and Phosphorylation

The divergence between smooth and skeletal muscle is perhaps most evident in how they utilize increases in $[\text{Ca}^{2+}]_i$ to turn on the contractile machinery. In skeletal muscle, once $[\text{Ca}^{2+}]_i$ reaches a threshold level Ca^{2+} binds to troponin C and induces conformational changes in thin filament proteins that allows the interaction of actin and myosin. However, troponin is not present in smooth muscle and therefore a different mechanism must be invoked following an increase in $[\text{Ca}^{2+}]_i$ to threshold levels. In smooth muscle it is the phosphorylation of myosin which provides the conformational

change necessary for its interaction with actin. Myosin is composed of six subunits of which two of these, the 20 kD light chains, are phosphorylated by the enzyme myosin light chain kinase (MLCK), usually at the serine-19 position and sometimes also at threonine-18 (Kamm & Stull, 1985).

The link between MLCK and threshold $[Ca^{2+}]_i$ levels that evoke contraction is the protein molecule calmodulin. The binding of Ca^{2+} to the calmodulin-MLCK complex induces conformational changes that cause the enzyme to phosphorylate myosin. The phosphorylated myosin in turn undergoes conformational changes that allow it to form cross-bridges with actin. Relaxation occurs when a dephosphorylase (phosphatase) enzyme removes the phosphate groups from myosin thereby uncoupling its association with actin (Hartshorne, 1987). Four isotypes of phosphatase have been isolated from gizzard smooth muscle, but it is still not known if phosphatase activity is physiologically regulated (Ikebe *et al.*, 1991). It is this phosphorylation-dephosphorylation that acts as the "on-off" switch tripped by threshold increases in $[Ca^{2+}]_i$. The initiation of contraction requires at least twice the rise in $[Ca^{2+}]_i$ as does maintenance of contraction (Gunst, 1989; Opie, 1991).

(D) The Latch-Bridge Hypothesis

Smooth muscles often require less than one percent of the ATP needed by skeletal muscle to sustain a comparable force under tonic conditions (Murphy, 1989). This observation is supported by previous studies on the mechanism of tonic smooth muscle contraction (Dillon *et al.*, 1981) which showed that the concept of degree of

phosphorylation determining the force of smooth muscle contraction is oversimplified. Following stimulation of swine carotid artery with K^+ , there was a reduction in the velocity of shortening and contractile force that could be maintained at low levels of phosphorylation by slowly cycling cross-bridges, termed "latch-bridges". It was postulated that latch-bridges are formed by dephosphorylation of the myosin component of a formed cross-bridge and that this would slow down the kinetics of dissociation, leading to a more sustained contractile state, but with little additional ATP expenditure (Paul, 1989). This reduction in the rate of contraction economizes the amount of ATP used by the muscle to sustain a tonic contraction, as in the major arteries (Ishii *et al.*, 1989). The observation of latch-bridges under K^+ stimulation indicates that this state is not conditional on a receptor-mediated event, and is thus a constitutive property of the contractile apparatus of smooth muscle. The initial contraction response is followed by a lower $[Ca^{2+}]_i$ level slightly above the initial resting level during the tonic phase when low energy steady-state force is maintained. It has been shown that the maintenance of latch-bridges is Ca^{2+} dependent. A smooth muscle in the latch state can be relaxed by the removal of extracellular Ca^{2+} (Hai & Murphy, 1989).

A number of protein kinases phosphorylate MLCK, e.g. protein kinase A (PKA), protein kinase C (PKC), indicating regulation pathways in addition to Ca^{2+} /calmodulin may be involved (Kamm & Stull, 1989). It has also been shown that force generated at comparable $[Ca^{2+}]_i$ levels can vary with different forms of smooth muscle stimulation (Morgan, 1990). In addition to the latch-bridge, additional receptor-mediated mechanisms

may be involved in the regulation of contraction. Some studies have implicated G proteins in the modulation of Ca^{2+} sensitivity (Somylo & Somylo, 1992).

(E) Correlation of $[\text{Ca}^{2+}]_i$ and Tension in Vascular Smooth Muscle

The contractile system of smooth muscle can be studied by a procedure termed "chemical skinning". This method permeabilizes the plasma membrane of the muscle cell thereby exposing the contractile proteins and SR to the extracellular environment. Chemical skinning was derived from the older technique of physical skinning (Natori, 1954) developed for larger and more rugged skeletal muscle fiber. A number of methods of chemically skinning smooth muscle have been developed using glycerin (Filo *et al.*, 1965), Triton X-100 (Gordon, 1978), and EDTA (Beguet & Marchand-Dumont, 1975).

Treatment with saponin is the most common method of skinning smooth muscle fibers (Endo *et al.*, 1977; Saida & Nonomura, 1978; Endo *et al.*, 1982). After saponin treatment, the substrate of the contractile reaction (MgATP), activating agents (usually Ca^{2+}), and relaxing agents (EGTA to chelate Ca^{2+}) may pass by diffusion through the permeabilized plasma membrane to interact with contractile proteins. Endo and colleagues (1982) measured relative tension in saponin-skinned smooth muscle fibres of guinea pig *taenia ceci* over the pCa (-log $[\text{Ca}^{2+}]$) range of 7 to 3 (100 nM to 1 mM free $[\text{Ca}^{2+}]$). An exponential relationship was recorded between tension development and pCa. Threshold for tension development was observed at pCa $\sim$ 6.9 (125 nM). Half-maximal tension was observed at pCa $\sim$ 6.2 (600 nM) with maximal tension developing at pCa $\sim$ 5.0 (10 μM). In guinea pig mesenteric artery the minimum $[\text{Ca}^{2+}]$ required

to evoke contraction was approximately 100-200 nM. Half-maximal tension occurred at $pCa \approx 6.35$ (440 nM). Maximal tension was observed at $pCa \approx 5.0$ (10 μM) (Itoh *et al.*, 1981). Results were similar using guinea pig *taenia coli* (Saida & Nonomura, 1978). Unlike skeletal muscle (Gordon *et al.*, 1973), the tension- pCa relationship in smooth muscle was not appreciably affected by pH changes (6.4-7.2), ionic strength (150-300 mM), or [MgATP] (0.4-8.0 mM). The slope of the tension- pCa relationship was also less steep than that of skeletal muscle (Endo *et al.*, 1977).

(F) Ca^{2+} -Independent Vascular Smooth Muscle Contraction

A number of studies indicate that smooth muscle contraction may not necessarily be conditional upon an increase in $[Ca^{2+}]_i$. In rabbit aorta, mesenteric artery, ear artery, and in rat tail artery, the application of phorbol ester induced a sustained contraction without increasing $[Ca^{2+}]_i$ (Sato *et al.*, 1992). Similar results have been obtained in ferret aorta (Jiang & Morgan, 1987), swine carotid (Rembold & Murphy, 1988) and coronary artery (Mori *et al.*, 1990). Furthermore, it has been shown that phorbol esters induce sustained contractions in the absence of external Ca^{2+} without increasing myosin light chain phosphorylation (Singer & Baker, 1987; Jiang & Morgan, 1989; Sato *et al.*, 1992). Studies of Ca^{2+} -independent intrinsic tone in ferret aorta are consistent with the presence of a constitutively active, calcium-independent form of MLCK (Pawlowski & Morgan, 1992). This may be responsible for basal levels of myosin light chain phosphorylation responsible for maintenance of intrinsic VSM tone. A Ca^{2+} -independent form of MLCK has been produced *in vitro* (Ikebe *et al.*, 1987). An

alternative explanation is that the enzyme is actually Ca^{2+} dependent with significant activity occurring at resting $[\text{Ca}^{2+}]_i$ levels (Pawlowski & Morgan, 1992).

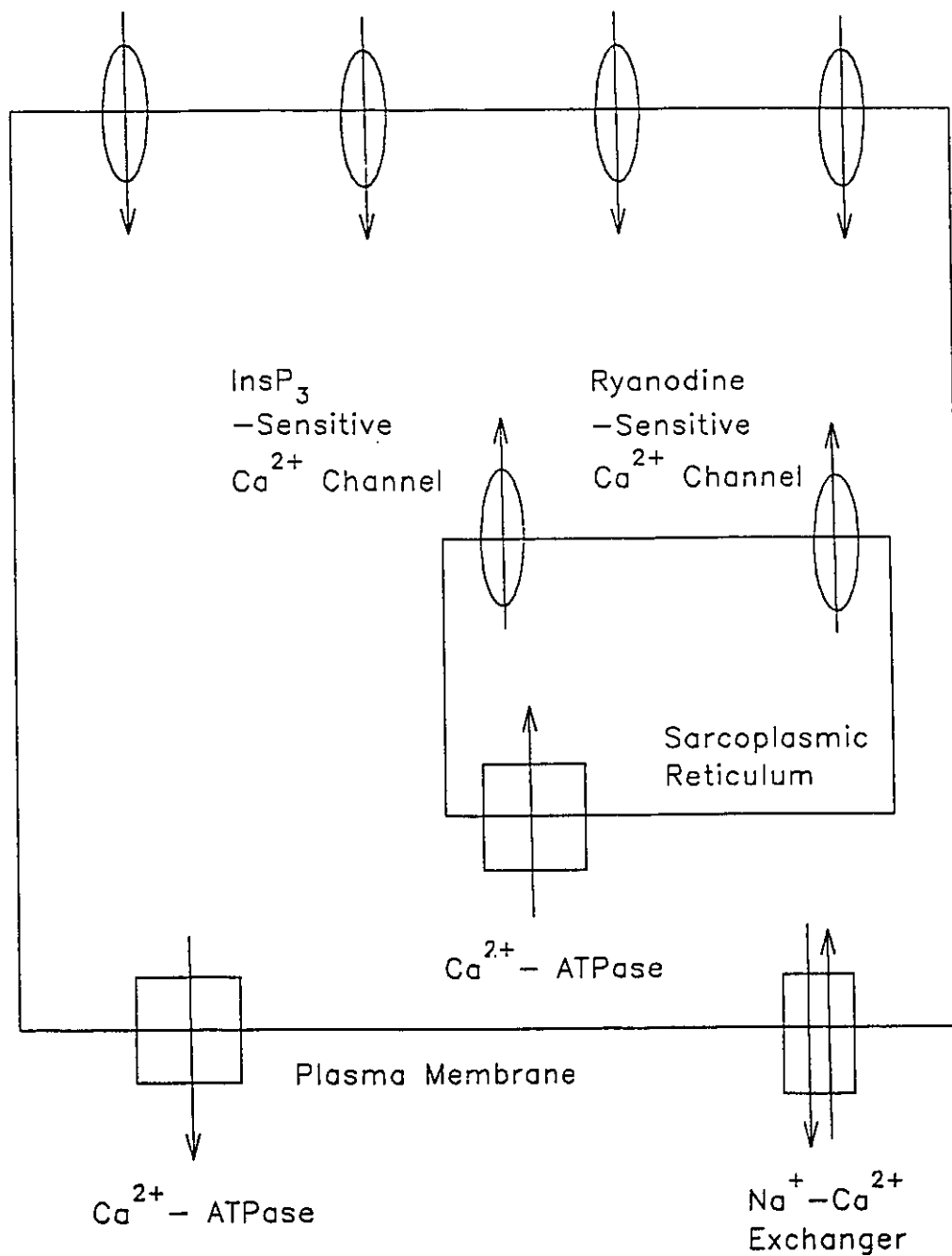
Part II. Ca^{2+} Regulation in Smooth Muscle: General Model

The smooth muscle cells maintains a 10,000 fold Ca^{2+} gradient across the plasma membrane between the extracellular environment and the cytoplasm. In addition, the highly sensitive nature of much of the cell's contractile proteins to changes in $[\text{Ca}^{2+}]_i$, requires uncompromising regulation of Ca^{2+} translocation throughout the cell. Figure 1 illustrates the principal mechanisms responsible for Ca^{2+} translocation, and therefore $[\text{Ca}^{2+}]_i$, in the smooth muscle cell. The Ca^{2+} translocation proteins are situated in two principal membrane systems which form an integrated system to control $[\text{Ca}^{2+}]_i$. In the plasma membrane: 1) voltage-dependent Ca^{2+} channel, 2) receptor-mediated Ca^{2+} channel, 3) Ca^{2+} leak channel, 4) stretch-activated Ca^{2+} channel, 5) Ca^{2+} -ATPase, 6) reversible Na^+ - Ca^{2+} exchanger, and in the sarcoplasmic reticulum membrane: 7) InsP_3 -sensitive Ca^{2+} channel, 8) ryanodine-sensitive Ca^{2+} channel, and 9) SR Ca^{2+} -ATPase. Ca^{2+} translocation across the plasma membrane is under the control of both membrane potential (E_m) and extracellularly-based agonists such as neurotransmitters, hormones and autocoids. The SR is the principal storage site of intracellular Ca^{2+} . The Ca^{2+} translocation proteins situated in the membrane of the SR are controlled by means of intracellularly-derived second messengers.

Figure 1. Ca^{2+} regulation in the smooth muscle cell.

Illustration of the principal mechanisms responsible for Ca^{2+} translocation, and therefore $[\text{Ca}^{2+}]_i$, in the smooth muscle cell. The Ca^{2+} translocation proteins are situated in two principal membrane systems which form an integrated system to control $[\text{Ca}^{2+}]_i$. In the plasma membrane: 1) voltage-dependent Ca^{2+} channel, 2) receptor-mediated Ca^{2+} channel, 3) Ca^{2+} leak channel, 4) stretch-activated Ca^{2+} channel, 5) Ca^{2+} -ATPase, 6) reversible Na^+ - Ca^{2+} exchanger, and in the sarcoplasmic reticulum membrane: 7) InsP_3 -sensitive Ca^{2+} channel, 8) ryanodine-sensitive Ca^{2+} channel, and 9) SR Ca^{2+} -ATPase. Ca^{2+} translocation across the plasma membrane is under the control of both membrane potential (E_m) and extracellularly-based agonists such as neurotransmitters, hormones and autocoids. The SR is the principal storage site of intracellular Ca^{2+} . The Ca^{2+} translocation proteins situated in the membrane of the SR are controlled by means of intracellularly-derived second messengers.

Stretch	Voltage		Receptor
-Activated	-Dependent	Ca^{2+} Leak	-Mediated
Ca^{2+} Channel	Ca^{2+} Channel	Channel	Ca^{2+} Channel



(A) **Plasma Membrane Regulation of Ca^{2+}**

(i) **Ca^{2+} Influx Across the Plasma Membrane**

(i) *Voltage-Dependent Ca^{2+} Influx*

Hinke *et al.* (1964) and Briggs (1962) were the first to show that K^{+} -induced contraction of smooth muscle was highly dependent on the presence of extracellular Ca^{2+} . As the principal determinant of membrane potential is $[\text{K}^{+}]$, it is now recognized that membrane depolarization is the cause of contraction. The correlation between membrane depolarization and tension development can be shown from simultaneous recordings of membrane potential with microelectrodes and contraction in isolated vascular segments (Cheung, 1984). In most vascular tissues, the threshold membrane potential for tension development is about -49 mV (Cheung, 1984).

The development of the patch-clamp recording technique (Hamill *et al.*, 1981) enabled the identification of two types of Ca^{2+} channel that mediate influx of Ca^{2+} with membrane depolarization: T- and the more predominant L-type Ca^{2+} channels. T(transient)-type Ca^{2+} channels have a low 8 pS conductance in 100 mM Ba^{2+} , low voltage activation threshold (positive to -70 mV) and rapid inactivation ($\tau > 20$ -50 ms) (Benham *et al.*, 1987; Yatani *et al.*, 1987). The role of these channels in smooth muscle is not clear. The lack of a specific pharmacological probe also makes it difficult to assess its physiological function. They are transient in nature and impart only a fraction of the total Ca^{2+} current in most vascular smooth muscle cells. L (for long lasting) -type Ca^{2+} channels have a 25 pS conductance in 100 mM Ba^{2+} and show a higher activation threshold (positive to -50 mV) and slow inactivation ($\tau > 500$ ms) producing a relatively

long lasting current. L-type Ca^{2+} channels are blocked specifically by dihydropyridine Ca^{2+} antagonists, and less specifically by other types of Ca^{2+} antagonists including phenylalkylamines and the benzothiazepines. The L-type Ca^{2+} channel is composed of five subunits. The 165 kDa α_1 subunit is capable of functioning as a Ca^{2+} channel and contains binding sites for Ca^{2+} channel blockers (Catterall, 1988).

(ii) *Receptor-Mediated Ca^{2+} Influx*

Agonist-induced contractions can be obtained in completely depolarized (Evans & Schild, 1957) as well as polarized (Su *et al.*, 1964) smooth muscle. In addition, maximal agonist-induced ^{45}Ca influx can be additive to maximal high K^+ -induced ^{45}Ca influx (Zschauer *et al.*, 1987). Ca^{2+} antagonists which are very effective in abolishing K^+ -induced contractions are capable, if at all, of only partially inhibiting agonist-induced contractions or ^{45}Ca influx. This indicates the presence of additional Ca^{2+} -influx pathways activated by receptor binding.

Patch-clamp recordings provide direct evidence of receptor-mediated Ca^{2+} entry from channels distinct from voltage-dependent Ca^{2+} channels. In smooth muscle cells of the rabbit ear artery, it was first demonstrated that ATP activates a cation channel with a unitary conductance of about 5 pS in Ca^{2+} or Ba^{2+} . It is relatively non-selective and is permeable to Ca^{2+} over Na^+ in a 3:1 ratio (Benham & Tsien, 1987). These channels are directly activated by ATP without involvement of a second messenger. Unlike voltage-gated Ca^{2+} channels, they are not blocked by nifedipine or cadmium. Cation

channels in vascular smooth muscle cells activated by noradrenaline (Loirand *et al.*, 1991) and endothelin (Chen & Wagoner, 1991) have also been observed.

Receptor-mediated Ca^{2+} influx in vascular smooth muscle cells can also be mediated by products of the signal transduction pathway (Himpens & Somlyo, 1988; Gill, 1992). Thus the non-selective cation channel in rabbit portal vein is activated by PKC (Oike *et al.*, 1993). Agonists such as neuropeptide Y (Xiong *et al.*, 1993), noradrenaline (Benham & Tsien, 1988; Nelson *et al.*, 1988) and histamine (Oike *et al.*, 1992) also potentiate current through voltage-dependent Ca^{2+} channels. Similar enhancement of Ca^{2+} current could be produced by phorbol esters, implicating a role for PKC (Fish *et al.*, 1988; Oike *et al.*, 1992). Recently, Ca^{2+} entry activated by inositol 1,3,4,5-tetrakisphosphate through yet unidentified channels has been observed in endothelial cells (Luckoff & Clapham, 1992). Ca^{2+} entry through a Ca^{2+} - and K^{+} -permeable channel activated by an unidentified diffusible messenger released upon depletion of InsP_3 stores has also been reported in a number of cell preparations (Parekh *et al.*, 1993; Randriamampita & Tsien, 1993).

(iii) Resting Influx of Ca^{2+} : Leak Channels

When smooth muscle is in the resting state neither voltage-gated nor receptor-mediated Ca^{2+} channels are open. However, the plasma membrane remains permeable to Ca^{2+} (van Breemen *et al.*, 1981; Scheid & Fay, 1985; van Breemen *et al.*, 1986). Owing to their small size and high surface to volume ratio, smooth muscle cells are more prone to passive flux of extracellular Ca^{2+} across the membrane. In rabbit aorta, the

rate of ^{45}Ca leakage was approximately 14 nmol Ca^{2+} per minute per gram of wet tissue. In terms of membrane surface area this represents a passive flux of $0.03 \text{ pmol/cm}^{-2}/\text{s}^{-1}$. Ca^{2+} leak channels are insensitive to calcium channel blockers. However, the form of a Ca^{2+} channel is inferred from the observation that Ca^{2+} leakage is sensitive to H^{+} and La^{3+} , which indicates negatively charged transport sites are involved. Application of La^{3+} maximally reduced passive Ca^{2+} leak to one-third its original value (van Breemen & Deth, 1976). High K^{+} depolarization in the presence of the Ca^{2+} antagonist D600 reduced Ca^{2+} influx 17%, indicating a partially electrogenic Ca^{2+} translocation (Mayer *et al.*, 1975). The opening of leak channels is also potentiated upon removal of extracellular Ca^{2+} (Casteels & van Breemen, 1975).

(iv) *Stretch-Induced Ca^{2+} Influx*

Vascular tissues develop myogenic tone which is dependent upon stretch of the vessel wall (Bevan, 1985). The Ca^{2+} entry pathway activated by stretch is not well understood. The stretch response may not be sensitive to Ca^{2+} antagonists and it appears that stretch-induced Ca^{2+} influx is a rather complex process. In cerebral arteries, stretch causes depolarization of the membrane and thereby activates Ca^{2+} entry through voltage-operated Ca^{2+} channels (Brayden & Nelson, 1992). Activation of a non-selective cation channel by stretch has also been reported in vascular smooth muscle cells (Davis *et al.*, 1992).

(ii) Ca^{2+} Efflux Across the Plasma Membrane

(i) *The Plasma Membrane Ca^{2+} -ATPase*

Smooth muscle utilizes a Ca^{2+} -ATPase situated in the plasma membrane to maintain a low $[\text{Ca}^{2+}]_i$. Biochemical evidence clearly shows a much lower density of Ca^{2+} -ATPase in the plasma membrane than is found in either striated skeletal or cardiac muscle. This is consistent with the lower rates of contraction and relaxation seen in smooth muscle. A recent review of Ca^{2+} -ATPase in smooth muscle has been made by Raeymaekers & Wuytack (1993). Knowledge of the smooth muscle plasma membrane Ca^{2+} -ATPase is derived mainly from studies of the SR Ca^{2+} -ATPase in skeletal muscle; however the general structural model is valid for the smooth muscle plasma membrane Ca^{2+} -ATPase. It is known to belong to the P-class of ion-motive ATPases: The Ca^{2+} -ATPase incorporates Ca^{2+} in a high-affinity conformational state ($K_m(\text{Ca}^{2+}) \sim 100\text{-}200 \text{ nM}$). It then relies on the binding of Mg^{2+} to transform it to a second, lower affinity, conformational state ($K_m(\text{Ca}^{2+}) \sim 145\text{-}180 \mu\text{M}$). This structural transition utilizes the energy of ATP hydrolysis to effectively transport Ca^{2+} from the inner to the outer side of the plasma membrane. The $\text{Ca}^{2+} : \text{ATP}$ binding stoichiometry of the reaction remains unclear. It may be 2-1 as in the case of the SR Ca^{2+} -ATPase or it may be 1-1 as seen with a purified reconstituted preparation (Carafoli, 1991). Ca^{2+} -ATPase has been purified from the plasma membrane of a variety of smooth muscle tissues such as porcine stomach (Wuytack *et al.*, 1984) and aorta of several species (Imai *et al.*, 1990). It has an apparent molecular weight of 130 kDa. Smooth muscle plasma membrane Ca^{2+} -ATPase has also been cloned and sequenced from a number of sources

(Dejaegere *et al.*, 1990). The Ca^{2+} -ATPase is encoded by at least four genes, with alternative splicing of the primary transcripts occurring at four sites (Strehler, 1991). However, a number of workers have recovered between 1-4 isoforms from smooth muscle cDNA libraries. One isoform appears to lack a cAMP responsive site. The different isoforms still need to be confirmed at the protein level, although their expression appears to be tissue- and developmental stage-dependent (Masuo *et al.*, 1991; Carafoli, 1992).

Regulation of the smooth muscle plasma membrane Ca^{2+} -ATPase is very complex. The pump is activated by calmodulin and evidence indicates it is also regulated by a cAMP-dependent protein kinase, a cGMP-dependent protein kinase, by PKC, by lipids, by proteolysis and by self-association. In the absence of calmodulin, the calmodulin-binding domain interacts with another part of the Ca^{2+} -ATPase and inhibits its activity (Enyedi *et al.*, 1989). Activation of the pump by calmodulin results in an increase of the V_{max} and an increase in its affinity for Ca^{2+} (Niggli *et al.*, 1981). Hydropathy analysis indicates the Ca^{2+} -ATPase consists of 10 transmembrane helices with approximately 80 % of the mass of the Ca^{2+} -ATPase protruding into the cytoplasmic side of the plasma membrane. The 4-5 transmembrane helix domain contains an ATP binding site (lys-601). The COOH-terminal intracellular tail contains the calmodulin-binding domain and a consensus sequence which indicates a PKC-binding site as well as a putative Ca^{2+} binding site in the form of an acidic peptide sequence. Several groups have provided both biochemical and intact cell evidence for the stimulation of Ca^{2+} -ATPase by both cGMP- and cAMP-dependent protein kinase. However, it is

not yet clear whether a direct phosphorylation of the protein occurs. Previous studies have suggested that cGMP acts on cellular Ca^{2+} activation via Ca^{2+} extrusion, in contrast to cAMP which is suggested to act by means of increased Ca^{2+} storage (Itoh *et al.*, 1985). The Ca^{2+} -ATPase is additionally up-regulated by, and appears to possess up to two specific binding sites for, acidic phospholipids such as PIP_2 and PIP (Brodin *et al.*, 1992). Acidic phospholipids increase both the $V_{\max}$ and the affinity for Ca^{2+} (Missiaen *et al.*, 1989). Ca^{2+} -ATPase activation by phospholipid exceeds levels attributable to reconstitution into the phospholipid environment of the native membrane (Niggli *et al.*, 1981).

(ii) *The Plasma Membrane Na^+ - Ca^{2+} Exchanger*

The Na^+ - Ca^{2+} exchanger mediates both the rate and direction of Ca^{2+} translocation across the plasma membrane (Blaustein, 1988). The Na^+ - Ca^{2+} exchanger is regulated by three principal factors. 1) Ca^{2+} entry and exit are activated by changes in $[\text{Ca}^{2+}]_i$, 2) the intracellular $[\text{Na}^+]$, and 3) the absolute difference between the membrane potential and the exchanger reversal potential. The exchanger typically operates at a stoichiometry of 3 Na^+ per Ca^{2+} exchanged and is therefore electrogenic (Rasgado-Flores & Blaustein, 1987). The large transmembrane Na^+ gradient, maintained by the ouabain-sensitive Na^+ - K^+ -ATPase, provides the driving force for the Na^+ - Ca^{2+} exchanger (Grover *et al.*, 1985; Ashida & Blaustein, 1987). The Na^+ - Ca^{2+} exchanger is co-distributed with the Na^+ - K^+ -ATPase on unique regions of the smooth muscle membrane in apposition to calsequestrin-containing regions of the SR (Moore *et al.*,

1993). This type of intimate *en face* contact might give the $\text{Na}^+ \text{-Ca}^{2+}$ exchanger preferred access to SR Ca^{2+} stores, despite its lower affinity for Ca^{2+} relative to the Ca^{2+} -ATPase. ATP does not fuel the exchange; however, in cardiac cells the exchanger is phosphorylated leading to alterations in binding kinetics (Reinlib *et al.*, 1987; Crespo *et al.*, 1990; Durkin *et al.*, 1991). The vascular smooth muscle $\text{Na}^+ \text{-Ca}^{2+}$ exchanger appears to be relatively dormant at resting $[\text{Ca}^{2+}]_i$ but is activated over the physiological $[\text{Ca}^{2+}]_i$ (0.1-1.0 μM) range (Reeves & Pronnilk, 1987; Rasgado-Flores *et al.*, 1988). Reduction in extracellular $[\text{Na}^+]$ has been shown to decrease Ca^{2+} extrusion by the $\text{Na}^+ \text{-Ca}^{2+}$ exchanger (Bova *et al.*, 1990). The $\text{Na}^+ \text{-Ca}^{2+}$ exchanger is fully reversible; with the direction of Ca^{2+} exchange depending on prevailing Na^+ and Ca^{2+} gradients (Schnetkamp *et al.*, 1989; Reeves, 1992).

(iii) *Relative Contribution of Various Mechanisms to Maintenance of Resting $[\text{Ca}^{2+}]_i$*

The relative contributions in $[\text{Ca}^{2+}]_i$ -lowering capacity between the plasma membrane Ca^{2+} -ATPase and its SR counterpart, or between the plasma membrane $\text{Na}^+ \text{-Ca}^{2+}$ exchanger, is of considerable interest. Eggermont and colleagues (1988) calculated that in a crude membrane fraction from bovine main pulmonary artery, about 57 % of the Ca^{2+} pumping activity would be due to the plasma membrane and 43 % to that of the SR. However, evidence would suggest that these ratios may differ widely between different types of smooth muscle. For example, the amount of 130 kDa (plasma membrane) and 100 kDa (SR) phosphoprotein intermediates in isolated membranes were

much higher in porcine stomach than in bovine aorta or main pulmonary artery (Eggermont *et al.*, 1988).

The plasma membrane Ca^{2+} -ATPase operates in parallel with the Na^{+} - Ca^{2+} exchanger. Evidence strongly suggests that in most types of smooth muscle the former plays a relatively more important role. In rat aortic smooth muscle, at the peak of the transient $[\text{Ca}^{2+}]_i$ increase induced by a stimulus, the amount of ^{45}Ca efflux in Na^{+} -free solution was about 60 % of that in Na^{+} -containing solution. This is in contrast to the reverse case seen in cardiac muscle (Furakawa *et al.*, 1988). This approximately equal Ca^{2+} translocation activity is comparable with data obtained from isolated plasma membrane vesicles from rat uterus, which indicated a similar ratio of Na^{+} -dependent and ATP-dependent Ca^{2+} uptake activity (Grover *et al.*, 1983).

Studies have also shown that the Ca^{2+} -ATPase activity in plasma membrane vesicles is roughly equal to the Na^{+} - K^{+} ATPase activity (Raeymaekers *et al.*, 1985; Eggermont *et al.*, 1988). This is important since the activity of the Na^{+} - K^{+} pump sets an upper limit to the amount of Ca^{2+} that can be extruded from the cell in exchange for Na^{+} . Thus prolonged Ca^{2+} extrusion at a high rate must occur at least partially by means of the Ca^{2+} -ATPase (Raeymaekers & Wuytack, 1993). It should also be kept in mind that a simplified comparison of relative concentrations of the various Ca^{2+} regulatory proteins, or comparison of *in vitro* kinetics, may not necessarily lead to the relative *in vivo* importance of each of the Ca^{2+} regulatory proteins.

(B) Intracellular Regulation of Ca^{2+}

(i) Ultrastructure of the Sarcoplasmic Reticulum (SR)

The ability of smooth muscle cells to contract in the absence of extracellular Ca^{2+} indicates the presence of intracellular sources of Ca^{2+} . Studies have shown the SR to be the principal organelle responsible for intracellular Ca^{2+} regulation (Somlyo, 1985). The ultrastructure of the SR has been revealed by a number of electron microscopy and electron probe microanalysis studies (Somlyo, 1980; Gabella, 1981; Somlyo *et al.*, 1985). SR in relation to Ca^{2+} regulation has been studied in intact and permeabilized smooth muscle and in isolated membrane vesicles. The SR of smooth muscle is composed of a system of tubules and, in some species, flattened cisternae of which the lumen appears to be contiguous (Somlyo & Franzini-Armstrong, 1985).

(ii) SR Ca^{2+} Storage

Smooth muscle SR content has been shown to comprise 2 to 7 % of the volume of the smooth muscle cell (Somlyo, 1985). Ca^{2+} accumulation studies in rabbit mesenteric artery (Leijten & van Breemen, 1986) and aorta (Leijten & van Breemen, 1984) have estimated the physiological SR $[\text{Ca}^{2+}]$ to be in the order of 5 mM. A value of 6 mM has been obtained by electron microprobe analysis of peripheral SR in guinea pig portal vein (Bond *et al.*, 1984). Electron microscopy studies have revealed that peripherally located SR forms special junctions with the plasma membrane, similar to the triadic junctions of skeletal muscle (Somlyo & Franzini-Armstrong, 1985). High capacity low affinity Ca^{2+} -binding proteins are localized close to these junctional elements as well

as throughout the SR (Lytton & MacLennan, 1991). The most well known of these proteins capable of holding large amounts of releasable Ca^{2+} is calsequestrin, first isolated from skeletal SR (MacLennan & Wong, 1971). It is a highly acidic 63 kDa protein capable of binding 40-50 moles of Ca^{2+} per mole of protein with a dissociation constant of about 1 mM. Other " Ca^{2+} sponges" known to be present in the SR are calreticulin, histidine-rich Ca^{2+} binding protein, and a number of both low and high molecular weight glycoproteins (Lytton & MacLennan, 1991).

(iii) SR Ca^{2+} Uptake

Ca^{2+} uptake by the SR is effected by a 110 kDa Mg^{2+} -dependent Ca^{2+} -ATPase (Raeymaekers & Wuytack, 1993). It is regulated by Ca^{2+} -dependent phosphorylation (Sumida *et al.*, 1984) and cAMP-dependent phosphorylation of phospholamban (Raeymaekers & Jones, 1986; Eggermont *et al.*, 1988). The SR Ca^{2+} -ATPase is not stimulated by calmodulin as is the case with its plasma membrane equivalent (Schatzmann, 1989). The SR Ca^{2+} -ATPase is additionally up-regulated by cGMP (Twort & van Breemen, 1988). Studies with saponin-skinned smooth muscle fibres show the SR Ca^{2+} -ATPase is able to load the SR at an ambient $[\text{Ca}^{2+}]$ of 1 μM , with half-maximal loading at an ambient $[\text{Ca}^{2+}]$ of 400 nM, indicating its high Ca^{2+} affinity (Saida & Nonomura, 1978; Yamamoto & van Breemen, 1986). The SR Ca^{2+} -ATPase is able to fully load the SR of permeabilized mesenteric artery in 3 minutes (Saida & van Breemen, 1984). Tension development initiated from influx of Ca^{2+} is dependent on the state of Ca^{2+} loading within the SR. A Ca^{2+} -depleted SR will take up all incoming

Ca^{2+} until it has attained its normal physiological $[\text{Ca}^{2+}]$ before tension can be developed (Loutzenhiser & van Breemen, 1983).

(iv) SR Ca^{2+} Release

The contribution of internal Ca^{2+} to contraction is proportional to the size of the SR (Cauvin *et al.*, 1983). Repeated challenges with agonist in the absence of extracellular Ca^{2+} will exhaust the SR Ca^{2+} store (van Breemen, 1969; van Breemen *et al.*, 1972). SR Ca^{2+} depletion may be effected by inhibition of the Ca^{2+} -ATPase. For example, cyclopiazonic acid inhibits the ATP-driven Ca^{2+} -sequestration mechanism resulting in an increase in $[\text{Ca}^{2+}]_i$ and an increase in smooth muscle tension (Shima & Blaustein, 1992). The SR Ca^{2+} store may also be depleted by opening Ca^{2+} release channels, effectively making the SR leaky to Ca^{2+} . For example ryanodine, thapsigargin, caffeine, ATP, and Ca^{2+} itself can deplete SR Ca^{2+} stores by acting on Ca^{2+} release channels (Shima & Blaustein, 1992).

(i) Ca^{2+} -Induced Ca^{2+} Release (CICR)

During muscle stimulation, the influx of a small amount of extracellular Ca^{2+} across the plasma membrane can trigger the release of a much greater amount of Ca^{2+} from the SR (Ito *et al.*, 1991). CICR has been demonstrated in skeletal (Endo *et al.*, 1970; Ford & Podolsky, 1970), smooth (Saida, 1981; Saida, 1982) and cardiac (Fabiato & Fabiato, 1972) muscle. CICR is effected by the activation of large (75 pA in 100 mM Ba^{2+}) cation channels. These channels can be activated by Ca^{2+} , caffeine, ATP, and

ryanodine (Smith *et al.*, 1985). Procaine blocks CICR (Ueno *et al.*, 1987) while cAMP is able to potentiate it (Saida & van Breemen, 1984). Studies on saponin-skinned cultured VSMCs have shown that caffeine-induced CICR requires the presence of very low concentrations of ambient Ca^{2+} (Yamamoto & van Breemen, 1986). Caffeine releases Ca^{2+} by acting exclusively on the SR in skeletal (Endo, 1975), cardiac (O'Neill *et al.*, 1990) and smooth muscle (Watanabe *et al.*, 1992). Caffeine produces CICR-induced contraction in skeletal muscle (Fleischer *et al.*, 1985), cardiac muscle (Rousseau & Meissner, 1989), and smooth muscle (Endo, 1977; Itoh *et al.*, 1981; Itoh *et al.*, 1982; Leijten & van Breemen, 1984). Caffeine-induced Ca^{2+} release does not require GTP and is insensitive to pertussis toxin (van Breemen *et al.*, 1988), indicating that a G protein is not involved. The agonists noradrenaline, histamine, angiotensin, vasopressin, and the prostaglandins all release Ca^{2+} from the same caffeine-sensitive intracellular pool (Deth & Casteels, 1977; Deth & van Breemen, 1977). Fura-2 studies of single smooth muscle cells of porcine coronary artery showed that ryanodine (50 μM) greatly inhibited the increase in Ca^{2+} induced by 5 mM caffeine or 10 μM acetylcholine (Katsuyama *et al.*, 1991). ^{45}Ca efflux studies employing rabbit aorta have showed that ryanodine also releases Ca^{2+} in smooth muscle (Hwang & van Breemen, 1987). This points to the possible presence of an analogous structure in smooth muscle, similar to the junctional foot proteins present in skeletal muscle (Lytton & MacLennan, 1991).

(ii) *InsP₃ -Induced Ca²⁺ Release (IICR)*

Inositol 1,4,5-trisphosphate (InsP₃) is released into the cytosol in response to plasma membrane-bound receptor activation of phospholipase C, which mediates hydrolysis of the InsP₃ parent compound phosphatidylinositol-4,5-bisphosphate (PIP₂). InsP₃ can then interact with its receptor to open cation channels situated in the SR leading to the release of Ca²⁺ (Berridge & Irvine, 1984; Berridge, 1993). The InsP₃-receptor-channel complex is mediated by a guanine nucleotide regulatory mechanism (Dawson, 1985; Gill *et al.*, 1986; Dawson *et al.*, 1987). Studies with skinned smooth muscle indicate IICR requires the presence of GTP (Saida & van Breemen, 1987; Saida *et al.*, 1988; van Breemen *et al.*, 1988) or its non-hydrolysable analog C₇ppNHp, and is inhibited by pertussis toxin (Saida *et al.*, 1988). Studies with skinned cultured smooth muscle cells have shown that up to 97% of SR Ca²⁺ is releasable by InsP₃ (Yamamoto & van Breemen, 1985).

Studies with caged InsP₃ showed that sub-micromolar concentrations are sufficient for initiating the rapid release of SR Ca²⁺ (Walker *et al.*, 1987). In addition, InsP₃ is both dephosphorylated and further phosphorylated to non-Ca²⁺ releasable products. Both membrane-bound (Rossier *et al.*, 1987) and soluble (Hansen *et al.*, 1987) phosphomonoesterases have been shown to inactivate InsP₃. InsP₃ is also metabolized by a calmodulin-dependent 3-kinase to inositol 1,3,4,5-tetrakisphosphate (Johanson *et al.*, 1988). The accumulation of InsP₃ after agonist stimulation has been found to be biphasic, peak increases occurring within 10-15 seconds, whereas PIP₂ hydrolysis is sustained for several minutes (Chahwala *et al.*, 1987).

The InsP₃ receptor from cerebellar neurons has been purified and functionally reconstituted into lipid vesicles (Ferris *et al.*, 1989), cloned and functionally expressed (Furuichi *et al.*, 1989). The InsP₃ receptor is similar in structure to the ryanodine receptor/Ca²⁺ channel complex found in skeletal muscle SR (Gill, 1992; Mignery *et al.*, 1989). The cDNA sequence indicates a protein of 2,749 amino acids of theoretical molecular weight of 313 kDa. Hydropathy studies indicate the protein contains up to nine membrane-spanning regions, however immuno-localization studies indicate that only seven transmembrane domains are likely to exist.

(iii) *Differences Between IICR and CICR*

The InsP₃-gated and the ryanodine-sensitive release channels display different kinetics. In studies of these channels from the Purkinje cells of cerebellum incorporated into lipid bilayers, it was shown that the open probabilities of both types of channel display a bell-shaped dependence on Ca²⁺ (Bezprozvanny *et al.*, 1991). For the InsP₃-gated channel, the active range is between 10 nM and 10 μ M Ca²⁺, with a maximum probability of opening occurring at 200 nM free Ca²⁺. In contrast, the maximum activity for the ryanodine-sensitive channels is maintained between 1 and 100 μ M. Under physiological levels of Ca²⁺, the narrow active range of the InsP₃-gated channels allows positive and negative feedback control for oscillatory Ca²⁺ release. The ryanodine-sensitive channels behave solely as a Ca²⁺-activated channel.

(iv) *Caffeine as a Tool to Study Intracellular Ca^{2+}*

Caffeine is a member of the methylxanthine family of alkaloids found naturally throughout the world (Rall, 1990; Graham, 1987). Caffeine is 1,3,7-trimethylxanthine. Xanthine is a dioxypurine and is structurally related to the nitrogenous compound uric acid. The order of potency for the three naturally occurring methylxanthines is: theophylline > caffeine > theobromine (Arnaud, 1987). Theophylline has the greatest efficacy and is the preferred methylxanthine for therapeutic purposes. The potency of theobromine is considered too low and is not used as a therapeutic. Caffeine is distributed into all body compartments; it can cross the blood-brain barrier, placenta, and pass into breast milk. It is metabolized primarily in the liver with a plasma half-life of 3 to 7 hours, depending on the individual (Becker *et al.*, 1984).

The three principal mechanisms of action of caffeine and the related methylxanthines are: 1) ability to affect intracellular calcium translocation, 2) increased accumulation of cyclic nucleotide via inhibition of cyclic nucleotide phosphodiesterase, and 3) antagonism of the receptor-mediated actions of adenosine (Rall, 1982). Less well known actions have been reported. These include the potentiation of inhibitors of prostaglandin synthesis (Vinegar *et al.*, 1976), reduction of non-neural uptake and/or metabolism of catecholamines (Kalsner *et al.*, 1975), and inhibition of DNA repair processes (Timson, 1977). It is interesting to consider the mechanisms of action of the methylxanthines in light of concentrations (20 to 50 μ M) used for therapy. As yet no particular mechanism has been confirmed to result in bronchodilation, their main therapeutic use.

Studies of the actions of caffeine and theophylline on intracellular Ca^{2+} have been performed on skeletal muscle (Berglund & Hemmingsson, 1982; Lopes *et al.*, 1983), cardiac muscle (Rall & West, 1963; Ogilvie *et al.*, 1977) and smooth muscle (Kalsner, 1971; Pfaffman & McFarland, 1978; see section (iv)(i)-CICR). Caffeine concentrations in the sub-millimolar range have been shown to affect Ca^{2+} regulation in the SR. Caffeine is more potent than theophylline in its ability to affect cellular Ca^{2+} translocation.

Methylxanthines have the ability to increase the accumulation of cyclic nucleotide in cells. However, theophylline concentrations of at least 500 μM are required, a level in excess of that used during therapy (Choi *et al.*, 1988). Concentrations of at least 100 to 200 μM are required for potentiation of cyclic AMP-based responses to stimuli (Peach, 1972). In addition, there is minimal inhibition of the hydrolysis of cyclic AMP (cAMP) at therapeutic concentrations (20 to 50 μM). Enhancement of the accumulation of cyclic GMP (cGMP) may be responsible for theophylline-induced relaxation of bronchial smooth muscle (Andersson & Persson, 1985). However, there is little evidence that the methylxanthines increase cGMP in heart and nerve tissue (Rall, 1990).

Methylxanthines act as competitive antagonists at adenosine receptors at concentrations similar to therapeutic levels (Fredholm & Persson, 1982). Caffeine and theophylline have nearly equal affinity for both adenosine receptor subtypes (A_1 , A_2). Adenosine receptors are linked via G proteins to a variety of effector systems such as adenylyl cyclase and K^+ channels (Carney & Katz, 1988). This could partially explain

the ability of the methylxanthines to evoke a variety of responses at micromolar concentrations.

Part III. Methods of Ca^{2+} Measurement in Smooth Muscle

(A) Non-fluorescent Methods of Ca^{2+} Measurement

(i) Introduction

A number of non-fluorescent methods are currently available to measure Ca^{2+} levels in cells. These are Ca^{2+} -selective microelectrodes, metallochromic Ca^{2+} indicators, ^{19}F NMR, ^{45}Ca analysis, and electron probe microanalysis. Both Ca^{2+} -selective electrodes and metallochromic Ca^{2+} indicators can be used to study real time $[\text{Ca}^{2+}]$ changes in single cells and are valuable alternatives to fluorescent studies of cell Ca^{2+} . ^{19}F NMR requires a large number of cells in suspension but its relatively high temporal resolution and potential *in vivo* application make it worthy of discussion. ^{45}Ca analysis was used extensively prior to development of fluorescent probes and will be discussed in light of the extensive literature on Ca^{2+} regulation employing this method. Electron probe microanalysis (Wendt-Gallitelli & Isenberg, 1991) has limited application in live preparations and will not be discussed.

(ii) Ca^{2+} -Selective Microelectrodes

Ca^{2+} -selective microelectrodes are capable of determining $[\text{Ca}^{2+}]$ both inside (cytoplasm) and outside (extracellular space) the cell (Orchard *et al.*, 1991). The sensitivity of Ca^{2+} -selective microelectrodes can be as low as 10 nM, even in the

presence of interfering ions normally present in physiological solutions. The principal difficulty in using ion-selective microelectrodes rests with ensuring that all other junction potential differences remain constant and do not drift during measurement of $[Ca^{2+}]_i$. The microelectrode contents may change over time through dialysis of ions in or out of the electrode, possibly resulting in potential changes and errant $[Ca^{2+}]$ measurements. Although Ca^{2+} -selective microelectrodes represent a powerful method of measuring $[Ca^{2+}]$, the method is not amenable to the study of $[Ca^{2+}]$ in single VSMCs. The dimensions of a typical VSMC are 110 μm long by only 8 μm wide. The size of a single VSMC is simply too small to accommodate the tip ($\sim 5 \mu m$) of an ion-sensitive microelectrode. Another limitation is the response time of the microelectrode is too slow to measure $[Ca^{2+}]_i$ transients and the method must therefore be relegated to monitoring changes in slower tonic cell $[Ca^{2+}]_i$ levels (Jensen *et al.*, 1993).

(iii) Metallochromic Indicators

The Ca^{2+} -sensitive metallochromic indicators were widely used prior to the development of fluorescent indicators (Thomas, 1991). Arsenazo-III, a derivative of chromotropic acid, and antipyrilazo-III (lower Ca^{2+} affinity) are prototypic metallochromic indicators, although many similar congeners are available. Detection of changes in $[Ca^{2+}]_i$ is similar in principle to fluorescent dyes, with Ca^{2+} binding producing a shift in light absorbency. The metallochromic indicators operate at relatively longer wavelengths (600-700 nm) compared to fluorescent dyes (300-500 nm). Background absorbency at these longer wavelengths is much higher than the background

encountered with fluorescent indicators. Metallochromic indicators also have relatively poor selectivity against competing ions, especially Mg^{2+} and H^{+} . In addition, Ca^{2+} -binding stoichiometry often varies, making calibration difficult. The metallochromic dyes are introduced into the cell by microinjection. This technique relies on a micropipette to inject a small quantity of the dye into the cell's interior. This rather intrusive method again limits application to larger and more rugged cell types.

(iv) ^{19}F Nuclear Magnetic Resonance

This method of measuring $[Ca^{2+}]_i$ (Metcalf & Smith, 1991) relies on a fluorine-19 labelled Ca^{2+} -selective probe (5-fluoro-BAPTA) introduced into cells and monitored at 376 Hz by ^{19}F NMR. This method utilizes a similar principal to measure $[Ca^{2+}]$ changes as do the fluorescent probes. Namely, the Ca^{2+} chelator BAPTA (Pethig *et al.*, 1989) is coupled to a reporter group composed of two fluorine-19 atoms. In the Ca^{2+} -free state the fluorine-19 atoms are situated in identical chemical environments, owing to the bilateral symmetry of the BAPTA molecule. Upon binding to Ca^{2+} , 5-fluoro-BAPTA undergoes a shift in the structural geometry of its four carboxylate groups. This differentially unshields the fluorine-19 atoms and causes a shift in their resonance. The degree of shift identifies the species of the complexing ion. For example, a shift of 5.7 p.p.m. down field identifies the bound ion as Ca^{2+} . It is the height of this peak which is correlated with $[Ca^{2+}]$. ^{19}F NMR cannot be used for single cells and currently a relatively large number ($\sim 3-5 \times 10^8 \text{ ml}^{-1}$) of loaded cells are required to obtain sufficient signal strength. 5-fluoro-BAPTA has an apparent K_d of 710 nM at 37 °C limiting its

Ca^{2+} sensitivity to 200 nM. This greatly reduces its suitability for the study of $[\text{Ca}^{2+}]_i$ in VSMCs since resting $[\text{Ca}^{2+}]_i$ levels are almost an order of magnitude lower. This is partially compensated by its high selectivity for Ca^{2+} over Mg^{2+} . The method can readily be used with physiological $[\text{Mg}^{2+}]$ between 0.1 and 1.0 mM. The kinetics of ^{19}F -BAPTA binding limits its temporal resolution to a minimum sampling time of 10 milliseconds.

A major advantage of ^{19}F NMR is that other ion-sensitive fluorine-labelled probes can be co-loaded and their unique resonance shifts used to simultaneously measure ions such as Mg^{2+} , Na^+ , and H^+ . 5-fluoro-BAPTA is loaded into cells by the method most commonly used for the fluorescent indicators i.e the cell-permeable acetoxymethyl (AM) ester conjugate. This is a more passive form of loading compared to the method of microinjection discussed above, and is especially useful for loading small and delicate cell types like VSMCs. One problem with this form of loading is incomplete hydrolysis. The unhydrolyzed ester of 5-fluoro-BAPTA produces a very broad ^{19}F NMR resonance, making it difficult to detect and possibly allowing incorrect measurement of $[\text{Ca}^{2+}]_i$. ^{19}F NMR is able to probe deeply into dense tissues and it is expected that this characteristic will one day allow real time *in vivo* studies in the intact animal. However, ^{19}F NMR requires both expensive and complex equipment which continues to impede its popularity.

(v) *Measurement of Cellular ^{45}Ca Flux*

The measurement of ^{45}Ca in cells is based on the fact that a lipid membrane barrier separates the intra- and extracellular spaces in any given tissue sample. The application of ^{45}Ca to smooth muscles, followed by stimulation, washing, and finally counting, allows the tracking of Ca^{2+} translocation (van Breemen *et al.*, 1982). Washing of a ^{45}Ca -loaded smooth muscle tissue first removes the radioactive isotope from the more accessible extracellular compartment. Vigorous washing followed by quenching is essential if intracellular ^{45}Ca levels are to be measured since 90 % of tissue calcium resides in the extracellular space in both bound and free forms. Washing is facilitated by the addition of La^{3+} to the washing solution. This inhibits transmembrane Ca^{2+} fluxes thus slowing down the loss of cellular Ca^{2+} while displacing it from extracellular sites. The use of La^{3+} was found to displace intracellular Ca^{2+} in some tissue preparations (Karaki & Weiss, 1981) prompting the use of EGTA in washing solutions (van Breemen *et al.*, 1982). Equally important to quenching is the need for careful dissection of the smooth muscle tissue. Even slight damage done during the removal of connective tissue can cause large ^{45}Ca spikes, thus obscuring the much lower physiological ^{45}Ca movements being investigated.

A number of drawbacks associated with ^{45}Ca measurements have reduced its use following the synthesis of fura-2 and other fluorescent Ca^{2+} -sensitive indicators. Whereas fura-2 allows continuous real-time recording of repeated agonist challenges for long periods of time (~hours), the ^{45}Ca method allows for only limited sample treatment. Fluorescent methods are equally amenable to the study of both Ca^{2+} influx, efflux, and

release, whereas the ^{45}Ca method requires multiple loadings of isotope if Ca^{2+} release is to be studied. ^{45}Ca studies can not be used to study fast Ca^{2+} kinetics associated with Ca^{2+} transients and oscillations. In addition, ^{45}Ca measurements are limited to bulk tissue preparations that must be rigorously cleaned of foreign tissue prior to stimulation. The elapsed time between stimulation of smooth muscle and counting of ^{45}Ca , and resultant equilibration across membranes, may lead to discrepancies between the calculated and actual Ca^{2+} fluxes in the tissue at the time of stimulation.

(B) Fluorescent Methods of Ca^{2+} Measurement

(i) Ca^{2+} -Sensitive Fluorescent Indicators

Fluorescent indicators (Thomas & Delaville, 1991) provide better spatial and temporal resolution when compared to the above methods (Tsien, 1989). Only fluorescent techniques may be used to monitor real time $[\text{Ca}^{2+}]_i$ changes below 200 nM. The photoprotein aequorin (Cobbald & Lee, 1991) was the first Ca^{2+} -selective fluorescent probe used to measure real time changes in cell $[\text{Ca}^{2+}]_i$, and has been in use for over twenty years. Aequorin is a 21 kD protein isolated from the jellyfish and contains three Ca^{2+} -binding consensus sequences. Aequorin emits blue-green light of 470 nm in the presence of Ca^{2+} , reverting to a non-luminescent form in the process. The higher the $[\text{Ca}^{2+}]_i$, the more aequorin is consumed and the brighter the luminescence. The lower limit of Ca^{2+} sensitivity is about 100 nM with the upper limit approaching 10 μM . High ionic strength and millimolar $[\text{Mg}^{2+}]$, but not physiological pH changes, depress aequorin sensitivity. The method of loading the protein into cells

relies on microinjection which limits the method to larger and hardier cell types. Passive loading techniques such as hypo-osmotic shock have had limited success with aquorin.

The synthesis of quin-2 by Tsien (1980; Tsien & Poenie, 1986) represented a major breakthrough in real time $[Ca^{2+}]_i$ measurement. Many earlier studies of $[Ca^{2+}]_i$ have utilized this dye (Morgan *et al.*, 1982). The synthesis of quin-2 involved combining a stilbene fluorophore with EGTA, an octacoordinate tetracarboxylate Ca^{2+} chelator. This elegant idea effectively coupled a Ca^{2+} -selective moiety (EGTA) with an efficient reporter group (the fluorophore). In addition, the development of the AM ester technique for loading these new dyes into living cells represented a major improvement over more invasive loading processes (Tsien & Pozzan, 1989).

(ii) Fura-2 as a Probe for Measurement of $[Ca^{2+}]_i$

Fura-2 was developed by Grynkiewicz *et al.* (1985) and was prototypic of a second generation of Ca^{2+} -selective fluorescent probes. The structure of fura-2 (fig. 2A) was again based on that of EGTA. However, additional modifications to the fluorophore conjugate were made. When fura-2 chelates Ca^{2+} its absorbtion maxima shifts from 380 nm to 340 nm (fig. 2B). This allowed the excitation wavelength ratio to be used as an indicator of the Ca^{2+} -bound state of the probe. Indicators for other ions including Mg^{2+} K^+ Na^+ and H^+ have since been introduced. A new generation of dyes, such as fluo-3 and indo-1 (Molecular Probes, 1991) are finding niche applications. However, fura-2 continues to be invaluable in a multitude of applications where $[Ca^{2+}]_i$ is to be measured (Molecular Probes, 1993).

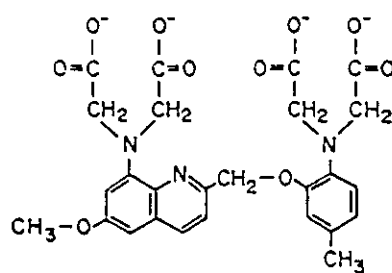
Figure 2. Structural and fluorescent characteristics of fura-2.

Panel A. Comparison of the structures of EGTA, quin-2 and fura-2. The design logic was to combine a Ca^{2+} chelator (EGTA) with a fluorophore able to report structural changes occurring as a result of Ca^{2+} binding to EGTA. Binding of Ca^{2+} withdraws the nitrogen lone pair electrons from the aromatic rings resulting in a shift in fluorescent properties. Structural modification of the aromatic nucleus to make it more or less electron withdrawing respectively increases or decreases its affinity for Ca^{2+} . Optimal measurement of Ca^{2+} is obtained if the affinity of the probe is in the same range as the $[\text{Ca}^{2+}]$ to be measured. (Thomas & Delaville, 1991).

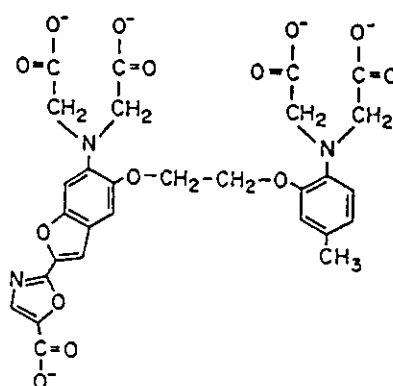
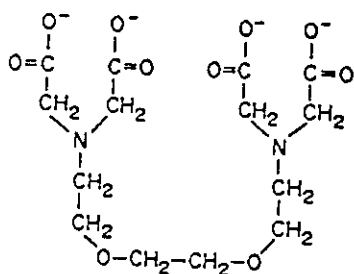
Panel B. Excitation spectra for 1 μM fura-2 at 20 °C in buffers with free Ca^{2+} values ranging from <1 nM to >10 μM . Emission was monitored at 505 nm for fluorescence excitation wavelengths of 340 nm and 380 nm. Note the increase in emission from the 340 nm excitation wavelength and the concomitant decrease in the 380 nm wavelength as $[\text{Ca}^{2+}]$ increases. The isobestic point is 360 nm. (Grynkiewicz *et al.*, 1985).

A

EGTA

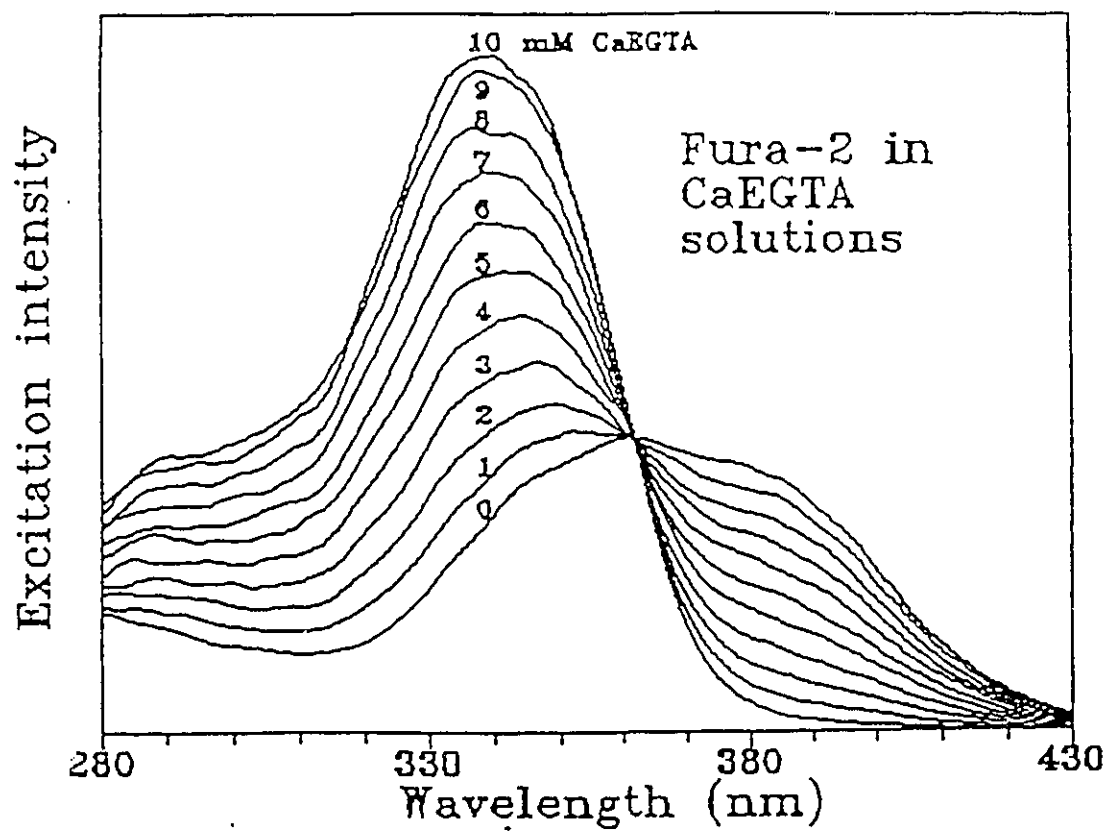


Quin-2



Fura-2

B



(iii) *Advantages of Fura-2 Measurement of $[Ca^{2+}]_i$*

Fura-2 superseded quin-2 as the method of choice for fluorescence-based measurement of $[Ca^{2+}]_i$ for a number of reasons. The quantum efficiency at half-saturation of fura-2 is approximately five times higher than it is for quin-2. Coupled with a six-fold higher quantum yield, this provides a fluorescence intensity 30 times greater than quin-2. This increased brightness allows smaller cytoplasmic volumes to be studied, e.g. single cells, or even certain areas of single cells by means of digital imaging microscopy (Williams *et al.*, 1985). Lower dye levels reduce the impact of potentially toxic metabolites e.g. formic and acetic acid from the AM ester form of fura-2. A lower dye concentration also reduces the probability of an effect known as Ca^{2+} buffering. This occurs when a Ca^{2+} -selective probe binds enough Ca^{2+} within the cytoplasm to lead to a reduction in $[Ca^{2+}]_i$ normally available to the cell, possibly suppressing $[Ca^{2+}]_i$ -based events. The affinity constant, K_d , of fura-2 is twice that of quin-2, which also reduces the chance of $[Ca^{2+}]_i$ buffering. Fura-2 shifts excitation wavelengths upon Ca^{2+} binding (fig. 2B). This has important advantages because the ratio of the bound versus unbound intensities is sufficient to calculate $[Ca^{2+}]_i$, independent of variations in dye concentration, path length, or instrument optics. Both Mn^{2+} and Fe^{2+} possess multiple unpaired electrons and will complex with fura-2, effectively quenching its fluorescence. Fura-2 has a Mn^{2+}/Ca^{2+} preference ratio of 510 versus 42 for quin-2, providing a better quench. The addition of $MnCl_2$ allows a simple determination of non-fura-2 background fluorescence (autofluorescence).

Use of the AM ester of fura-2 to load the dye represents a non-invasive means of introducing the dye into the cell's interior. The hydrophobic nature of the ester form allows it to pass across the cell membrane. The AM esters are cleaved inside the cell by non-specific cell hydrolases. This generates the ionized form of fura-2 and effectively traps it inside the cell. The integrity of the membrane is not compromised during the cell loading process as it would be for delivery systems such as microinjection. This is important because a damaged membrane might leak Ca^{2+} . The ester loading technique allows a number of cells to be simultaneously loaded. Other loading techniques such as microinjection must load one cell at a time. Both fragile and very small cells e.g. platelets (diameter $<3\ \mu\text{m}$) can also be loaded.

Fura-2 may be continuously monitored for periods of hours with little change in signal strength due to bleaching of the dye. This is unlike some of the newer dyes such as fluo-3 that rapidly bleach with continuous monitoring. A large number of studies have successfully employed fura-2 to study $[\text{Ca}^{2+}]_i$ and an extensive bibliography is available from the manufacturer (Molecular Probes, 1993).

(iv) Disadvantages of Fura-2 Measurement of $[\text{Ca}^{2+}]_i$

Use of the ratio method for determining $[\text{Ca}^{2+}]$ has two main disadvantages. Fura-2 independent changes in fluorescence (autofluorescence) must first be subtracted from each of the 340 nm and 380 nm wavelengths before forming a ratio. This can be accomplished by quenching with Mn^{2+} to obtain autofluorescence, then subtracting and ratioing the values with a computer. Secondly, measurement of two separate wavelengths

emissions means twice as much data must be collected per unit of time to form the resultant 340 nm/380 nm ratio. This has purportedly been circumvented by measuring only one excitation wavelength (Vranesic & Knöpfel, 1991). The development of precision light chopper and beam splitter technology, in addition to faster computers, has allowed equipment for real time measurement of fura-2 fluorescence to become readily available commercially.

Once inside the cell the AM form of fura-2 may not be completely hydrolysed (Roe *et al.*, 1990). Fura-2/AM is still highly fluorescent but does not respond to $[Ca^{2+}]_i$ fluctuations. This could lead to errors in determining $[Ca^{2+}]_i$. Sequestration of fura-2 in organelles such as the SR or mitochondria may render fura-2 unavailable to the cytoplasm and therefore measurement of $[Ca^{2+}]_i$. The $[Ca^{2+}]$ may be as high as 5 mM in these organelles leading to overestimation of $[Ca^{2+}]_i$. Reports of dye leakage, especially in enzyme treated cells, have also occurred with the use of fura-2. However, evidence of Ca^{2+} -tolerance in a cell indicates an intact membrane capable of containing fura-2. Fura-2 may also be excreted from the cell by anion transport mechanisms. Use of fluorescence implies that an optical system must be built around loaded cell(s). This limits the method to single cells, cell suspensions, or very thin tissue sections.

(v) *Validity of Fura-2 Measurement of $[Ca^{2+}]_i$*

As with many techniques, accuracy is determined to a large part on the calibration procedure. In the case of fura-2, this may be done by clamping $[Ca^{2+}]$ within the cytoplasm, measuring preloaded fura-2 fluorescence, and constructing an *in situ* standard

curve. This *in situ* method (Williams & Fay, 1990) employs a series of Ca^{2+} -EGTA buffers outside the cell to control $[\text{Ca}^{2+}]$ inside the cell. Cell membranes are treated to allow equilibration between outside $[\text{Ca}^{2+}]$ and the cell interior. $[\text{Ca}^{2+}]$ equilibration across the cell membrane may be done by either treating the cell with mild detergent solubilization of the membrane (e.g. Triton X-100, Digitonin, Saponin) or by means of a Ca^{2+} ionophore (4-Br-A23187 or Ionomycin). The *in situ* calibration has the important advantage of measuring fluorescence in an environment that most closely approximates the ionic strength, protein composition, and viscosity of the environment where fura-2 fluorescence will ultimately be measured. Cytoplasmic $[\text{Ca}^{2+}]$ must be known exactly. There must be equilibrium of $[\text{Ca}^{2+}]$ between the buffer and the cell cytoplasm.

Calibration may also be achieved by conducting non-cellular measurements with a series of buffers of known $[\text{Ca}^{2+}]$ that mimic the cell cytoplasm composition. A number of studies have tried to duplicate the cytoplasmic environment e.g. 100 mM KCl (Molecular Probes, 1991), in a buffer that the pentapotassium salt of fura-2 can be readily introduced into. The main problem with non-cellular methods of calibration relates to differences in fura-2 fluorescence in extracellular buffers compared to the cytoplasm (Owen, 1991). Differences in viscosity (Poenie, 1990), pH (Miller *et al.*, 1984), ionic strength (Grodén *et al.*, 1991), optics (Uto *et al.*, 1991), and temperature may affect the calibration.

A simpler calibration method employs only two nominally zero $[\text{Ca}^{2+}]$ and saturating $[\text{Ca}^{2+}]$ solutions in the presence of membrane equilibrating conditions

(detergent or Ca^{2+} ionophore). This allows determination of the minimum and maximum limits of the standard curve while assuming a linear relationship for intermediate points (Grynkiewicz *et al.*, 1985). Both minimum and maximum $[\text{Ca}^{2+}]$ values are dependent only on attaining essentially zero $[\text{Ca}^{2+}]$ and saturating $[\text{Ca}^{2+}]$ across the cell membrane. This is technically easier and more reliable than the necessity of $[\text{Ca}^{2+}]$ equilibration needed with the *in situ* method of calibration. Table 1 shows the equation and parameters needed to calculate $[\text{Ca}^{2+}]_i$ with this method. A survey of literature values for these parameters demonstrates a large variation between systems, hence caution must be used with this technique. Variations in K_d may arise from the effect that differences in temperature, pH, ionic strength, and viscosity, have on fura-2 fluorescence. Grynkiewicz *et al.* (1985) found K_d changed from 135 nM at 22 °C to 224 nM at 37 °C. However, Uto *et al.* (1991) found that K_d was only minimally affected by temperature. Viscosity effects may reduce the 380 nm signal by 15% (Poenie, 1990). Variations may also arise from fluctuations in the excitation light source, misalignment of the light paths, dust accumulation, or ageing of the fura-2 dye during storage or ageing of the rhodamine B quantum counter used to detect emission signals. Cell loading of fura-2 results in higher cytoplasmic concentrations than the loading concentration, and cannot precisely be determined. This underlines the utility of dual wavelength dye since the resultant ratio is independent of the dye concentration.

Because of limitations in the calibration of live cells the fura-2 method can only be considered a semi-quantitative one. Incomplete hydrolysis, dye leakage, and sequestration of fura-2 can lead to incorrect fluorescence readings. Calibration

Table 1. Determination of $[Ca^{2+}]_i$ by the minimum-maximum method

Grynkiewicz *et al.* (1985) suggested $[Ca^{2+}]_i$ could be obtained as follows:

$$[Ca^{2+}]_i = K_d \frac{(R - R_{min})}{(R_{max} - R)} \times \beta$$

where: $[Ca^{2+}]_i$ is intracellular free calcium concentration; K_d is the dissociation constant for Ca^{2+} -fura-2; R is the ratio of 340 nm/380 nm fluorescent signal; R_{min} is the minimum obtainable fluorescent ratio; R_{max} is the maximum obtainable fluorescent ratio; and β is the ratio of unsaturated/saturated 380 nm signal.

A survey of K_d , R_{min} , R_{max} , and β values in the literature is shown below:

K_d	R_{min}	R_{max}	β	Reference
135 nM	0.77	35.1	15.3	(Grynkiewicz <i>et al.</i> , 1985), at 22 °C
224	0.77	35.1	15.3	<i>Ibid.</i> , at 37 °C
224	0.76	35.3	27.5	(Meininger <i>et al.</i> , 1991), <i>in vitro</i>
224	0.87	6.41	1.04	<i>Ibid.</i> , <i>in situ</i>
210	0.25	5.5	4.85	(Bruschi <i>et al.</i> , 1988)
239	1.36	3.48	4.26	(Erne & Hermsmeyer, 1989)
287	1.24	40.7	11.7	(Uto <i>et al.</i> , 1991) ¹
236	0.26	2.59	5.00	(Grodén <i>et al.</i> , 1991), at 20 °C
285	0.28	2.51	5.55	<i>Ibid.</i> , at 37 °C
342	0.93	24.9	15.3	(Jensen <i>et al.</i> , 1993), at 37 °C

¹ Found that K_d was moderately changed by pH, but minimally affected by temperature.

procedures, that allow determination of $[Ca^{2+}]_i$ based on the fluorescence ratios, are prone to error and may be inaccurate. Some studies now report the fluorescence ratio of fura-2 without transforming these into actual $[Ca^{2+}]_i$ values. However, it may not be so much the absolute $[Ca^{2+}]_i$ levels that are so important, but rather the relative changes in $[Ca^{2+}]_i$ or fluorescence ratios, that occur. In spite of these shortcomings, fura-2 continues to represent a powerful and relatively non-invasive means of obtaining real time $[Ca^{2+}]_i$ in live VSMCs.

The value of the fura-2 technique in studying Ca^{2+} mobilization in VSMCs was validated by a recent comparative study which showed good accordance between fura-2 and Ca^{2+} -selective microelectrode determinations of $[Ca^{2+}]_i$ (Jensen *et al.*, 1993). The resting cell $[Ca^{2+}]_i$ in rat mesenteric resistance arteries was found to be 129 nM and 115 nM for fura-2 and Ca^{2+} -selective microelectrode measurements respectively. Application of 10 μ M noradrenaline resulted in contraction and elevation of $[Ca^{2+}]_i$. The response time of the Ca^{2+} -selective microelectrode was too slow to measure the initial $[Ca^{2+}]_i$ transient peak; however, a value of 656 nM was measured using fura-2. After five minutes $[Ca^{2+}]_i$ was found to be 537 nM and 708 nM for fura-2 and Ca^{2+} -selective microelectrode measurements respectively.

OBJECTIVES

1. To establish the fura-2 technique in determining $[Ca^{2+}]_i$ in single, enzymatically-isolated, vascular smooth muscle cells (VSMCs) from the rat tail artery.
2. To study the relationship between VSMC shortening and Ca^{2+} mobilization by caffeine.
3. To determine the source(s) of Ca^{2+} mobilization by caffeine and the interaction with pharmacological agents that influence Ca^{2+} regulation in VSMCs.
4. To determine the possible physiological role of the caffeine-sensitive Ca^{2+} store in VSMCs.

MATERIALS AND METHODS

(A) Materials

The pentapotassium salt and the acetoxymethyl ester form of fura-2, and $[Ca^{2+}]$ calibration kit were obtained from Molecular Probes Inc. (Eugene, OR). Caffeine, and Papain (from papaya juice, EC 3.4.22.2), and HEPES were from B.D.H. Inc. (Toronto, ON). Noradrenaline (bitartrate salt, approx. 98%), Collogenase (Clostridio-peptidase A, EC 3.4.24.3, Type II, collagen digestion activity 490 units/mg solid), dithiothreitol (DTT) (approx. 98%), 4-Br-A23187, thapsigargin, and TMB-8 (8-(Diethylamino)octyl ester of 3,4,5-Trimethoxybenzoic acid, approx. 97%) were purchased from Sigma Chemical Co (St. Louis, MO). Ryanodine (HPLC 30% Ryanodine, 90% Ryanodine & Didehydro-ryanodine) was purchased from Calbiochem Corp. (La Jolla, CA). MOPS was purchased from GIBCO Laboratories (Grand Island, NY). EGTA (microselect grade, 99% pure) was purchased from Fluka Chemie AG (Buchs, England). Nifedipine was purchased from Miles Laboratories (New York, NY). All other chemicals were of analytical or comparable grade.

(B) Stock Solutions

Table 2 shows details of stock solutions employed in the study. The stock concentration, solvent, storage temperature, and final working concentration are shown.

Table 2. Stock solutions

	<i>Stock</i>	<i>Solvent</i>	<i>Storage</i>	<i>Final</i>
Fura-2/K ₅ ¹	1 mM	ddH ₂ O	-80 °C	1 µM
Fura-2/AM ²	1 mM	DMSO ³	-80 °C	320 nM
MnCl ₂	100 mM	ddH ₂ O	4 °C	1 mM
CaCl ₂	500 mM	ddH ₂ O	4 °C	5 mM
4-Br-A23187	10 mM	DMSO ³	-20 °C	1 µM
TMB-8	10 mM	ddH ₂ O	-20 °C	10 µM
ryanodine	100 mM	Methanol ³	-20 °C	10 µM
nifedipine	2 mM	DMSO ³	-20 °C	1 µM
thapsigargin	1 mM	ddH ₂ O	-20 °C	1 µM

¹pentapotassium salt

²acetoxymethyl ester

³Final concentration <0.1 %; no effect on VSMCs or fluorescence signal.

(C) Buffers

The composition of buffers used in experiments is shown in table 3. The majority of experiments were performed using *Hanks* buffer. The vascular smooth muscle cell (VSMC) isolation used Ca^{2+} -free *Hanks* buffer. The $[\text{Ca}^{2+}]_i$ calibration procedure used *EGTA-Hanks* buffer. $[\text{Ca}^{2+}]$ standard curves were made using combinations of Ca^{2+} -, *EGTA* and *EGTA* buffers in varying proportions. All experiments were performed at room temperature.

(D) Isolation of Single Vascular Smooth Muscle Cells (VSMCs)

Single VSMCs were isolated using a technique developed in our laboratory (Bolzon & Cheung, 1989). Tissue originated from a 2 mm proximal section of caudal rat tail artery isolated from 10-12 week old male Wistar rats (Charles River Labs Inc., Wilmington, Mass.). The artery was cannulated by tying each end with 3-0 silk (Ethicon Inc.) to polyethylene tubing (Intramedic PE-60, Clay Adams Inc., Parsippany, NJ, USA). The artery was internally perfused with Ca^{2+} -free Hanks by connecting one opening to a 20 gauge needle fitted to a 10 ml syringe filled with Ca^{2+} -free Hanks buffer. The artery was checked for leaks by maintaining internal pressure via a clamp on the distal tubing throughout the perfusion. Distension of the artery indicated there were no leaks. The outside of the artery was superfused with Ca^{2+} -free Hanks at a temperature of 35-37 °C. The artery was equilibrated for a period of no less than 30 minutes with Ca^{2+} -free Hanks at a rate of 2 ml/min by means of a syringe infusion pump (Model 22, Harvard Apparatus, South Natick, MA, USA).

Table 3. Buffer composition (mM).

	<i>Hanks</i>	<i>Ca²⁺-free Hanks</i>	<i>EGTA-Hanks</i>	<i>Ca²⁺-EGTA</i>	<i>EGTA</i>
NaCl	137.0	137.0	137.0		
KCl	5.4	5.4	5.4	100.0	100.0
KH ₂ PO ₄	0.4	0.4	0.4		
NaHCO ₃	4.2	4.2	4.2		
NaH ₂ PO ₄	0.4	0.4	0.4		
CaCl ₂	2.0			10.0 ^I	
MgCl ₂	1.0				
glucose	5.6	5.6	5.6		
Hepes	10.0	10.0	10.0		
EGTA			1.0	10.0	10.0
MOPS				10.0	10.0
gas phase					
% O ₂	100	100	100		
pH	7.4	7.4	8.0	7.2	7.2

^IExact equivalence to quantity of EGTA determined by "pHimetric" method.

Following equilibration for 90 minutes, an enzyme mixture was substituted for the perfusate. This consisted of 0.02% (w/v) collagenase type II, 0.02% (w/v) DTT, and 0.1% (w/v) papain in Ca^{2+} -free Hanks, filtered through a 0.45 μm nylon membrane syringe filter (Nalgene Products, Sybron Corp., Rochester, NY). The enzyme mixture was perfused at a temperature of 35-37 °C. A cooled (4 °C initially) outer jacket surrounded the enzyme perfusate to maximize enzyme activity over the 60-70 minute perfusion period. Perfusion was maintained under pressure allowing the artery to enlarge to almost twice its original width. Digestion of the artery was indicated by an increase in transparency of the tissue. The artery was then cut longitudinally and a pair of fine forceps was used to tease out single VSMCs.

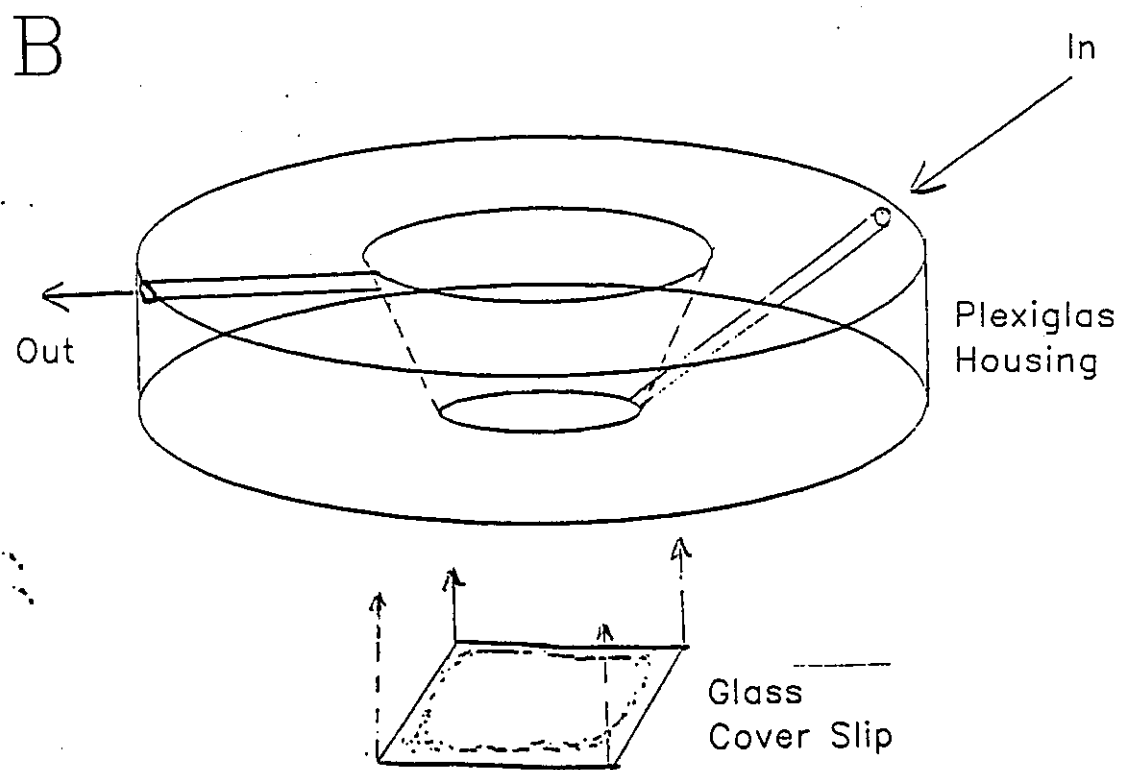
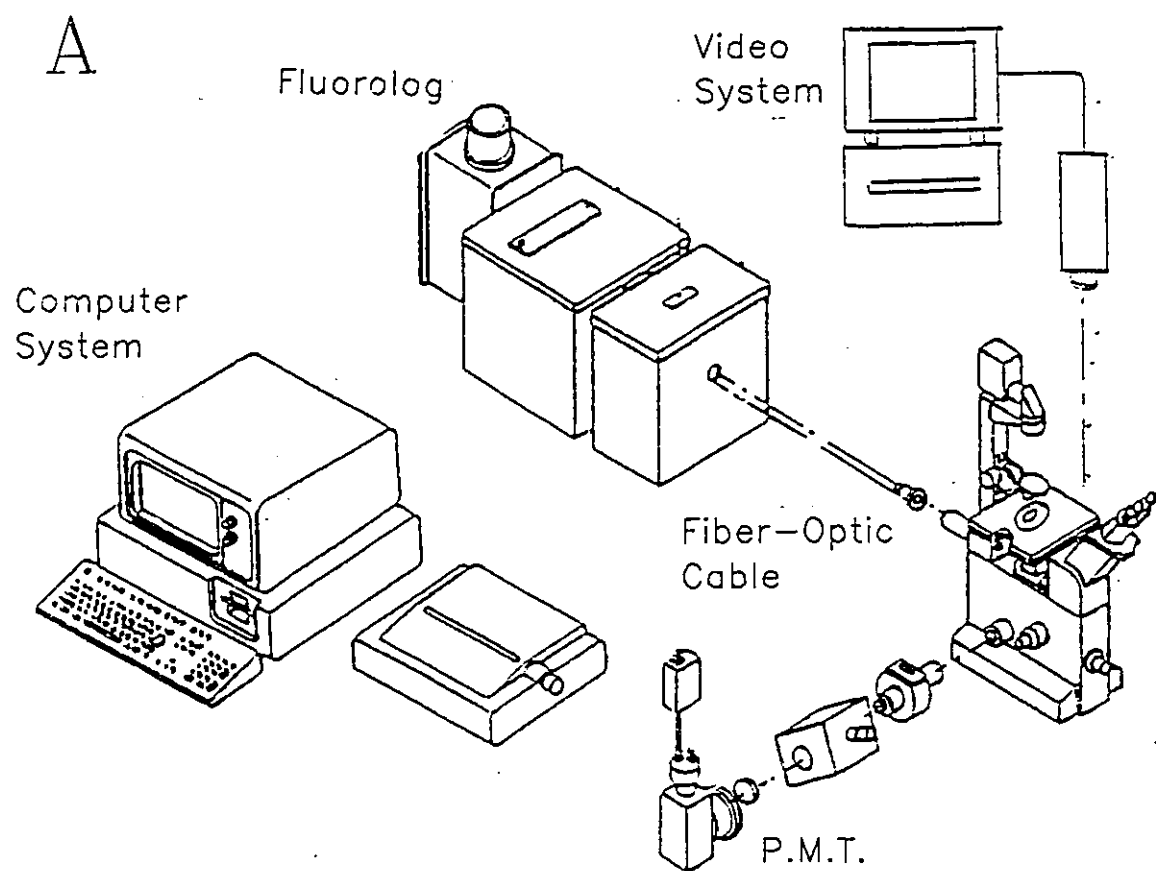
(E) Experimental Setup

Figure 3A shows a diagram of the experimental setup used in the study. Fluorescence measurements were made using a Spex Fluorolog (Model AR-CM-MAC, Spex Industries, Inc., Edison, N.J.) driven with Spex DM3000CM software Version 2.5. The light source was an air-cooled xenon arc lamp connected to a 150 watt power supply (Model ELXE, Electronic Measurements Inc. Edison, NJ). Light was passed through two narrow band-pass filters to produce two excitation wavelengths of 340 nm and 380 nm. These excitation wavelengths of fura-2 were alternated by means of a beam-splitter and a light chopper operating at a frequency of 400 Hz. The Spex Fluorolog was connected via a fibre-optic cable to a Carl Zeiss Jena Sedival inverted microscope (VEB Carl Zeiss

Figure 3. Experimental set-up

Panel A. Experiments were performed using a Spex Industries Fluorolog fluorometer consisting of a 150 watt Xenon lamp, chopper, beam splitter, 340 nm and 380 nm narrow band-pass filters, and a shuttering system. Excitation light was passed to an epifluorescence microscope via a fiber-optic cable. Emission light was collected on a microscope-mounted photomultiplier tube (P.M.T.) fitted with a 515 nm narrow band-pass filter. Still video images of VSMCs were obtained using a video camera connected to a video monitor. Video hard copies were obtained through an attached video printer. A shuttering system present in the microscope allowed for three possible configurations: 1) epifluorescence for monitoring $[Ca^{2+}]_i$, 2) video mode for taking VSMC pictures for cell length recording, and 3) visual inspection of the image through binocular lenses attached to the microscope. Computer-based proprietary software (Spex DM3000CM Version 2.5) controlled the Spex Fluorolog system (shutter, voltage, chopper) and was used to analyze and print data.

Panel B. Fura-2 loaded VSMCs were superfused through a home made perfusion chamber. The chamber consisted of a drilled angled plexiglas ring (4 cm diameter) with ports for input and output of cell superfusate. Fluid level was maintained by a vacuum line connected to the output port. A clean glass coverslip (Corning No. 1) was attached to the base of the chamber each day by means of a thin ring of silicone vacuum grease. The steady state volume of the chamber was 500 μ l.



JENA, DDR-6900, Jena, Carl-Zeiss-Str.1, Germany) in an epifluorescence configuration. Measurements were made through a Carl Zeiss Jena 50X/0.95 infinity/0.17-A Planachromat fl objective lens. Fura-2 fluorescence emission signals passed through a third narrow band-pass filter and were monitored at 515 nm, with an integration time of one second, by a microscope-mounted photomultiplier tube. Still video images of VSMCs were acquired through a Hitachi (Model KP-111) solid state video camera and (Model VM-901C) monitor, and hard copies obtained by a Mitsubishi (Model P50C) video printer. A shuttering system present in the microscope allowed for three possible configurations: 1) epifluorescence for monitoring $[Ca^{2+}]_i$, 2) video mode for taking VSMC pictures for cell length recording, and 3) visual inspection of the image through binocular lenses attached to the microscope. A computer was used to analyze and print data.

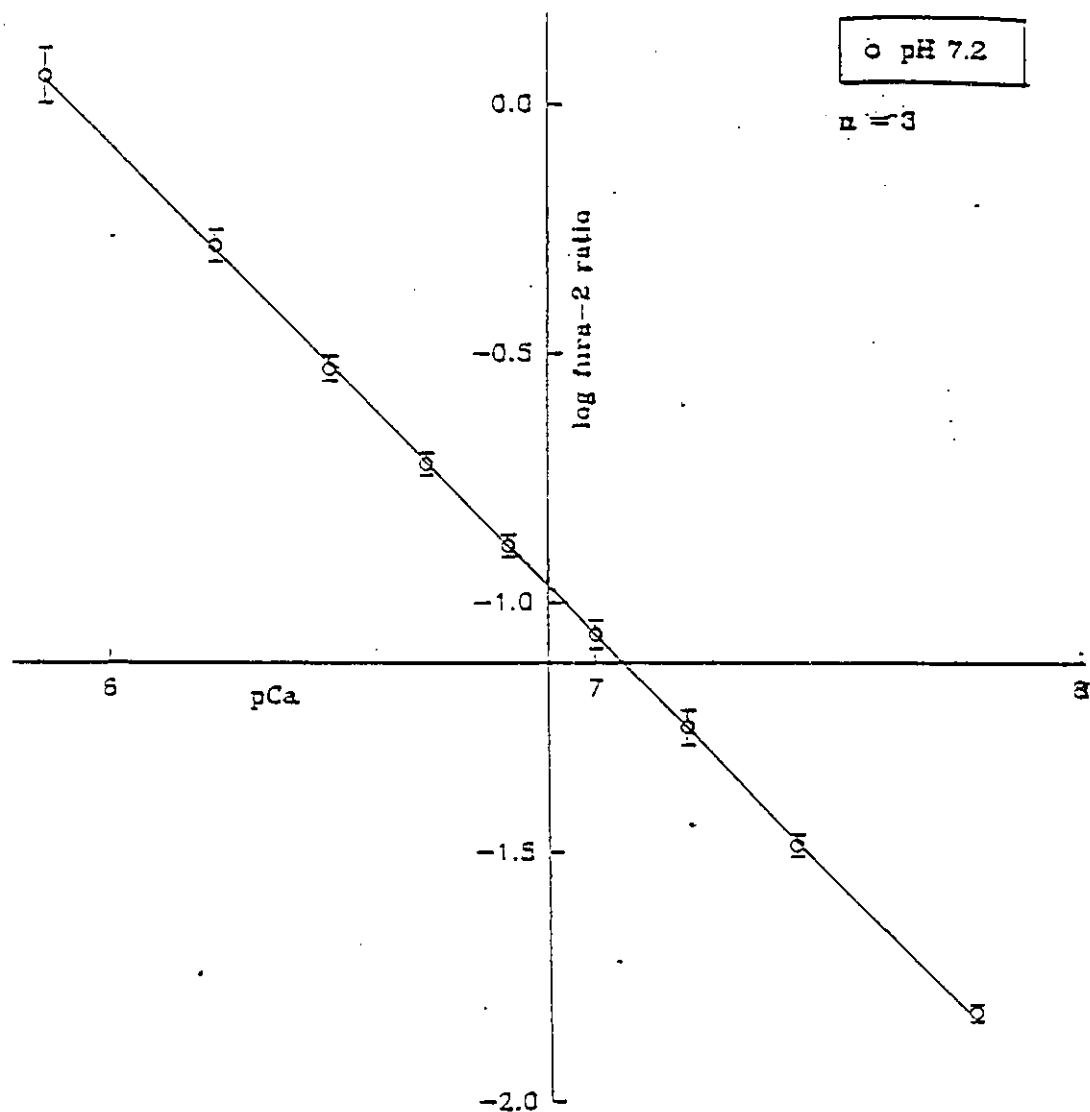
Fura-2 loaded VSMCs were added to a home made superfusion chamber (fig. 3B) consisting of a drilled angled plexiglas ring with input and output ports for the cell perfusate. The epifluorescence configuration employs an inverted microscope objective lens which carries both the excitation and emission light signals, and lies just below the glass cover slip base of the superfusion chamber. Cells were continuously superfused by means of a gravity-fed micro drip line at a rate of 2 ml per minute. Solutions were changed by moving the end of the tubing into the appropriate container. The void volume of the tubing was approximately 1.5 ml.

(F) Calibration of Ca^{2+} Signal

Prior to use in VSMCs it was important to determine if fura-2 fluorescence response was proportional to changes in free Ca^{2+} . This was tested by using the pentapotassium salt of fura-2 in a series of known $[\text{Ca}^{2+}]$ standard solutions. A volume of 150 μl each of eleven Ca^{2+} standard solutions containing 1 μM pentapotassium fura-2 was measured using the superfusion chamber and the Spex epifluorescence apparatus. Figure 4 is a plot of the emission signal from the fura-2 340 nm/380 nm excitation ratio versus pCa for a series of Ca^{2+} -EGTA buffers. $[\text{Ca}^{2+}]$ standard curve solutions were prepared using the "pHimetric" method of Neher (1989). Free $[\text{Ca}^{2+}]$ solutions ranged from nominally zero $[\text{Ca}^{2+}]$ to a fura-2 saturating concentration (38 μM free $[\text{Ca}^{2+}]$). A linear relationship was found over the full range of fura-2 fluorescence. The equation of the fitted line was: $\log (340 \text{ nm}/380 \text{ nm}) = -0.99 \times \text{pCa} + 5.84$ ($n=3$). The slope of one indicates a 1:1 binding ratio between Ca^{2+} and fura-2. The dissociation constant for fura-2, K_d , was determined from the half maximal ratio obtained between nominally zero $[\text{Ca}^{2+}]$ and saturating $[\text{Ca}^{2+}]$. A value of 153 nM was obtained from "home made" Ca^{2+} -EGTA buffers prepared using the pHimetric method (Neher, 1989) as well as purchased proprietary buffers (Molecular Probes, 1991). The value of 153 nM compares favourably with the literature value of 151 nM cited by the source of the proprietary buffers (Molecular Probes, 1991). The conditions for constructing the standard curve were as follows: pH 7.20, room temperature, 1 μM pentapotassium salt of fura-2 with combinations of Ca^{2+} -EGTA and EGTA buffers to effect proper $[\text{Ca}^{2+}]$.

Figure 4. Standard curve for fura-2

Logarithmic plot of fura-2 340 nm/380 nm fluorescence ratio as a function of pCa. $[Ca^{2+}]$ standard curve solutions were prepared using the "pHimetric" method of Neher (1989). Free $[Ca^{2+}]$ solutions ranged from nominally zero $[Ca^{2+}]$ to a fura-2 saturating concentration (38 μ M free $[Ca^{2+}]$). A linear relationship was found over the full range of fura-2 fluorescence. The equation of the fitted line was: $\log (340 \text{ nm}/380 \text{ nm}) = -0.99 \times pCa + 5.84$. The slope of one indicates a 1:1 binding ratio between Ca^{2+} and fura-2. The dissociation constant for fura-2, K_d , of 153 nM was determined from the half maximal ratio obtained between nominally zero $[Ca^{2+}]$ and saturating $[Ca^{2+}]$. The conditions for constructing the standard curve were as follows: pH 7.20, room temperature, 1 μ M pentapotassium salt of fura-2 with combinations of Ca^{2+} -EGTA and EGTA buffers to effect correct $[Ca^{2+}]$. (n=3).



Equation of line is $y = -0.99x + 5.84$
 slope of one means 1:1 binding ratio
 $pK_d = x \text{ intercept} + \log B$
 this curve gives $K_d = 153 \text{ nM}$
 lit. value for fura-2 is $K_d = 151 \text{ nM}$

The fura-2 fluorescence response to Ca^{2+} is linear over pCa 6 to 8, a range that encompasses pCa values encountered in the cytoplasmic environment.

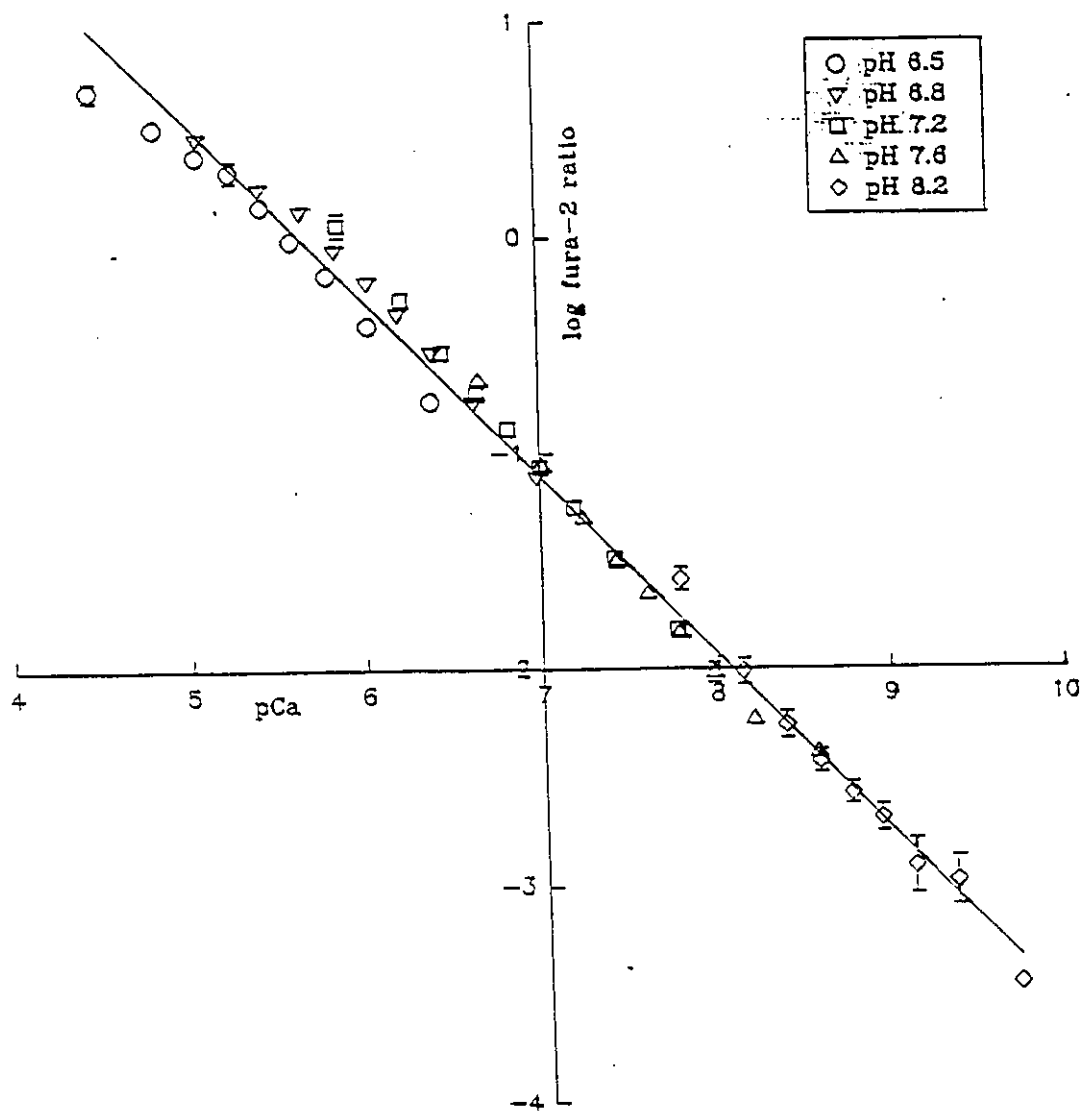
Figure 5 shows a series of fura-2 standard curves for a pH range of 6.5 to 8.2. This shows that the fura-2 fluorescence response remains linear over this pH range, which would include in excess of all possible pH values encountered in the cytoplasmic environment. Higher pH increases the binding affinity of EGTA for Ca^{2+} , effectively lowering the available Ca^{2+} and thus raising the pCa. As seen in the plot, "zero Ca^{2+} " EGTA buffer at pH 8.2 can effect a free pCa as low as 9.6. This illustrates the reason for the use of pH 8.0 in the EGTA-Hanks buffer used to obtain low $[\text{Ca}^{2+}]_i$ during the calibration procedure.

(G) Fura-2 Loading into Single VSMCs

Cells were loaded with the lipid soluble acetoxymethyl ester (AM) form of fura-2 (fura-2/AM). The fura-2/AM loading solution was prepared fresh daily from 1 mM dry DMSO aliquots maintained in the dark at -80°C . Aliquots were diluted in Ca^{2+} -free Hanks, sonicated for ten seconds, and added to the superfusion chamber to effect a final concentration of 320 nM. The final concentration of DMSO was 0.032 % and did not affect VSMCs or the fluorescence signal. Cells were incubated with fura-2/AM at room temperature for 30 minutes followed by superfusion with Ca^{2+} -free Hanks at a flow rate of 2 ml per minute. The steady state volume of the cell superfusion chamber was 500 μl . Cells were subjected to a slowly increasing $[\text{Ca}^{2+}]$ gradient by replacing Ca^{2+} -free Hanks with normal Hanks buffer over a 20 minute period. Only cells that maintained at

Figure 5. Standard curve for fura-2 over pH 6.5 to 8.2

A series of fura-2 standard curves for a pH range of 6.5 to 8.2. The fura-2 fluorescence response remains linear over this pH range. Higher pH increases the binding affinity of EGTA for Ca^{2+} . This results in a lowering of free Ca^{2+} available for binding to the fura-2 dye. Zero Ca^{2+} (EGTA buffer) at pH 8.2 results in a pCa value of 9.6. (n=3).



least eighty percent of their original length upon addition of Ca^{2+} to the buffer were studied. Cells were equilibrated in Hanks for a minimum 15 minutes prior to study.

(H) Effect of Fura-2 on Single VSMC Contraction to Noradrenaline

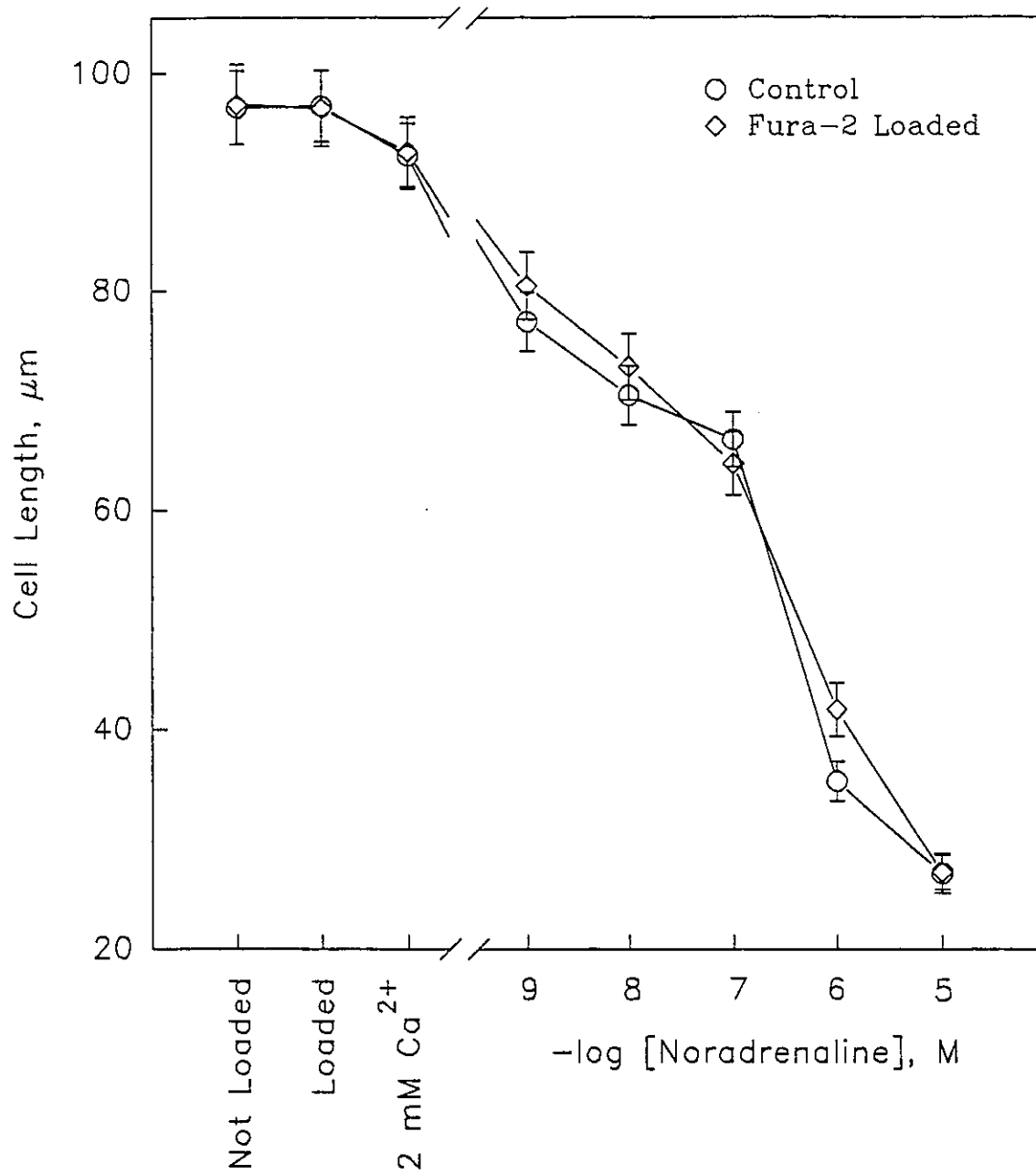
It was important to determine if the presence of fura-2 affected the cell's ability to contract. Figure 6 shows the effect of fura-2 loading on VSMC contraction response to noradrenaline. Contraction of individual VSMCs was measured as a function of cell length. There was no significant difference in the response to noradrenaline between fura-2 loaded and the paired unloaded cells ($n = 80$, 4 preps.).

(I) Background Fluorescence

Prior to calculation of $[\text{Ca}^{2+}]_i$ it was necessary to subtract from the data all signal components not attributable to $[\text{Ca}^{2+}]_i$ -dependent fura-2 fluorescence. This background signal arose mostly from ambient room light reaching the photomultiplier tube. Reduction in background light levels not associated with the single VSMC under study was effected by optically isolating the cell via a home made adjustable shutter. This formed a "window" through which the cell signal could pass and be monitored. After the window was sized, the signal was measured with no cell present within the window. The resultant no cell or "blank" represented the amount of ambient light contributing to background. Comparison of the signal with and without a cell present in the window allowed quick determination of whether the cell was properly loaded with fura-2. At the end of each experiment fura-2 fluorescence was eliminated by the addition of MnCl_2 .

Figure 6. Effect of fura-2 loading on VSMC contraction response

Contraction of individual VSMCs was measured as a function of cell length. Paired study of VSMC response to cumulative doses of noradrenaline between fura-2 loaded versus unloaded cells. Note the slight contraction of cells upon addition of Ca^{2+} . (n= 80, 4 preps).



The resultant "quenched" autofluorescence signal represented the amount of background to be subtracted.

Figure 7 shows the relationship between the 340 nm and 380 nm signals for both the blank and autofluorescence methods of background determination. There was a significant correlation between the blank and autofluorescence values for both the 340 nm ($r = 0.668$, $p < 0.001$, $n = 57$) and 380 nm ($r = 0.613$, $p < 0.001$, $n = 57$) signals. As the size of the window surrounding the cell was increased to accommodate larger cells, the absolute value of both the blank and autofluorescence signals increased (fig. 7B) as expected.

(J) Calculation of $[Ca^{2+}]_i$ from Fluorescence Signals

Fluorescence signals were converted into cell $[Ca^{2+}]_i$ using the equation originally developed by Grynkiewicz *et al.* (1985):

$$[Ca^{2+}]_i = K_d \frac{(R - R_{min})}{(R_{max} - R)} \times \beta$$

where: $[Ca^{2+}]_i$ is intracellular free calcium concentration

K_d is the dissociation constant for the Ca^{2+} -fura-2 complex

R is the ratio of 340 nm/380 nm fluorescent signal

R_{min} is the minimum obtainable fluorescent ratio

R_{max} is the maximum obtainable fluorescent ratio

β is the ratio of minimum/maximum 380 nm signal

Figure 7. Relationship between blank and autofluorescence methods of background determination

Panel A. Plot of the blank signal versus corresponding autofluorescence value for both the 340 and 380 nm signals. There was a significant correlation between the blank and autofluorescence values for both the 340 nm ($r = 0.668$, $p < 0.001$) and 380 nm ($r = 0.613$, $p < 0.001$) signals. ($n=57$).

Panel B. As the size of the window surrounding the cell was increased to accommodate larger cells, the absolute values of both the blank and autofluorescence signals increased.

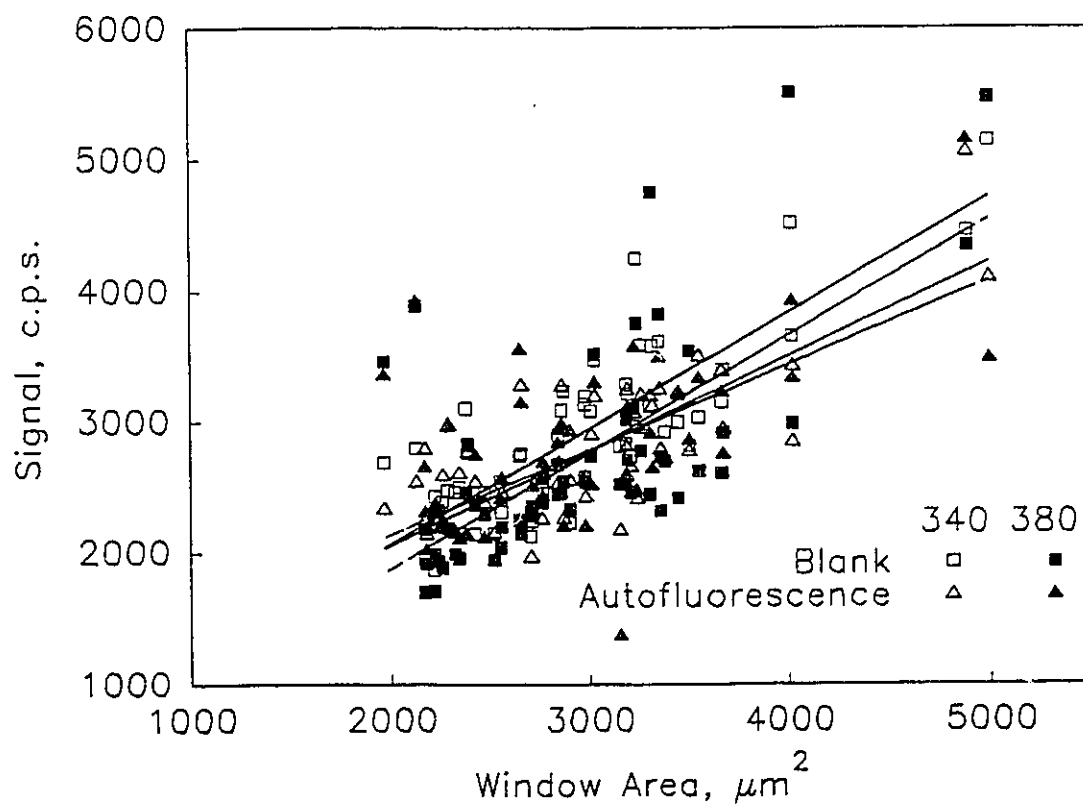
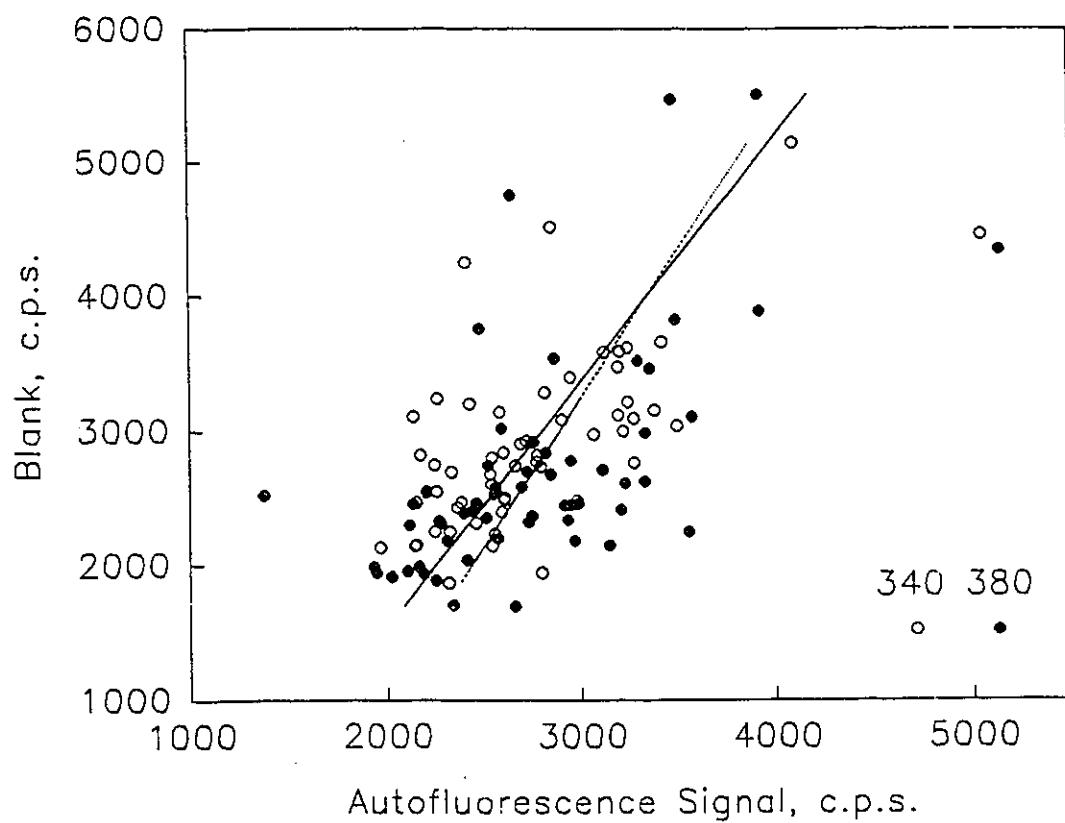


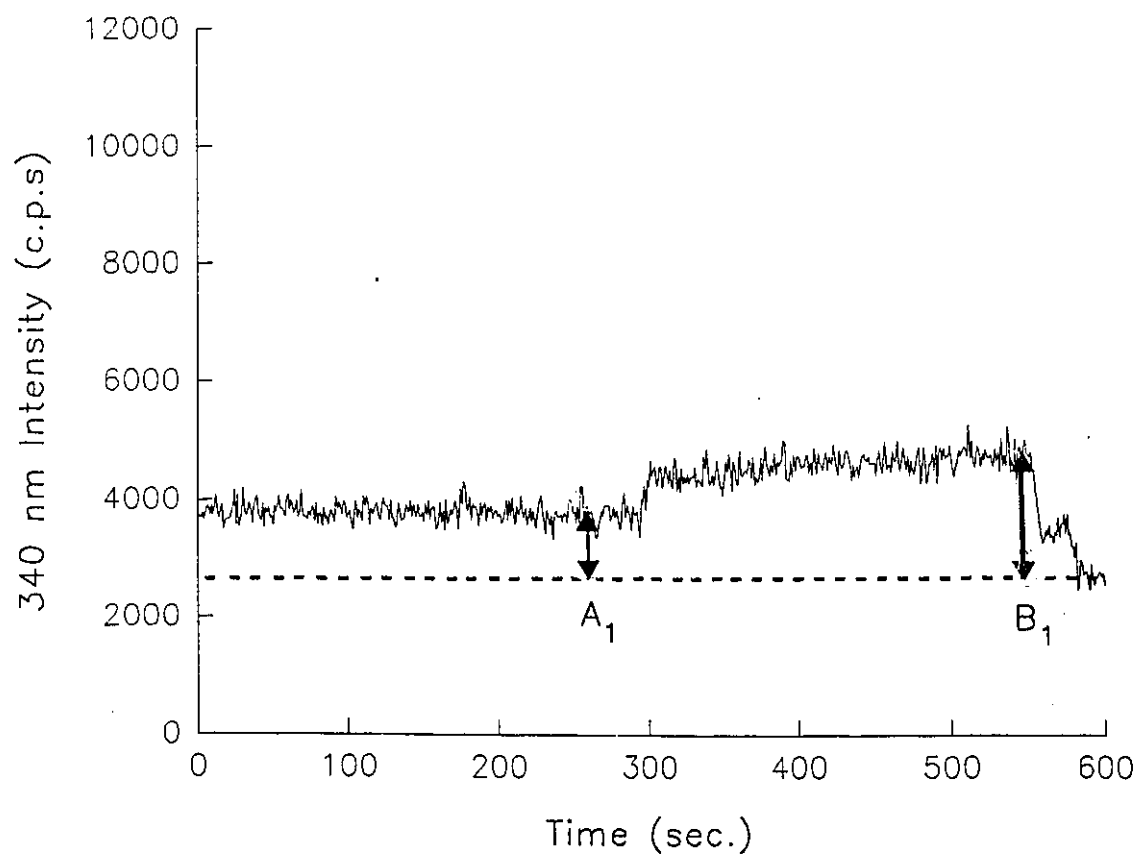
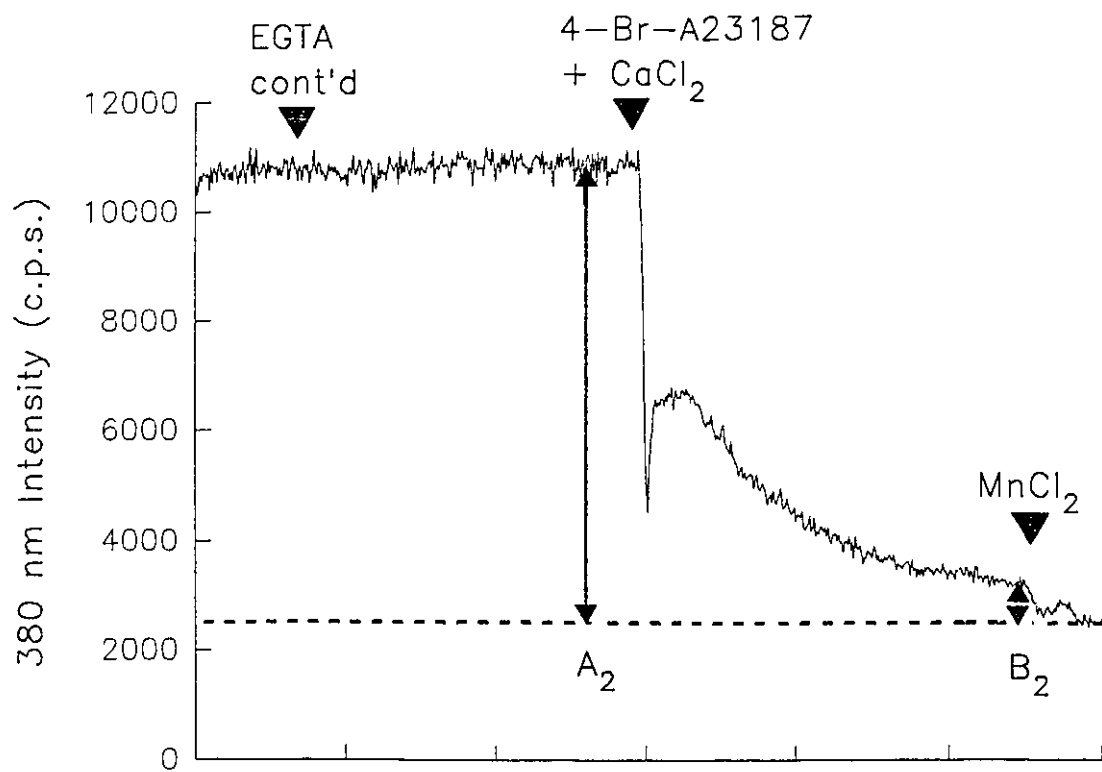
Figure 8 shows an example trace of changes in the 340 nm and 380 nm signals resulting from determination of the R_{min} , R_{max} , β , and autofluorescence values. R_{min} is obtained by perfusing the fura-2 loaded cell in EGTA-Hanks until a stable minimum ratio occurs, usually over a 10-30 minute period. R_{max} is determined by adding 12 μ M 4-Br-A23187 and high Ca^{2+} (10-20 mM $CaCl_2$) and was generally effected within 5 minutes. β represents the dynamic range of the 380 nm signal and is obtained from the R_{min} and R_{max} 380 nm signals. Note that calculation of all ratios and therefore $[Ca^{2+}]_i$ occurs after subtraction of the 340 nm and 380 nm autofluorescence values determined after addition of $MnCl_2$. The $[Ca^{2+}]_i$ values obtained for transient responses represent the maximal values attained during the response. All other $[Ca^{2+}]_i$ values, including resting, tonic, and washout levels, represent an average of the signals obtained over the period of measurement. Values for parameters used in Grynkiewicz's equation to obtain $[Ca^{2+}]_i$ were collected during the study (n = 58) and are as follows: $R_{min} = 0.126 \pm 0.003$; $R_{max} = 2.073 \pm 0.071$; $\beta = 7.4 \pm 0.3$. A K_d of 153 nM was determined using the standard curve studies discussed above.

(K) Cell Length Analysis

Single VSMC lengths were measured using the mid-line distance from tip to tip using a digitizing tablet (Jandel Scientific 2210) and Sigma Scan Version 2.5 software. Cell lengths were calculated by taking a video image of known length from a calibrated glass slide. The experimental setup determined that either fluorescence or video images,

Figure 8. Determination of R_{min} , R_{max} , β , and autofluorescence values

The R_{min} , R_{max} , β , and autofluorescence values are obtained at the end of each experiment. Superfusion of the cell with EGTA-Hanks buffer results in a maximal increase in the 380 nm signal (A_2) and a corresponding maximal decrease in the 340 nm signal (A_1). The ratio of 340 nm/380 nm (A_1/A_2) gives R_{min} . Addition of 12 μ M 4-Br-A23187 and 10-20 mM CaCl_2 produces the opposite effect, producing a maximal decrease in the 380 nm signal (B_2) and a maximal increase in the 340 nm signal (B_1). The ratio of B_1/B_2 gives R_{max} . The dynamic range of the 380 nm signal, expressed as β , is obtained from A_2/B_2 . Addition of 2 mM MnCl_2 quenches all fluorescence. The remaining 340 nm and 380 nm signals, known as a autofluorescence, are subtracted from each of the 340 nm and 380 nm signals prior to calculation of any of the above ratios.



but not both, could be observed at any particular moment. Therefore, pictures were taken only after cell $[Ca^{2+}]_i$ had stabilized, and for the briefest period of time possible.

(L) Statistical Analysis

Results are expressed as the mean $\pm$ standard error. Student's t-test was employed to determine significance with values of $p < 0.05$ considered to be statistically significant. One way ANOVA was used to analyze variations between experimental groups. The program Sigma Stat 1.01 was used for statistical analyses. Data were prepared graphically by using the Sigma Plot 5.0 program.

RESULTS

Part I. The Caffeine-Sensitive Ca^{2+} Store in VSMCs

(A) Effect of Caffeine on Vascular Smooth Muscle Cells (VSMCs)

The mean resting length of VSMCs used in this study was $134 \pm 3 \mu\text{m}$ ($n = 58$). The mean resting $[\text{Ca}^{2+}]_i$ in these cells was $44 \pm 3 \text{ nM}$ ($n = 58$). Figure 9 shows the effects of caffeine on cell length and $[\text{Ca}^{2+}]_i$. Each caffeine challenge was separated by a washout period of at least 5 minutes. The initial length of the cell was $142 \mu\text{m}$ and the resting $[\text{Ca}^{2+}]_i$ was 42 nM . Addition of 0.05 mM caffeine caused no significant change in cell length or $[\text{Ca}^{2+}]_i$. At 0.2 mM caffeine, there was no change in cell length although a small transient increase in $[\text{Ca}^{2+}]_i$ to 68 nM was observed. At 0.6 mM caffeine the cell length shortened to $116 \mu\text{m}$. $[\text{Ca}^{2+}]_i$ increased transiently to 138 nM and declined more gradually. A caffeine concentration of 2.0 mM further shortened the cell to $81 \mu\text{m}$. There was a transient $[\text{Ca}^{2+}]_i$ increase to 177 nM . At this caffeine concentration a sustained, or tonic, increase in $[\text{Ca}^{2+}]_i$ to 114 nM was maintained following the transient increase. At 10.0 mM caffeine the cell shortened to $62 \mu\text{m}$. Both the transient and tonic $[\text{Ca}^{2+}]_i$ increased to 383 nM and 124 nM respectively.

Following application of 10.0 mM caffeine the mean length of VSMCs decreased to $57 \pm 2 \mu\text{m}$ ($44 \pm 1 \%$ of the resting length, $n = 32$). This is $86 \pm 2 \%$ of the maximum contraction of $45 \pm 2 \mu\text{m}$ ($35 \pm 2 \%$ of the resting length, $n = 32$) obtained upon exposure of the cells to Ca^{2+} ionophore 4-Br-A23187 and high Ca^{2+} ($10\text{-}20 \text{ mM}$ CaCl_2). Results of 38 cells following application of 10 mM caffeine are described in figure 10. The mean transient $[\text{Ca}^{2+}]_i$ increase (panel A) was $392 \pm 24 \text{ nM}$ ($n = 38$).

Figure 9. Trace showing effect of caffeine on cell length and $[Ca^{2+}]_i$

Panel A. Contraction of single VSMC in response to single dose (0.05 to 10.0 mM) increases in caffeine. The first picture represents the length (142 μ m) prior to caffeine challenge. Each cell is shown bordered by a size-adjusted "window" used to optically isolate the cell. Cell lengths were digitized by tracing the mid-line cell length from tip to tip. A wash period in excess of five minutes was allowed between caffeine challenges.

Panel B. Corresponding $[Ca^{2+}]_i$ changes in the same cell. The resting $[Ca^{2+}]_i$ was 42 nM. The initial response to caffeine was a transient $[Ca^{2+}]_i$ increase which decayed back to the resting level even in the continued presence of caffeine. At higher caffeine concentrations a sustained, or tonic, increase was observed following the transient increase. After washing the $[Ca^{2+}]_i$ level returned to the resting level.

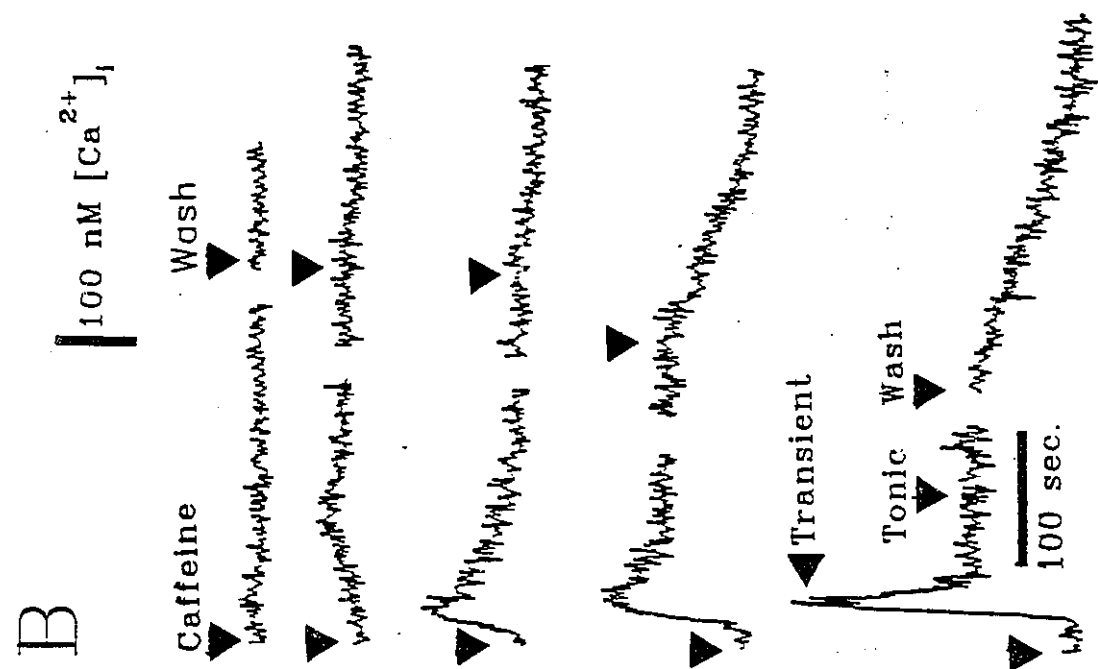
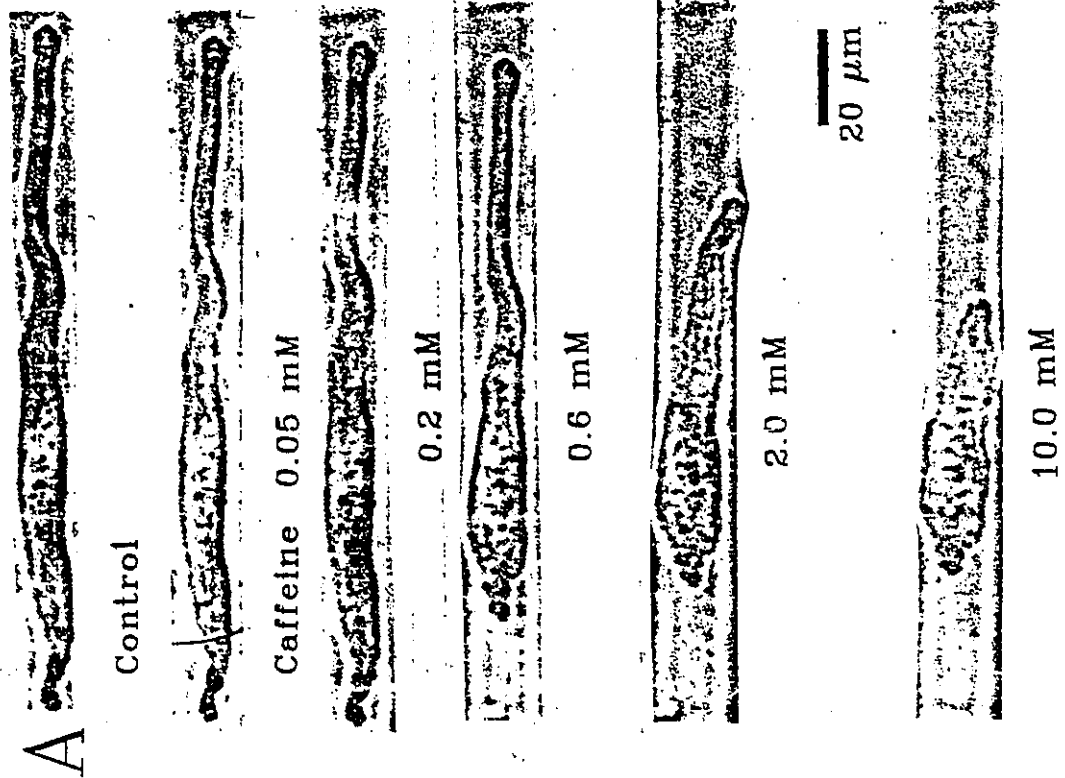
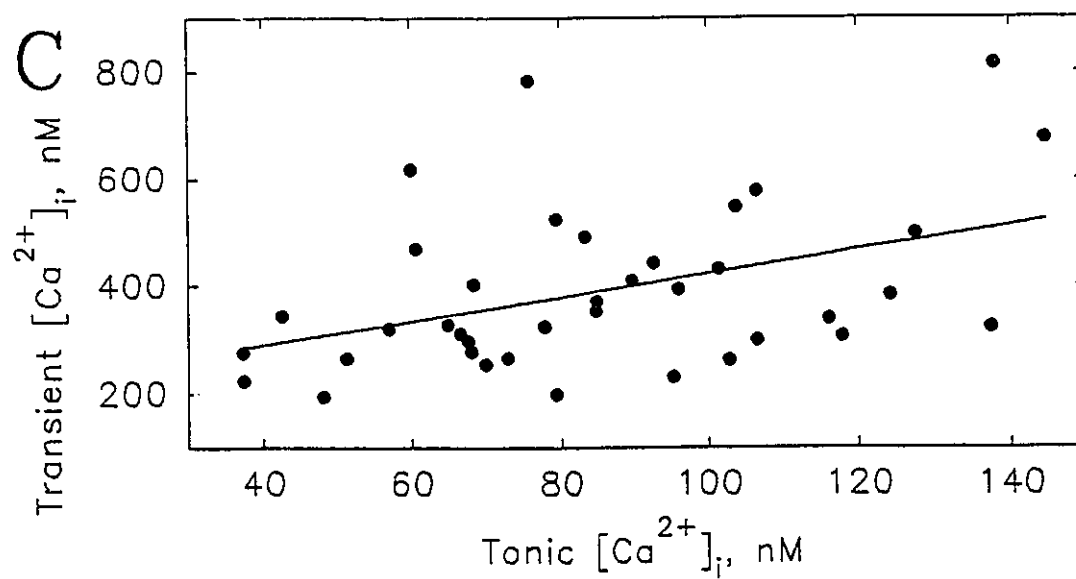
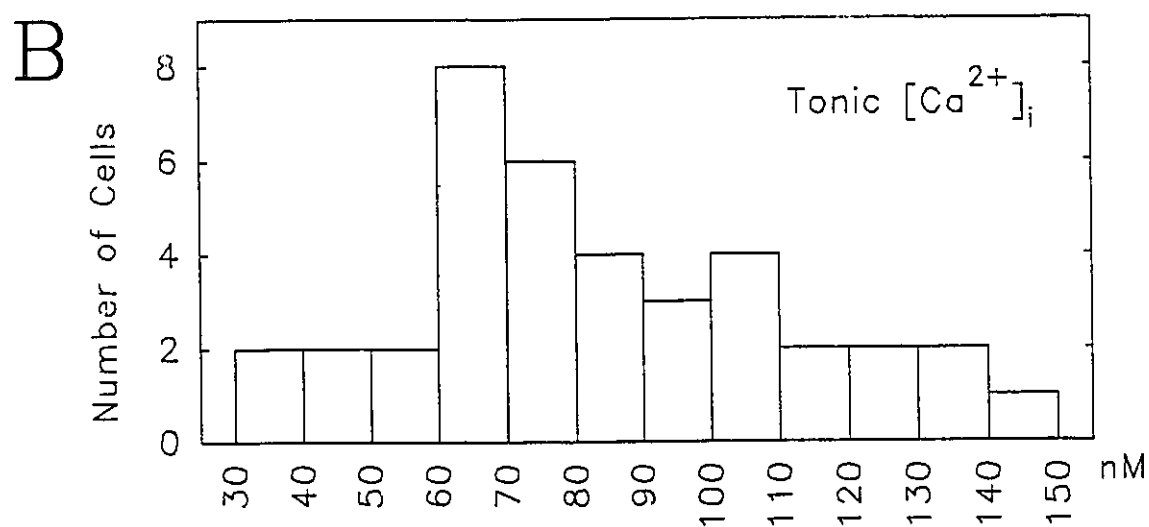
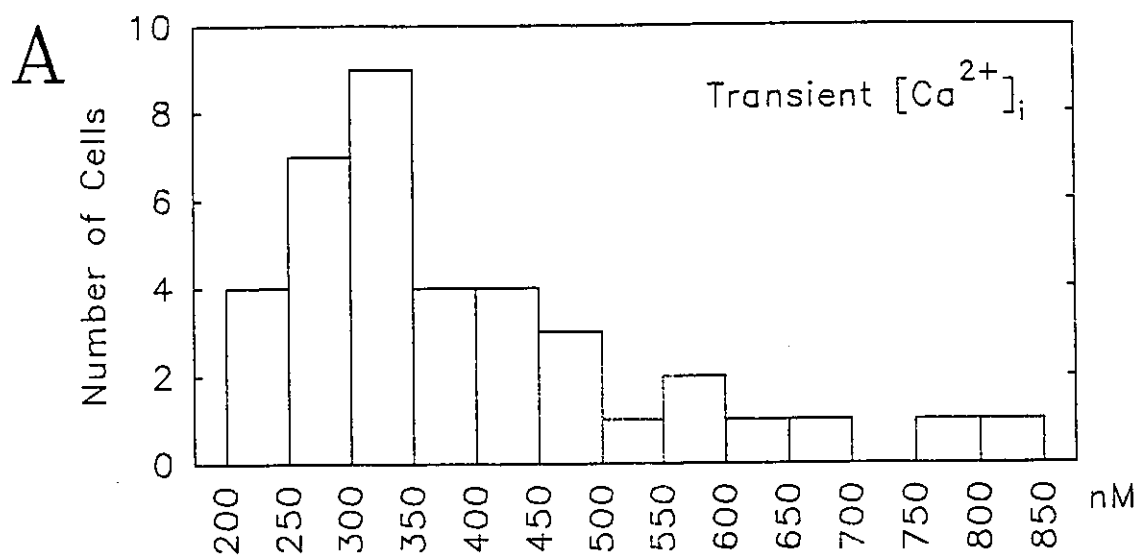


Figure 10. Graph of the $[Ca^{2+}]_i$ transient and tonic response to caffeine

Panel A. Frequency distribution for transient $[Ca^{2+}]_i$ in response to 10 mM caffeine. Resultant $[Ca^{2+}]_i$ transients were segregated into 50 nM increments, and the frequency of occurrence counted for each. The distribution of transient $[Ca^{2+}]_i$ responses ranged from 200 to 815 nM, with a median of 306 nM and a mode of 300 - 350 nM. The mean transient $[Ca^{2+}]_i$ increase was 392 ± 24 nM ($n = 38$). Note that the distribution is skewed in favour of the lower ranges.

Panel B. Frequency distribution for tonic $[Ca^{2+}]_i$ in response to 10 mM caffeine. Tonic $[Ca^{2+}]_i$ responses were divided into steps of 10 nM, and the frequency of occurrence counted for each. The distribution of tonic $[Ca^{2+}]_i$ responses ranged from 37 to 145 nM, with a median of 54 nM and a mode of 60 - 70 nM. The mean tonic $[Ca^{2+}]_i$ increase (panel B) was 85 ± 5 nM ($n = 38$).

Panel C. The transient $[Ca^{2+}]_i$ component was plotted as a function of the associated tonic $[Ca^{2+}]_i$ in response to 10 mM caffeine. A correlation coefficient of 0.414 ($p = 0.01$) was calculated. The equation of the fitted line is: transient $[Ca^{2+}]_i = 203 + 2.2 \times \text{tonic } [Ca^{2+}]_i$. ($n = 38$).



The distribution of transient $[Ca^{2+}]_i$ responses ranged from 200 to 815 nM, with a median of 306 nM and a mode of 300 - 350 nM. The mean tonic $[Ca^{2+}]_i$ increase (panel B) was 85 ± 5 nM ($n = 38$). Distribution of tonic $[Ca^{2+}]_i$ responses ranged from 37 to 145 nM, with a median of 54 nM and a mode of 60 - 70 nM. There was a significant ($p = 0.01$) correlation ($r = 0.414$) between the transient and tonic $[Ca^{2+}]_i$ increase (panel C). Following a washout period the cell $[Ca^{2+}]_i$ returned to 28 ± 1 nM ($n = 38$), with a range from 14 - 57 nM.

Figure 11 shows the relationship between peak $[Ca^{2+}]_i$ and length of VSMCs during the contraction response to caffeine. There was a significant ($p < 0.001$) correlation ($r = 0.696$) between highest $[Ca^{2+}]_i$ reached and the degree of cell shortening. The threshold $[Ca^{2+}]_i$ level required to produce cell shortening was 53 nM. Maximal shortening occurred at $[Ca^{2+}]_i = 326$ nM. Half maximal shortening occurred at $[Ca^{2+}]_i = 189$ nM.

(B) Effect of Repeated Caffeine Challenge on $[Ca^{2+}]_i$ and Cell Length

Single vascular smooth muscle cells do not relax after contraction, even with prolonged washing. The next series of experiments was designed to test if the $[Ca^{2+}]_i$ response to caffeine is dependent on the length of the cell. The cell shown in figure 9 remained shortened at 62 μ m after washing for more than 10 minutes following the first series of caffeine challenges. Both transient and tonic $[Ca^{2+}]_i$ responses to a second set of caffeine challenges to the same cell were not significantly affected by previous exposure to caffeine (fig. 12). For example, a small transient increase in $[Ca^{2+}]_i$ to 48

Figure 11. Graph of VSMC shortening and peak $[Ca^{2+}]_i$ relationship

Plot showing the relationship between highest $[Ca^{2+}]_i$ reached and percent VSMC shortening. A correlation coefficient of 0.696 ($p < 0.001$) was calculated. The threshold $[Ca^{2+}]_i$ level required to produce cell shortening was 53 nM. Maximal shortening occurred at $[Ca^{2+}]_i = 326$ nM. Half maximal shortening occurred at $[Ca^{2+}]_i = 189$ nM. The plot represents 130 measurements from a total of 22 cell preparations. Linear regression analysis was performed using only the intermediate data points between 0 % and 100 % cell shortening, representing 86 measurements from 22 cell preparations. The mean resting $[Ca^{2+}]_i$ was 45 ± 2 nM; the resting mean cell length was 134 ± 3 μ m, and the maximal contracted length observed was 45 ± 2 μ m.

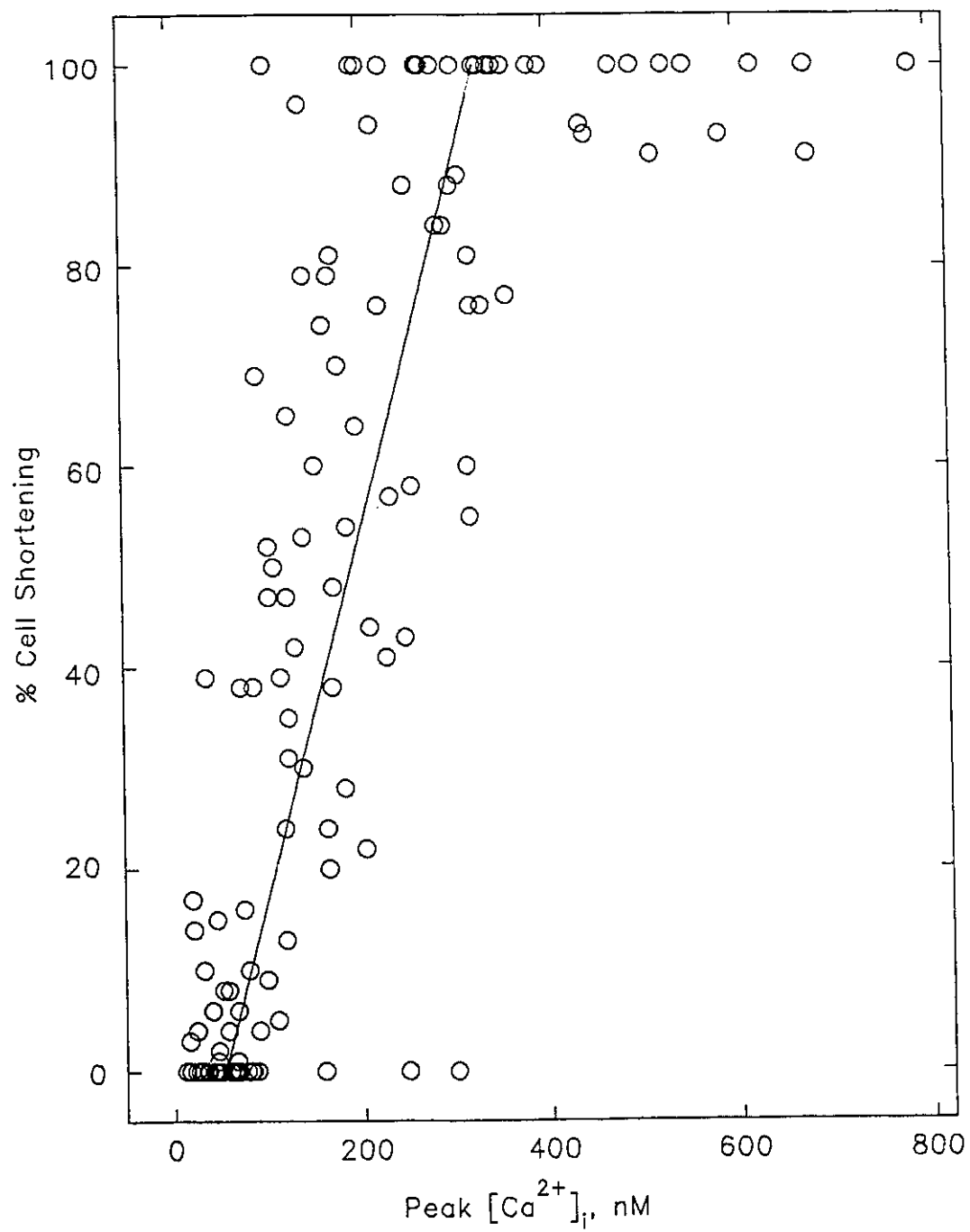


Figure 12. Trace showing effect of repeated caffeine challenge on cell length and $[Ca^{2+}]_i$

Panel A. Contraction of VSMC previously exposed to caffeine (see fig. 9) in response to a second set of 0.05 to 10.0 mM caffeine challenges. The first video printout shows the cell remained contracted (62 μm) from the first caffeine challenge series, even with prolonged washing (> 10 minutes). The cell shortened further to 52 μm in response to the second set of caffeine challenges.

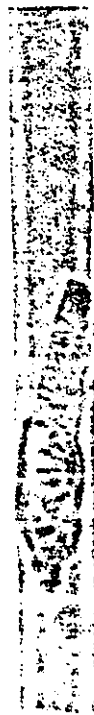
Panel B. Corresponding $[Ca^{2+}]_i$ changes in the same cell. Even though the cell remained contracted, the resting $[Ca^{2+}]_i$ returned to 20 nM after washing. Both transient and tonic $[Ca^{2+}]_i$ responses to the second set of caffeine challenges were not significantly affected by previous exposure to the first caffeine challenge set. The trace following the break in each trace represents the return to resting $[Ca^{2+}]_i$ levels following washout of caffeine.



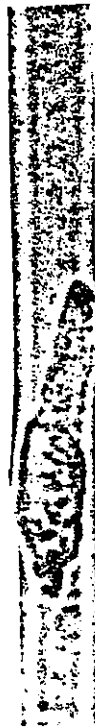
Control



Caffeine 0.05 mM



0.2 mM



0.6 mM

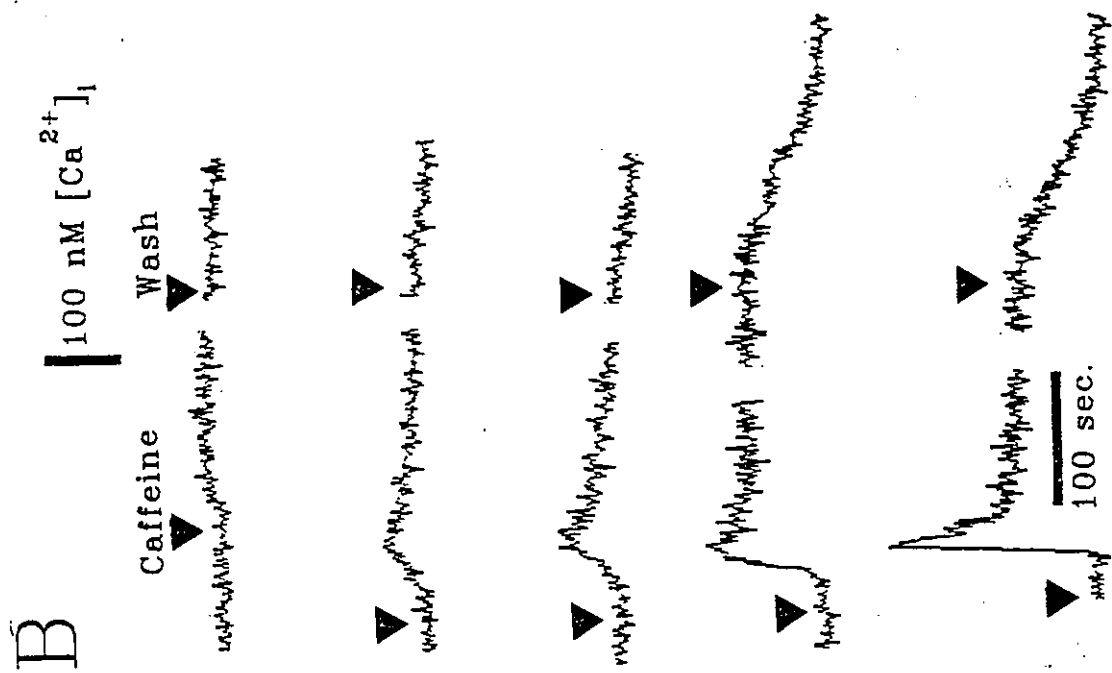


2.0 mM

20 μ m



10.0 mM



nM was observed upon challenge with the second 0.05 mM caffeine concentration. Again, at higher caffeine concentrations (2.0 and 10.0 mM) a tonic $[Ca^{2+}]_i$ increase followed the $[Ca^{2+}]_i$ transient. Upon washing the resting cell $[Ca^{2+}]_i$ could be restored to pre-challenge levels. Following application of 10 mM caffeine, the cell shortened further to 52 μ m. Figure 13 presents results of five cells comparing the effects of caffeine on the dose-response relationship from the two series of experiments described above. There were no significant differences in the increase in transient and tonic $[Ca^{2+}]_i$ between the control and second caffeine dose-response sets, even though the cells were already contracted in the second set. Thus changes in $[Ca^{2+}]_i$ in response to caffeine are not affected by repeated caffeine challenge or cell length.

(C) Effect of Cumulative Addition of Caffeine

Figure 14 illustrates results of cumulative addition (i.e no washing between increasing doses) of 0.05 mM to 10.0 mM caffeine to a single VSMC. The initial length of the cell was 144 μ m. Resting $[Ca^{2+}]_i$ observed prior to caffeine challenge was 23 nM. Upon addition of 0.05 mM caffeine, there were no significant changes in length or $[Ca^{2+}]_i$. After five minutes, the caffeine concentration was increased to 0.2 mM. The cell responded by shortening to 75 μ m and $[Ca^{2+}]_i$ increased transiently to 136 nM. Raising caffeine concentration to 0.6 mM further shortened the cell length to 55 μ m. A transient $[Ca^{2+}]_i$ increase to 124 nM was also observed. An increase in caffeine concentration to 2.0 mM invoked a large transient $[Ca^{2+}]_i$ increase of 370 nM followed by maintenance of tonic $[Ca^{2+}]_i$ to 42 nM. There was no further shortening of the cell

Figure 13. Graph of $[Ca^{2+}]_i$ and cell length changes in response to repeated caffeine challenges

Panel A. Comparison of the effect of caffeine on $[Ca^{2+}]_i$ in the control and 2nd set of caffeine challenges. Results are presented as increases in $[Ca^{2+}]_i$ above and beyond $[Ca^{2+}]_i$ washout levels. There were no significant differences in either transient or tonic $[Ca^{2+}]_i$ increases between the control and second caffeine dose-response sets ($n = 5$).

Panel B. Comparison of cell length changes between the control and 2nd set of caffeine challenges. The initial cell length prior to caffeine challenge in the control set was $135 \pm 3 \mu m$ ($n = 5$). Note that the cells were already contracted at the start of the 2nd set of caffeine challenges.

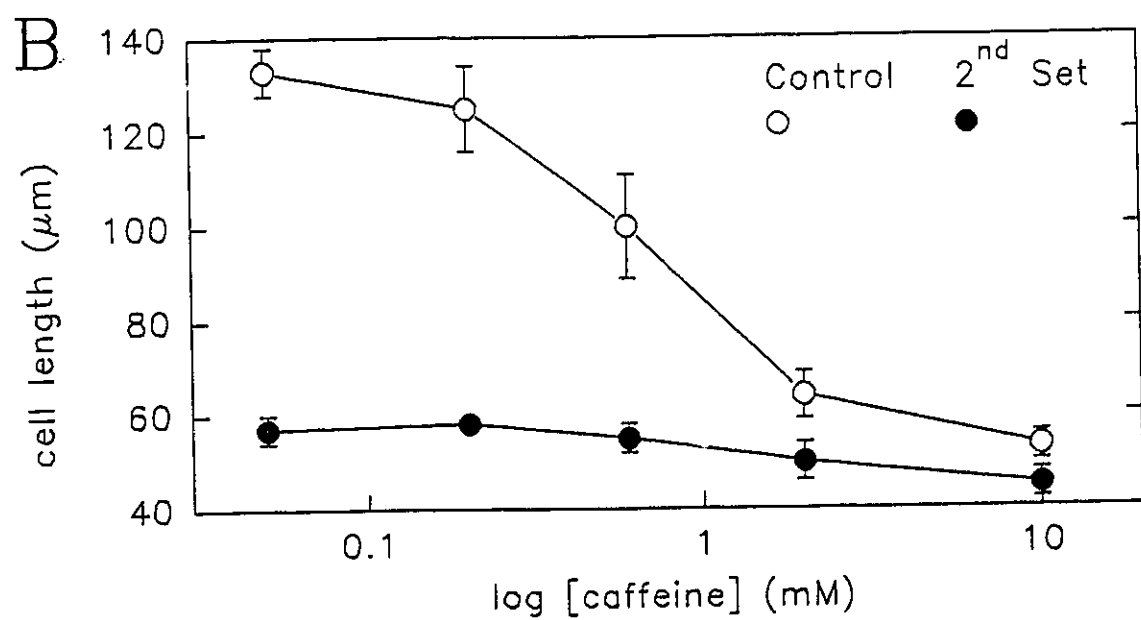
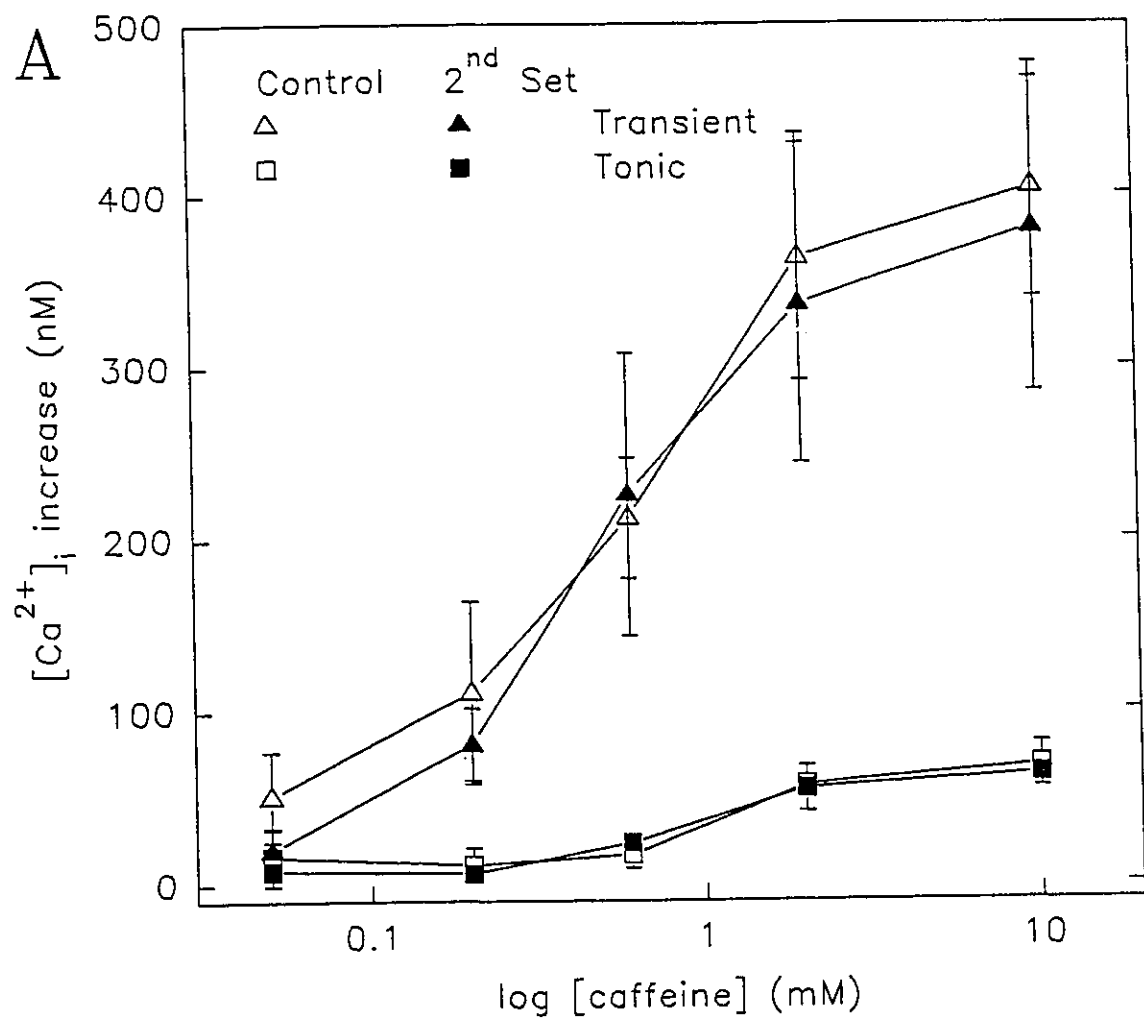


Figure 14. Trace showing effect of cumulative caffeine increases on cell length and $[Ca^{2+}]_i$

Panel A. Contraction of VSMC in response to cumulative (0.05 to 10.0 mM) increases in caffeine. VSMCs were not allowed to "recover" by denying the usual five minute wash between increasing caffeine challenges. The first picture represents the initial length (144 μ m) of the cell before application of caffeine.

Panel B. Corresponding change in single VSMC $[Ca^{2+}]_i$ in response to cumulative concentrations of caffeine. The initial $[Ca^{2+}]_i$ level was 23 nM. The trace following the break in the 10.0 mM caffeine trace represents the return to resting $[Ca^{2+}]_i$ levels (23 nM) following washout of caffeine. Note that the transient $[Ca^{2+}]_i$ rise in the 10.0 mM caffeine challenge was smaller than the 2.0 mM caffeine challenge.

A



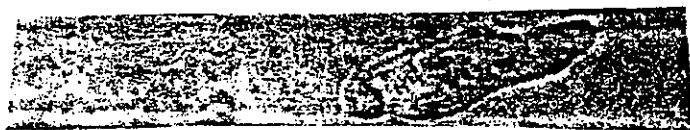
Control



Caffeine 0.05 mM



0.2 mM



0.6 mM



2.0 mM

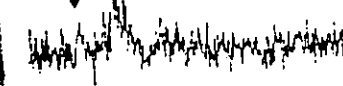
20 μm 

10.0 mM

B

100 nM $[\text{Ca}^{2+}]_i$

Caffeine



Wash



100 sec.

length (54 μm). At 10.0 mM caffeine, there was a reduction in the transient $[\text{Ca}^{2+}]_i$ response to 147 nM.

Figure 15 is a plot of both transient and tonic $[\text{Ca}^{2+}]_i$ increase (panel A) and cell length (panel B) as a function of cumulative caffeine concentration. Similar to that observed with single doses of caffeine, cumulative additions shortened the VSMC from $139 \pm 9 \mu\text{m}$ to a final length of $56 \pm 15 \mu\text{m}$ ($n = 4$). Unlike that observed with single doses, the greatest increases in transient and tonic $[\text{Ca}^{2+}]_i$ occurred at 2.0 mM caffeine, with elevations of $229 \pm 36 \text{ nM}$ and $14 \pm 7 \text{ nM}$ respectively. The 10.0 mM caffeine response was smaller than the preceding response with transient $[\text{Ca}^{2+}]_i$ rising to only $113 \pm 15 \text{ nM}$ while the tonic $[\text{Ca}^{2+}]_i$ increased to $15 \pm 4 \text{ nM}$.

(D) Effect of Ca^{2+} -Free Extracellular Environment on Caffeine Response

Figure 16 is a representative $[\text{Ca}^{2+}]_i$ trace illustrating the effect of removing Ca^{2+} from the VSMC extracellular environment. Caffeine (10 mM) induced a transient $[\text{Ca}^{2+}]_i$ increase from 37 nM to a maximum of 263 nM, followed by a tonic $[\text{Ca}^{2+}]_i$ increase to 66 nM. Washing brought $[\text{Ca}^{2+}]_i$ to the pre-challenge level of 37 nM. Following a five minute transition to Ca^{2+} -free solution containing 1 mM EGTA, resting $[\text{Ca}^{2+}]_i$ dropped to 20 nM where it remained stable (range 20-23 nM) for the rest of the experiment. The first caffeine challenge in Ca^{2+} -free solution evoked a maximum transient $[\text{Ca}^{2+}]_i$ to 132 nM. After washing in Ca^{2+} -free solution, the second caffeine challenge failed to elicit any increase in $[\text{Ca}^{2+}]_i$ levels. Reintroduction of Ca^{2+} to the external media restored the caffeine-induced increase in the $[\text{Ca}^{2+}]_i$ transient to a

Figure 15. Graph of $[Ca^{2+}]_i$ and cell length changes in response to cumulative caffeine increases

Panel A. Increase in transient and tonic $[Ca^{2+}]_i$ in response to cumulative caffeine increases (0.05 to 10.0 mM). There was no wash between caffeine doses. Basal $[Ca^{2+}]_i$ was 28 ± 3 nM ($n = 4$). Results are presented as increase in $[Ca^{2+}]_i$ above the previous tonic level. Final $[Ca^{2+}]_i$ following washout of caffeine was 25 ± 3 nM.

Panel B. Corresponding changes in cell length associated with the above changes in $[Ca^{2+}]_i$. The initial cell length prior to caffeine challenge was 139 ± 9 μ m ($n = 4$). Maximum contraction (66 ± 14 μ m, $n = 4$) occurred after challenge with 2.0 mM caffeine.

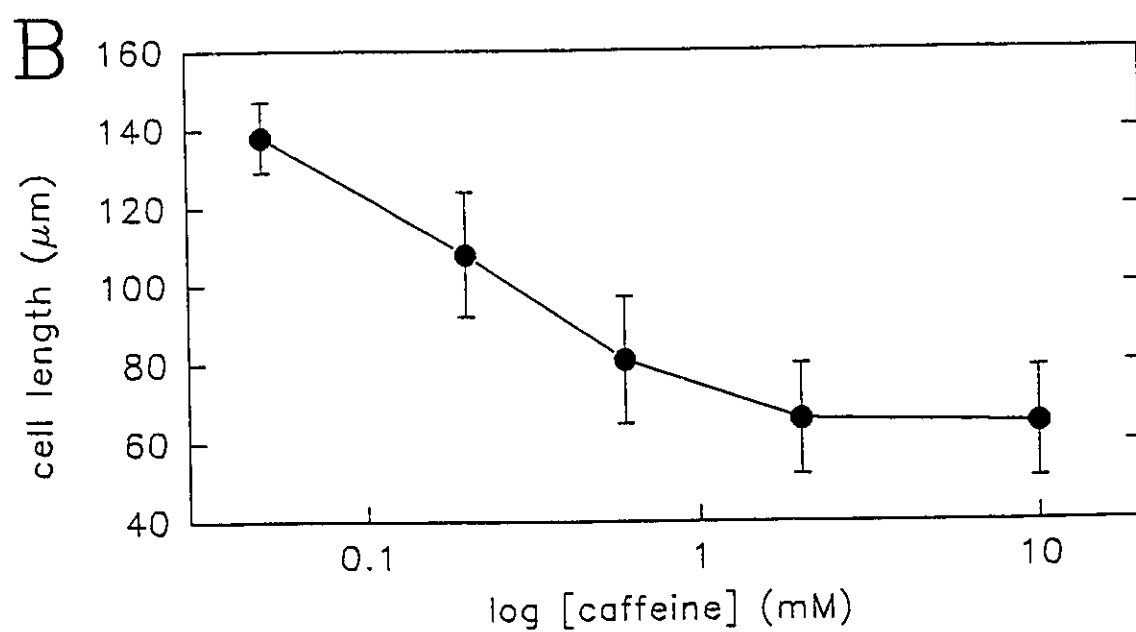
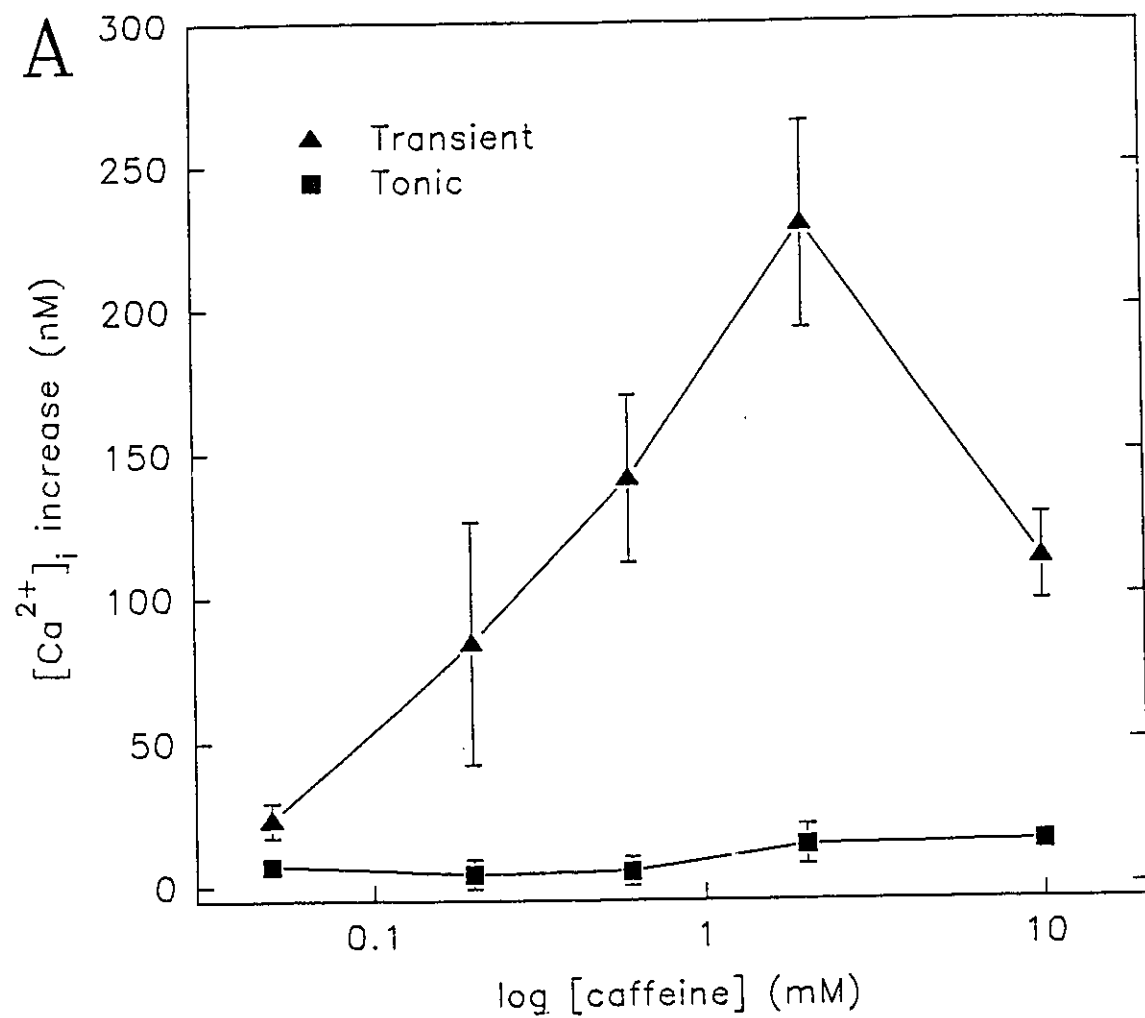
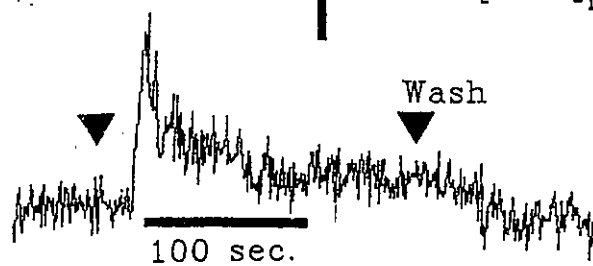


Figure 16. Trace showing effect of removal of extracellular Ca^{2+} on VSMC response to caffeine challenge

The control trace illustrates transient and tonic $[\text{Ca}^{2+}]_i$ increases following application of caffeine and the return to resting $[\text{Ca}^{2+}]_i$ levels following washout. Ca^{2+} -Hanks was then replaced with EGTA-Hanks for a five minute period. The second trace shows the response to caffeine in Ca^{2+} -free conditions. The $[\text{Ca}^{2+}]_i$ transient was reduced and there was an absence of any tonic $[\text{Ca}^{2+}]_i$ component. The $[\text{Ca}^{2+}]_i$ increase was abolished by a 2nd caffeine challenge in Ca^{2+} -free solution (3rd trace). The final scan shows the return to typical responses following re-addition of Ca^{2+} . The basal $[\text{Ca}^{2+}]_i$ was 37 nM and the final washout $[\text{Ca}^{2+}]_i$ was 23 nM. All four traces are from the same cell.

Control

100 nM $[Ca^{2+}]_i$



1st Caffeine in Ca^{2+} -free



2nd Caffeine in Ca^{2+} -free



Caffeine in Ca^{2+}



maximum of 257 nM, and the tonic $[Ca^{2+}]_i$ to 73 nM. The resting $[Ca^{2+}]_i$ also recovered to the pre-challenge level of 37 nM.

Figure 17 presents results of five cells comparing effects of Ca^{2+} removal on the resting $[Ca^{2+}]_i$ and the increase in $[Ca^{2+}]_i$ to 10 mM caffeine. In Ca^{2+} -free solution the basal $[Ca^{2+}]_i$ was reduced significantly from the control level of 45 ± 6 nM to 21 ± 4 nM ($p = 0.008$, $n = 10$). Reintroduction of extracellular Ca^{2+} restored $[Ca^{2+}]_i$ to 63 ± 15 nM ($n = 10$). In Ca^{2+} -free solution, response to the first 10 mM caffeine challenge resulted in a small but insignificant decrease in the $[Ca^{2+}]_i$ transient from the control level of 327 ± 27 nM ($n = 10$) to 259 ± 54 nM ($n = 10$) (fig. 17). After washing in Ca^{2+} -free solution, the second caffeine challenge failed to elicit a $[Ca^{2+}]_i$ increase. The transient $[Ca^{2+}]_i$ component was restored after reintroduction of extracellular Ca^{2+} to 296 ± 38 nM. Tonic $[Ca^{2+}]_i$ increase in response to 10 mM caffeine was also affected by removal of extracellular Ca^{2+} , but in a different way. Removal of extracellular Ca^{2+} abolished the tonic $[Ca^{2+}]_i$ increase in both the first ($p = 0.001$) and second ($p = 0.0001$) caffeine challenge (fig. 17). Reintroduction of extracellular Ca^{2+} restored the tonic $[Ca^{2+}]_i$ component.

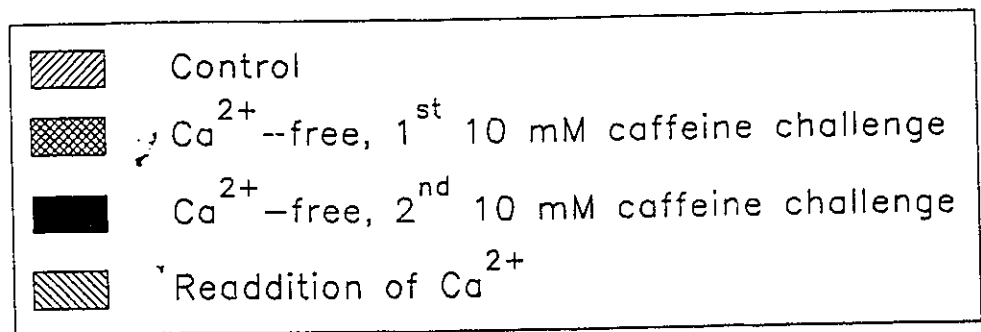
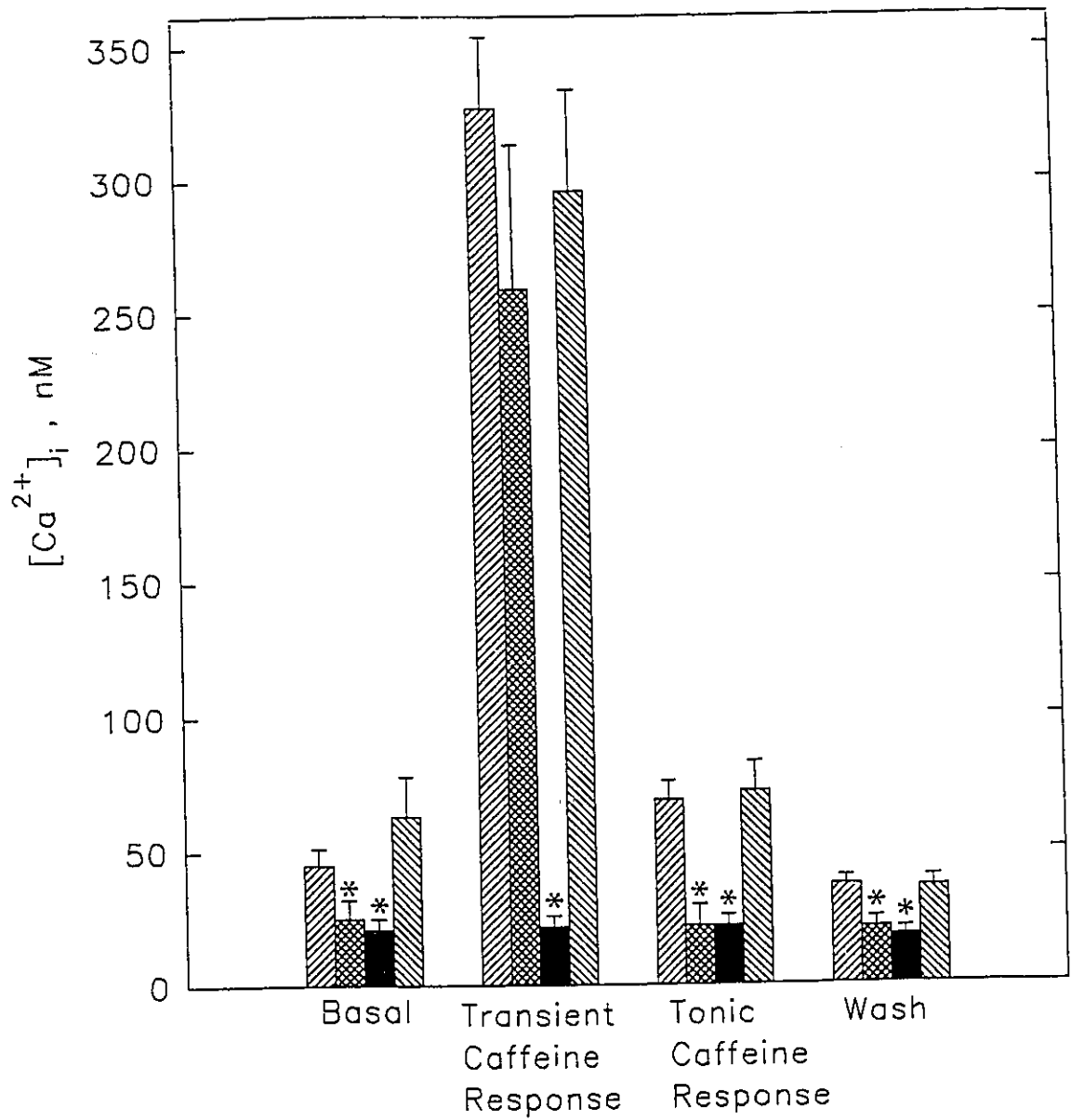
Part II. Drug Effects on the Caffeine-Sensitive Ca^{2+} Store in VSMCs

(A) Effect of Ryanodine on Caffeine Dose-Response in VSMCs

Application of $10 \mu M$ ryanodine to vascular smooth muscle cells for a minimum 5 minute period did not significantly change either resting $[Ca^{2+}]_i$ (control = 42 ± 5 nM; ryanodine-treated = 51 ± 11 nM, $n = 4$) or cell length (control = $156 \pm 3 \mu m$;

Figure 17. Graph of $[Ca^{2+}]_i$ increases in response to caffeine: Dependence on extracellular Ca^{2+}

The results of five cells are shown indicating changes in $[Ca^{2+}]_i$ in response to 10.0 mM caffeine with and without extracellular Ca^{2+} . Basal, transient, tonic, and washout $[Ca^{2+}]_i$ levels are shown for control and three different treatments. The first caffeine challenge following removal of extracellular Ca^{2+} resulted in $[Ca^{2+}]_i$ returning to basal levels following a slightly reduced transient response. No tonic $[Ca^{2+}]_i$ component was present. A second caffeine challenge in the absence of extracellular Ca^{2+} eliminated both the transient and tonic $[Ca^{2+}]_i$ components of the caffeine response. Removal of extracellular Ca^{2+} also results in a reduction in the resting Ca^{2+} level. Re-addition of Ca^{2+} to the extracellular environment enabled a return of transient, tonic, and resting $[Ca^{2+}]_i$ to typical values. Note that results are plotted as absolute $[Ca^{2+}]_i$, thus the resting $[Ca^{2+}]_i$ level is included in each response.



ryanodine-treated = $152 \pm 8 \mu\text{m}$, $n = 4$). However, ryanodine significantly reduced the $[\text{Ca}^{2+}]_i$ transient component in response to caffeine (fig. 18). The transient $[\text{Ca}^{2+}]_i$ increase resulting from 0.6 mM caffeine was reduced from 91 ± 9 to 8 ± 3 nM ($p = 0.0008$, $n = 4$). Similarly, the 2.0 mM caffeine $[\text{Ca}^{2+}]_i$ transient was reduced from 155 ± 18 nM to 25 ± 7 nM ($p = 0.009$, $n = 4$). The $[\text{Ca}^{2+}]_i$ transient response to 10 mM caffeine decreased from 234 ± 22 nM to 13 ± 12 nM ($p = 0.006$, $n = 4$). Tonic $[\text{Ca}^{2+}]_i$ was not significantly changed.

To study the effects of 10 μM ryanodine treatment on contraction response to caffeine, cells that had not been prechallenged, and thus not precontracted, were used. In the presence of 10 μM ryanodine (fig. 19) contraction of the cells decreased, with cell lengths shortening to only $64\% \pm 3\%$ of original length ($156 \pm 2 \mu\text{m}$ to $99 \pm 6 \mu\text{m}$, $n = 4$) at 10 mM caffeine. This is significantly ($p = 0.001$) less than the $48\% \pm 1\%$ decrease in control cells ($127 \pm 7 \mu\text{m}$ to $61 \pm 3 \mu\text{m}$, $n = 4$) (fig. 18B). The transient and tonic $[\text{Ca}^{2+}]_i$ increases to caffeine were significantly depressed (fig. 19A).

(B) Effect of TMB-8 on Caffeine Dose-Response in VSMCs

The addition of 10 μM TMB-8 for a minimum 5 minute period did not significantly change either the resting $[\text{Ca}^{2+}]_i$ (40 ± 7 nM versus 48 ± 7 nM, $n = 4$) or cell length ($128 \pm 4 \mu\text{m}$ versus $127 \pm 4 \mu\text{m}$, $n = 4$). TMB-8 (10 μM) did not significantly affect either transient or tonic $[\text{Ca}^{2+}]_i$ increase in response to 0.05 to 10.0 mM caffeine (fig. 20).

Figure 18. Graph of effect of ryanodine on caffeine-induced changes in $[Ca^{2+}]_i$ and cell length

Panel A. After control responses to caffeine were obtained, VSMCs were incubated with 10 μ M ryanodine for five minutes prior to each caffeine challenge in the 2nd set. Cells were washed between each caffeine dose. Ryanodine produced a significant decrease ($p < 0.01$) in the VSMC response to caffeine for the 0.6, 2.0, and 10.0 mM doses of caffeine. The resting cell $[Ca^{2+}]_i$ for the ryanodine treatment was 27 ± 2 nM and was not significantly different from the $[Ca^{2+}]_i = 55 \pm 15$ nM of the control.

Panel B. Resultant changes in cell length associated with the above changes in $[Ca^{2+}]_i$. The initial cell length prior to caffeine challenge was 127 ± 7 μ m ($n = 4$). Cell lengths did not shorten further following ryanodine treatment.

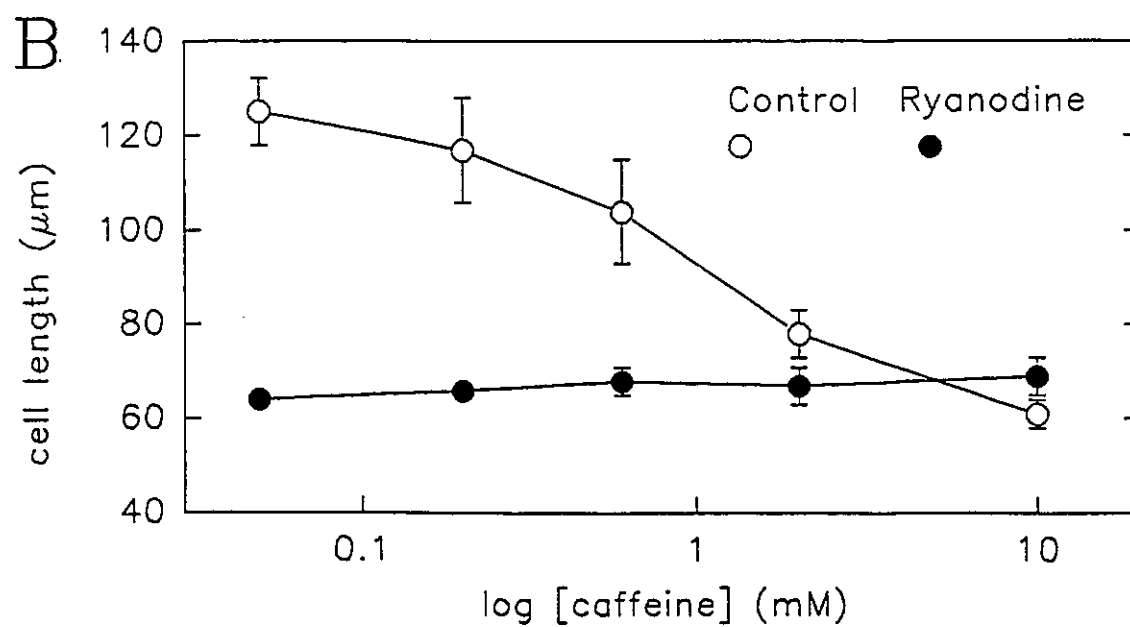
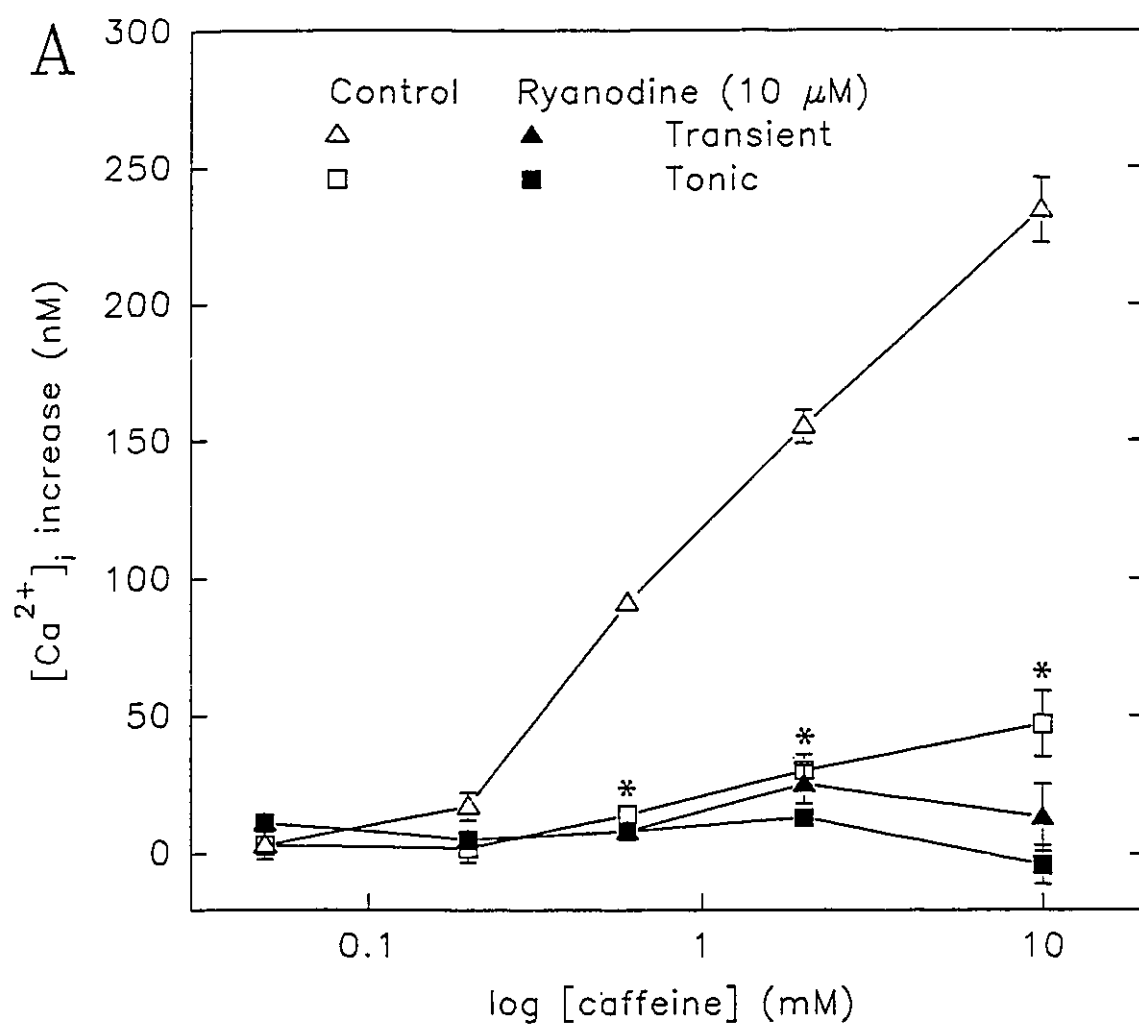


Figure 19. Graph of effect of ryanodine on caffeine-induced changes in $[Ca^{2+}]_i$ and cell length in naive cells

Panel A. Naive non-contracted VSMCs were incubated with 10 μ M ryanodine for 5 minutes prior to each increasing caffeine concentration. Cells were washed between each caffeine dose. The transient and tonic $[Ca^{2+}]_i$ increases to caffeine were significantly decreased. Prior to treatment the resting cell $[Ca^{2+}]_i$ was 53 ± 9 nM (n = 4).

Panel B. Effect of ryanodine on contraction. The initial cell length prior to treatment was 156 ± 2 μ m (n = 4). Ryanodine significantly (p = 0.001) depressed the caffeine-induced contraction of cells: Ryanodine-treated cells contracted to $64 \pm 3\%$ (156 ± 3 μ m to 99 ± 6 μ m, n = 4) of their original length compared the $48 \pm 1\%$ (127 ± 7 μ m to 61 ± 3 μ m, n = 4) in control cells in response to 10.0 mM caffeine.

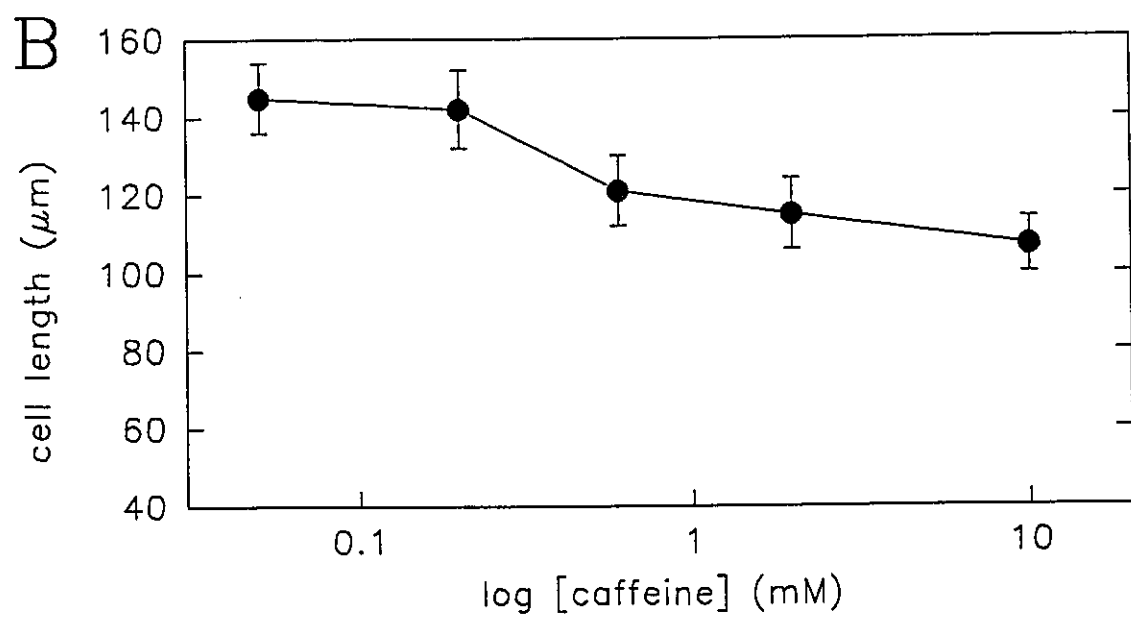
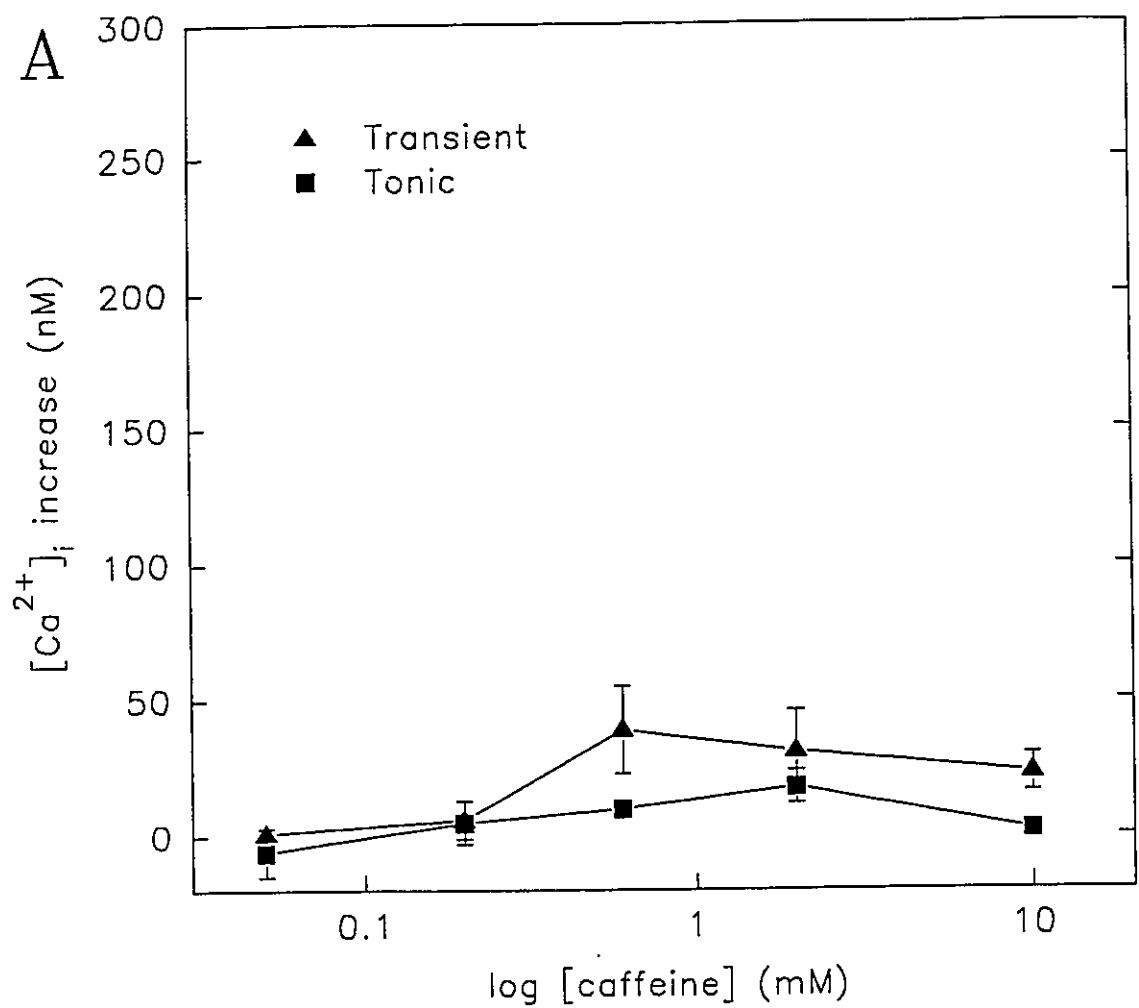
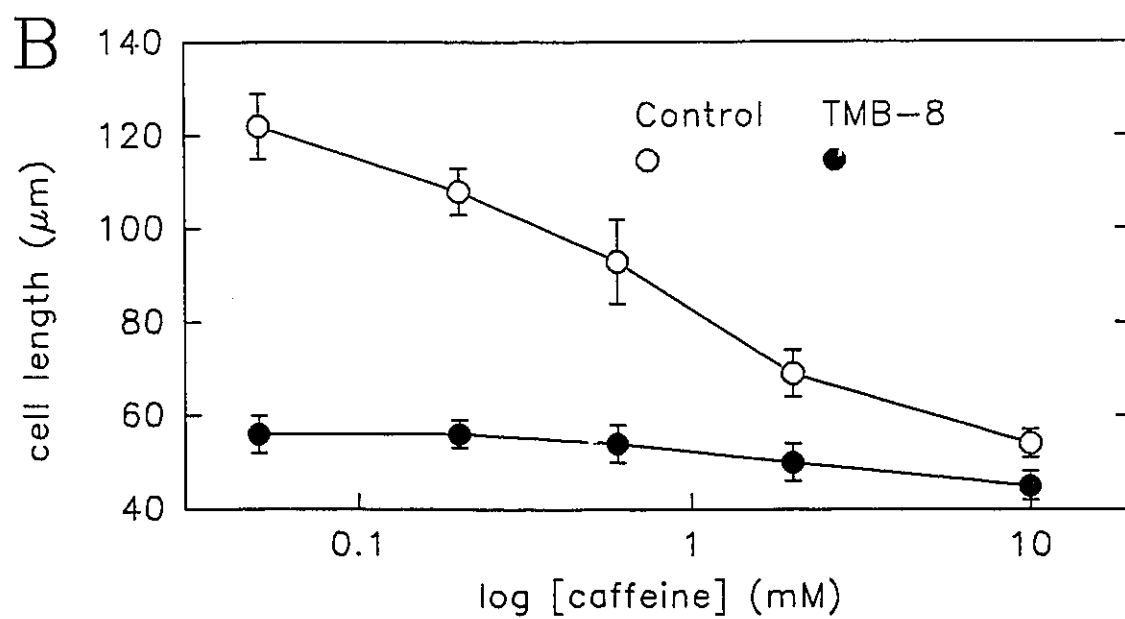
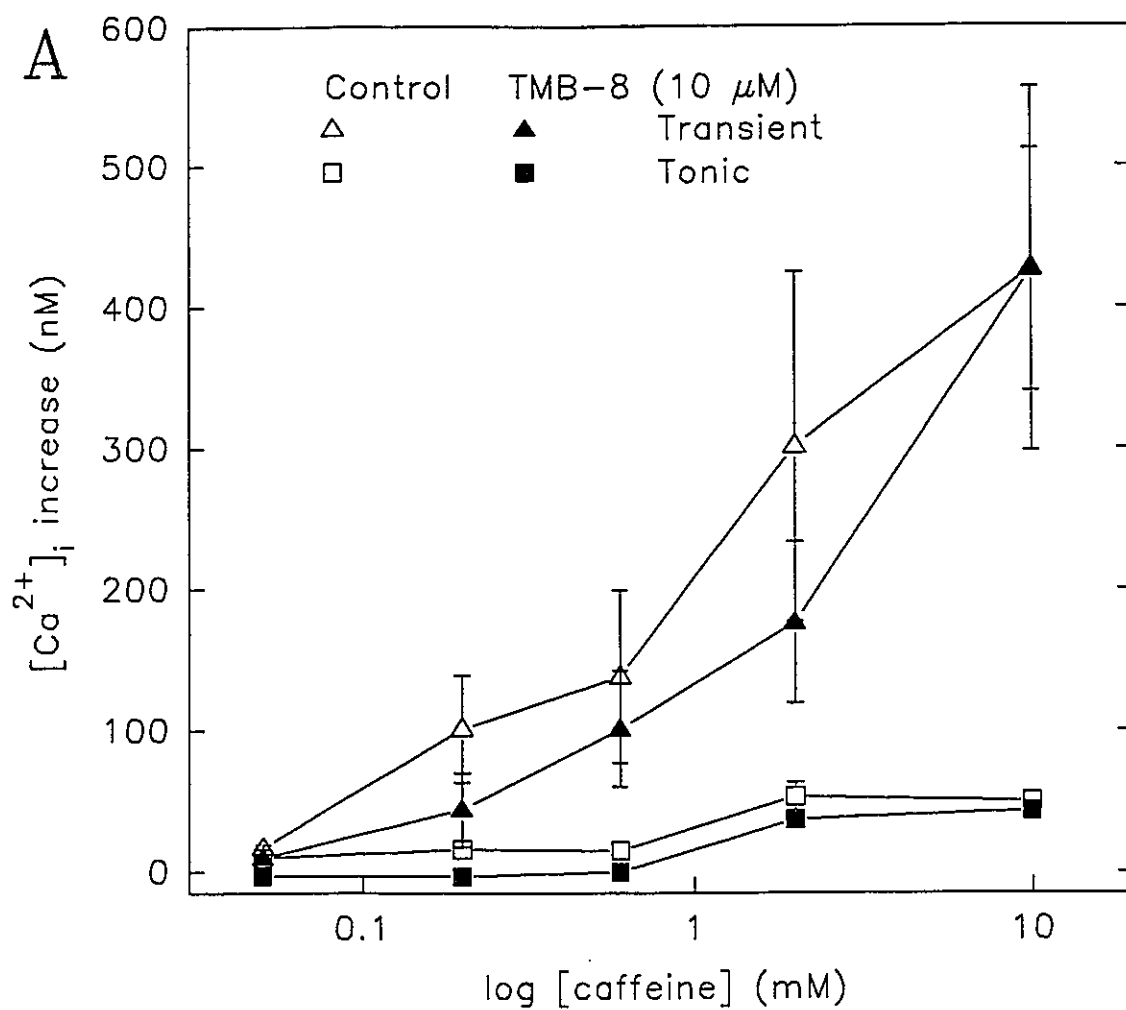


Figure 20. Graph of effect of TMB-8 on caffeine-induced changes in $[Ca^{2+}]_i$ and cell length

Panel A. After control responses to caffeine were obtained, VSMCs were incubated with 10 μ M TMB-8 for five minutes prior to each caffeine challenge in the 2nd set. TMB-8 had no significant effect on either transient or tonic $[Ca^{2+}]_i$ response to caffeine. Prior to caffeine challenge the resting cell $[Ca^{2+}]_i$ for the control was 43 ± 15 nM. This was not significantly different from the treatment $[Ca^{2+}]_i$ level of 35 ± 9 nM.

Panel B. Corresponding changes in cell length associated with the above changes in $[Ca^{2+}]_i$. The initial cell length prior to caffeine challenge was 124 ± 7 μ m (n = 4). Caffeine contracted TMB-8 treated cells further from 54 ± 3 μ m to 45 ± 3 μ m (n = 4).



The effect of 10 μ M TMB-8 treatment on contraction response to caffeine (fig. 21) was tested on cells that had not been prechallenged, and thus not precontracted. Cell contraction was not significantly different to the control response. Both transient and tonic $[Ca^{2+}]_i$ increases to caffeine were similar to control responses.

(C) Effect of Nifedipine on Caffeine Dose-Response in VSMCs

Application of 1 μ M nifedipine for a minimum 5 minute period did not significantly change either the resting $[Ca^{2+}]_i$ (38 ± 10 nM versus 44 ± 12 nM, $n = 4$) or cell length (157 ± 14 μ m versus 152 ± 15 μ m, $n = 4$). The effect of nifedipine on transient and tonic $[Ca^{2+}]_i$ increases in response to caffeine is shown in figure 22. Nifedipine (1 μ M) did not significantly affect either transient or tonic $[Ca^{2+}]_i$ increase in response to 0.05 to 10.0 mM caffeine (fig. 22).

The effect of 1 μ M nifedipine treatment on VSMC contraction response to caffeine was tested on cells that were not prechallenged, and thus not precontracted (fig. 23). Cell contraction was not significantly different to the control response.

(D) Effect of Thapsigargin on Caffeine Response in VSMCs

Figure 24 shows the effect of thapsigargin on $[Ca^{2+}]_i$ in VSMCs. A sample trace (panel A) shows that prolonged exposure to 1 μ M thapsigargin raises $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ increased after 5 minutes in the presence of 1 μ M thapsigargin from a resting level of 47 nM to 151 nM. After 10 minutes, $[Ca^{2+}]_i$ was maintained at 87 nM. Increasing the concentration of thapsigargin to 100 μ M had no further effect on the $[Ca^{2+}]_i$. There

Figure 21. Graph of effect of TMB-8 on caffeine-induced changes in $[Ca^{2+}]_i$ and cell length in naive cells

Panel A. Naive relaxed single VSMCs were incubated with 10 μ M TMB-8 for 5 minutes prior to each increasing caffeine concentration. Cells were washed between each caffeine dose. The transient and tonic $[Ca^{2+}]_i$ increases in response to caffeine were not significantly affected by TMB-8. Prior to treatment the resting cell $[Ca^{2+}]_i$ was 39 ± 4 nM (n = 4).

Panel B. Corresponding changes in cell length associated with the above changes in $[Ca^{2+}]_i$. The initial cell length prior to treatment was 128 ± 4 μ m (n = 4). TMB-8 did not significantly alter the cell length decrease in response to caffeine.

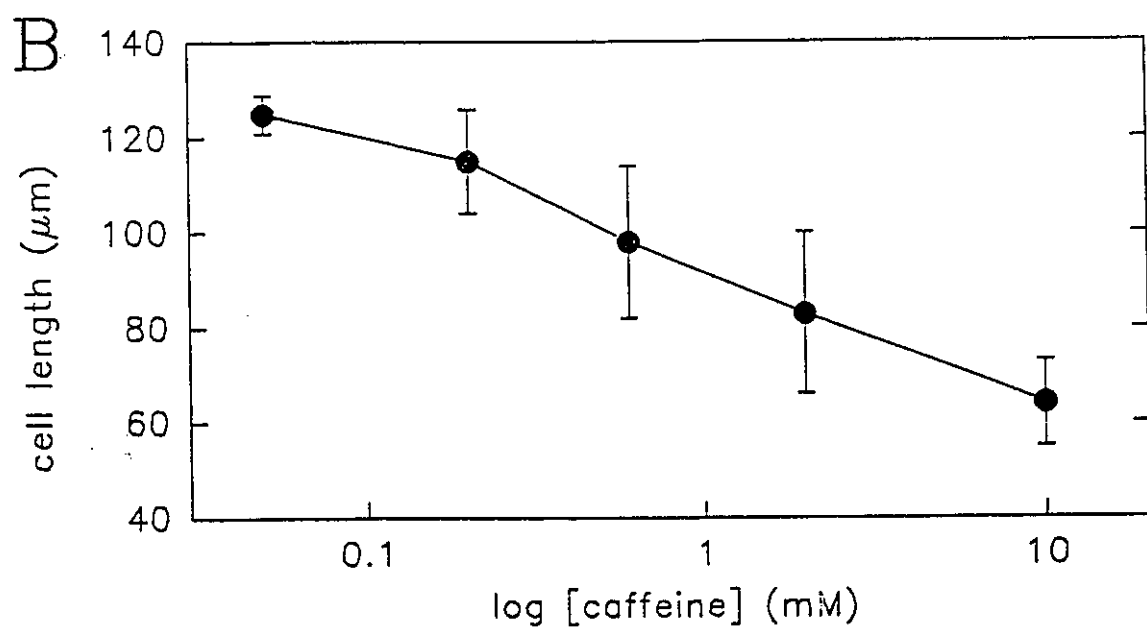
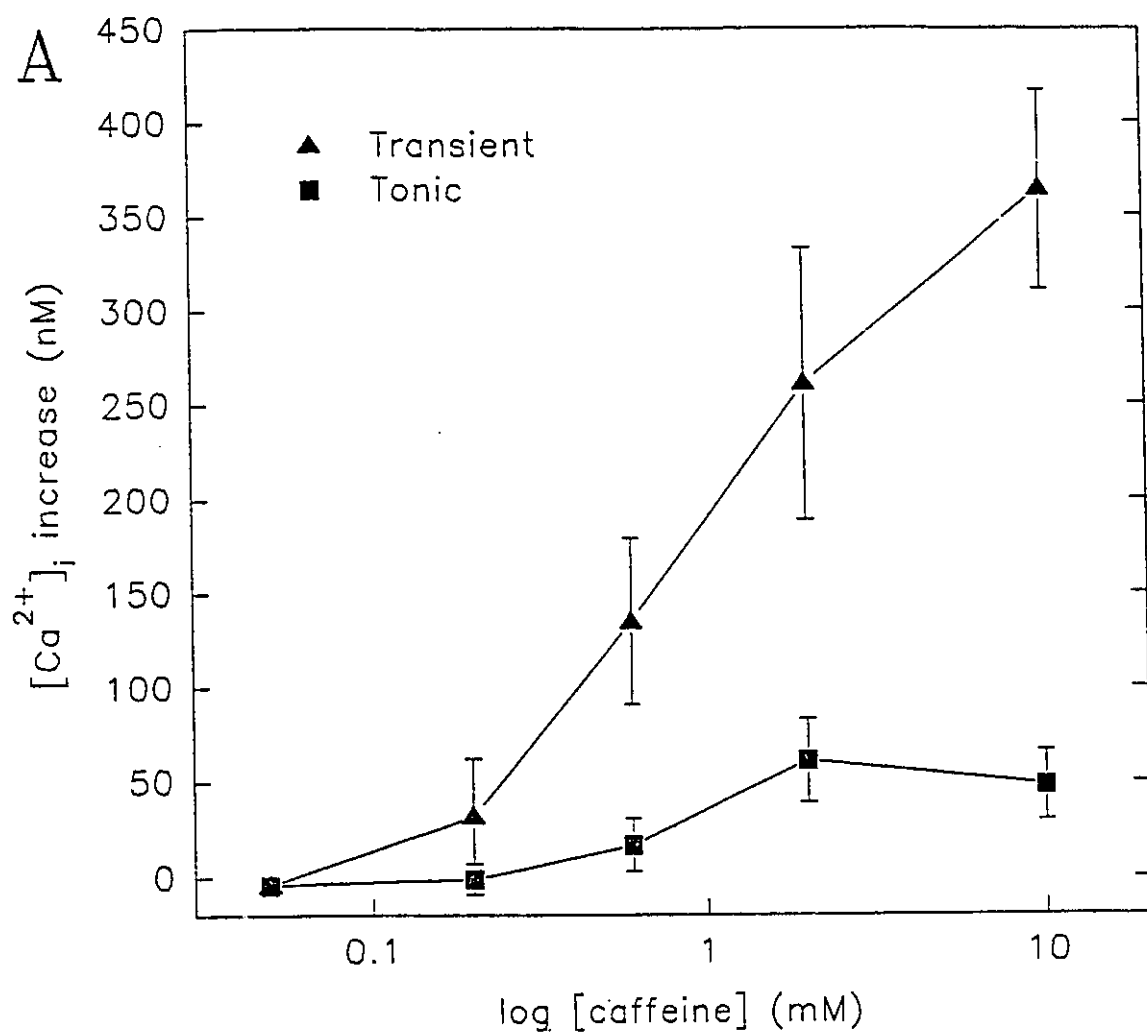


Figure 22. Graph of effect of nifedipine on caffeine-induced changes in $[Ca^{2+}]_i$ and cell length

Panel A. After control responses to caffeine were obtained VSMCs were incubated with 1 μ M Nifedipine for five minutes prior to each caffeine challenge in the 2nd set. Nifedipine had no significant effect on either transient or tonic $[Ca^{2+}]_i$ in response to 0.05 to 10.0 mM caffeine. Prior to caffeine challenge the resting cell $[Ca^{2+}]_i$ for the control was 33 ± 5 nM. This was not significantly different from the nifedipine-treated cell $[Ca^{2+}]_i$ level of 26 ± 3 nM.

Panel B. Corresponding changes in cell length associated with the above changes in $[Ca^{2+}]_i$. The initial cell length prior to caffeine challenge was 131 ± 8 μ m (n = 4). Caffeine further contracted the nifedipine treated cells from 57 ± 10 μ m to 43 ± 7 μ m (n = 4).

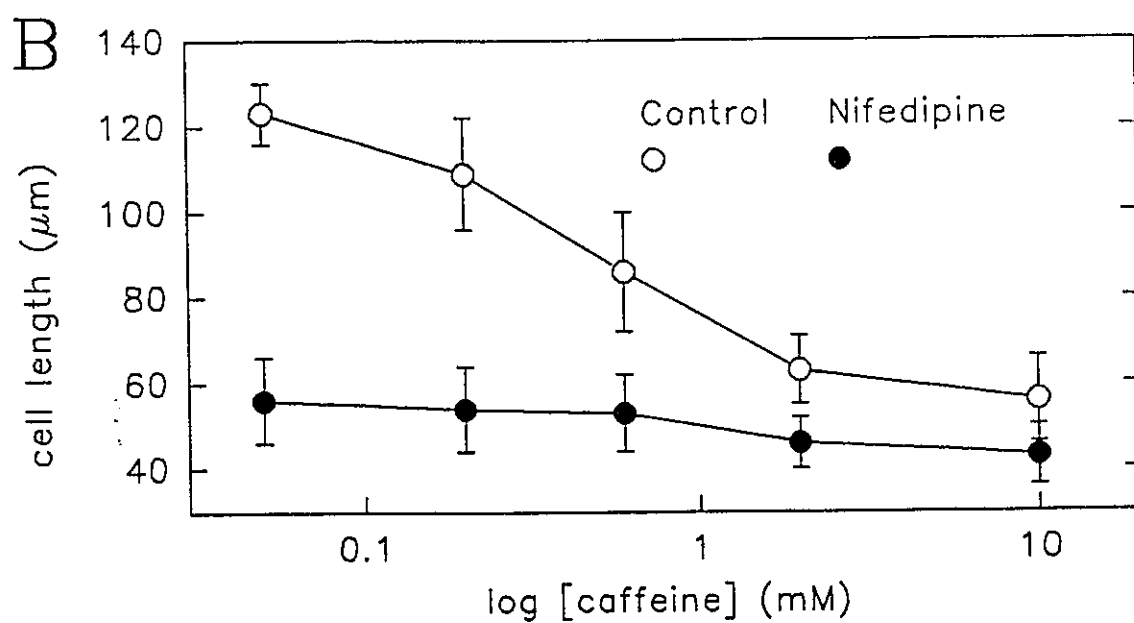
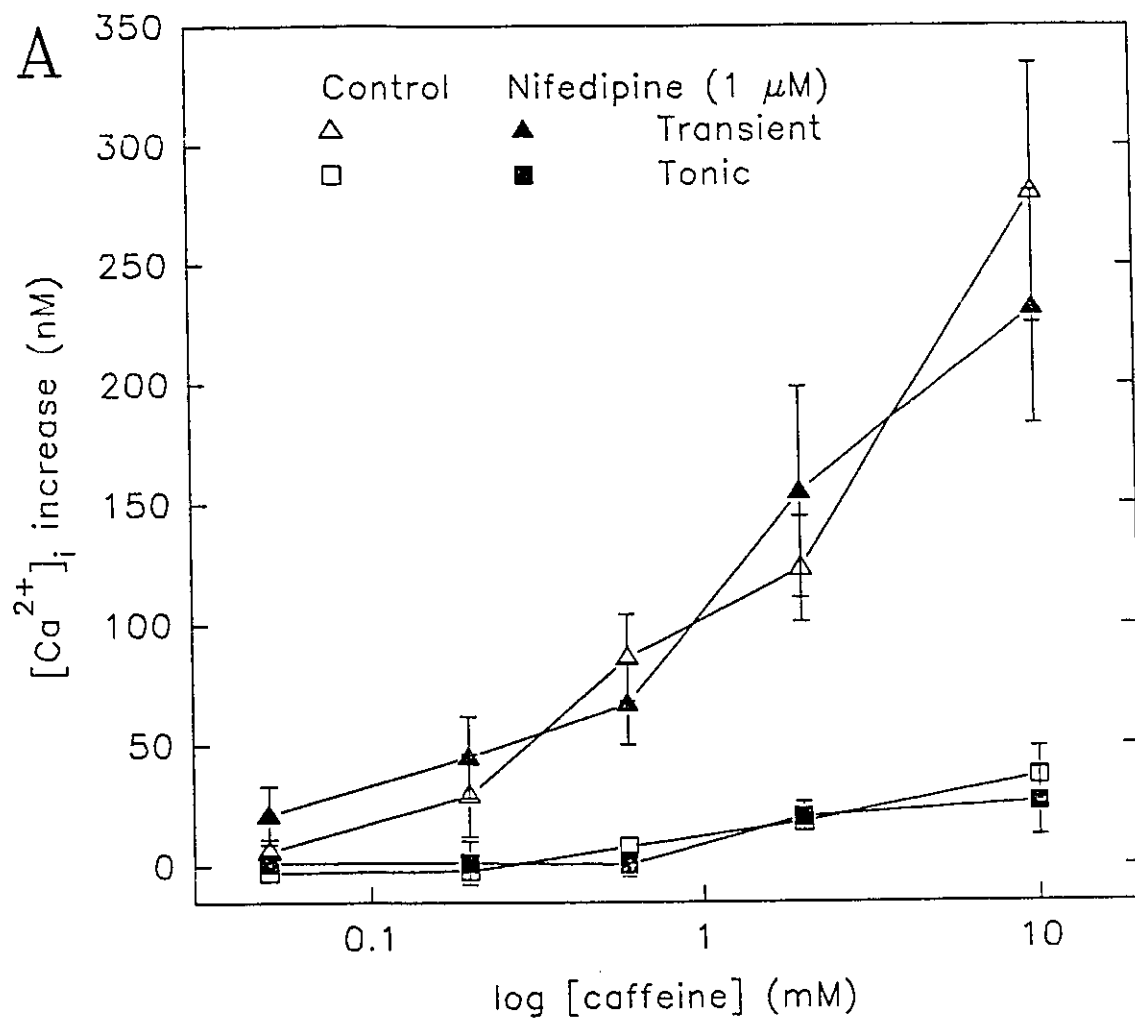


Figure 23. Graph of effect of nifedipine on caffeine-induced changes in $[Ca^{2+}]_i$ and cell length in naive cells

Panel A. Naive relaxed single VSMCs were incubated with 1 μ M nifedipine for 5 minutes prior to each increasing caffeine concentration. Cells were washed between each caffeine dose. The transient and tonic $[Ca^{2+}]_i$ increases to caffeine were not significantly affected by nifedipine. Prior to treatment the resting cell $[Ca^{2+}]_i$ was 38 ± 10 nM (n = 4).

Panel B. Corresponding changes in cell length associated with the above changes in $[Ca^{2+}]_i$. The initial cell length prior to treatment was 157 ± 14 μ m (n = 4). Nifedipine did not significantly alter the cell length decrease in response to caffeine.

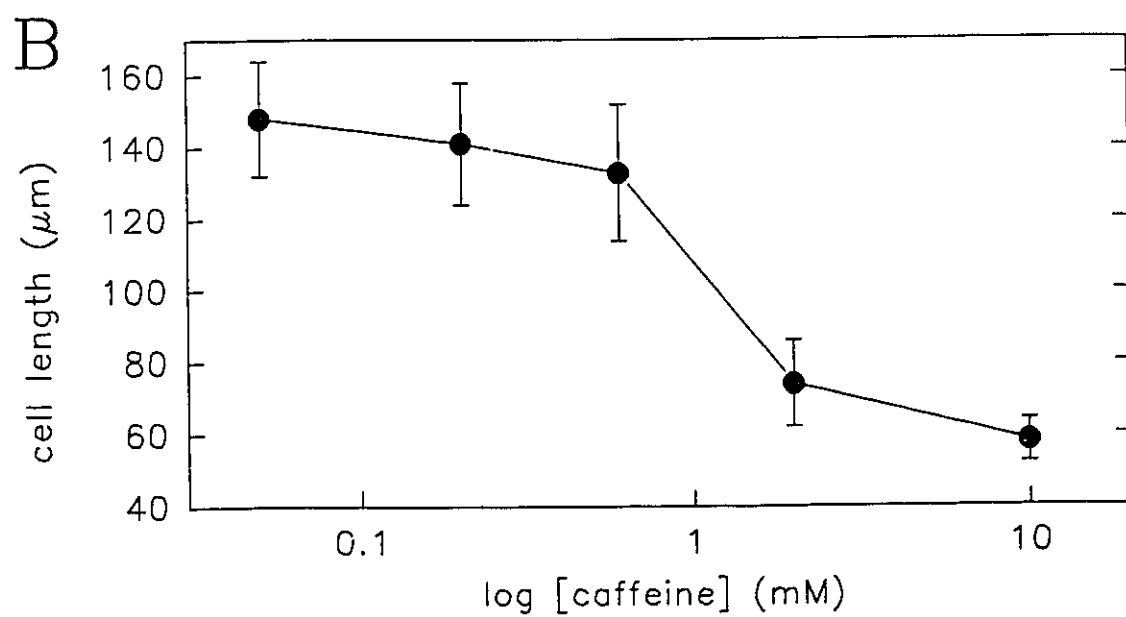
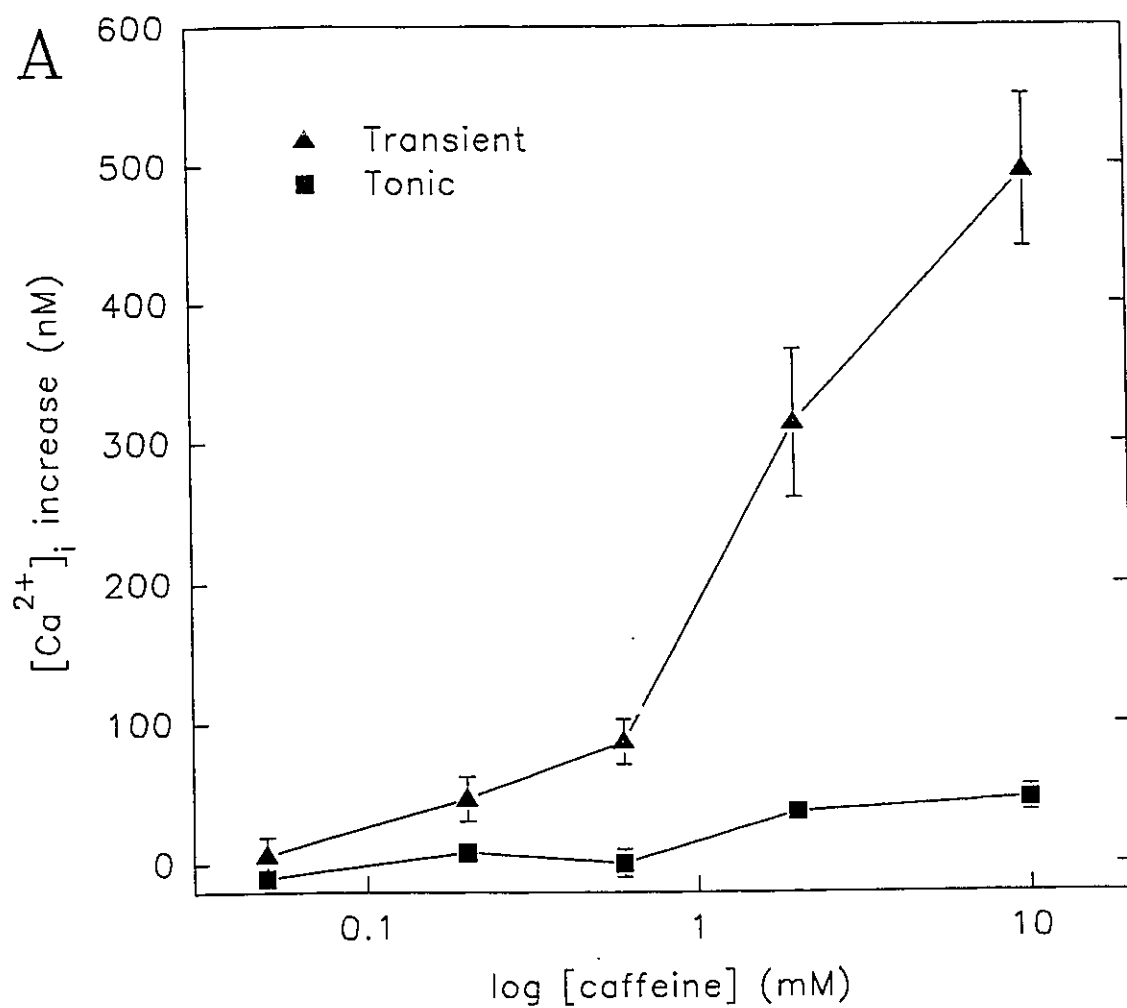
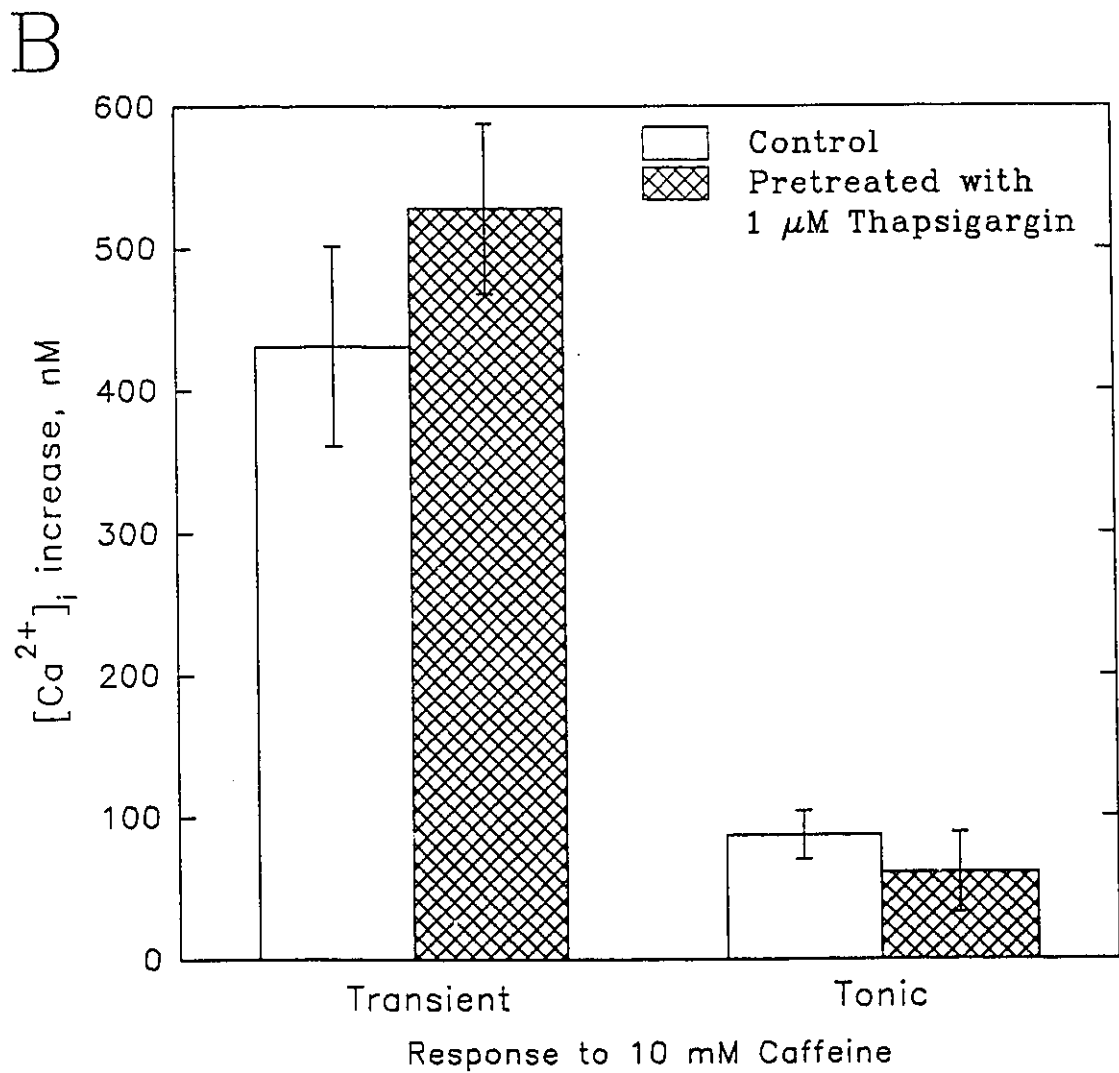
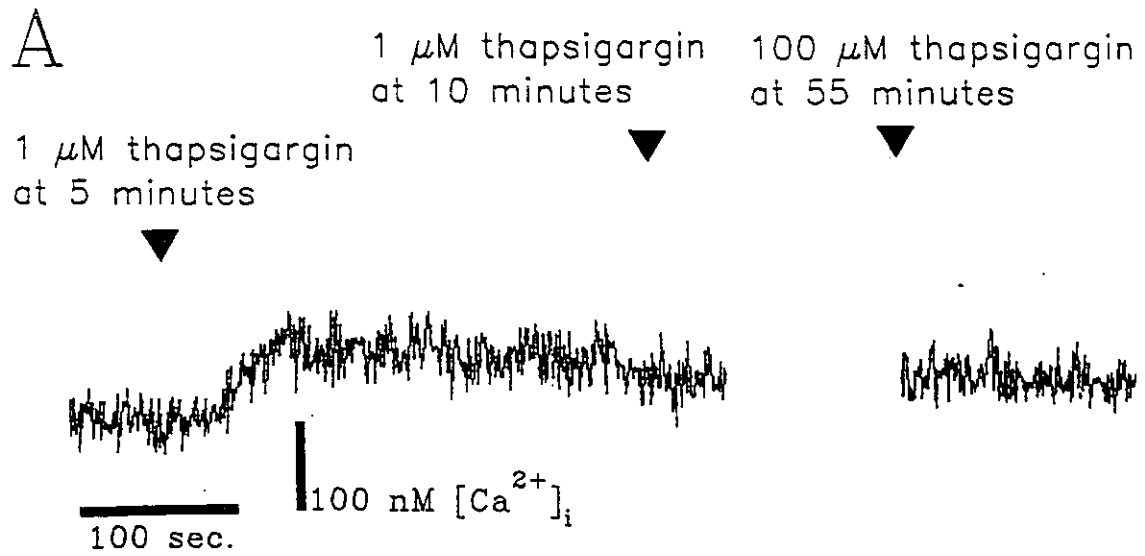


Figure 24. Trace and graph showing effect of thapsigargin on $[Ca^{2+}]_i$ and caffeine-induced changes in $[Ca^{2+}]_i$

Panel A. Representative trace showing the slow rise in $[Ca^{2+}]_i$ levels induced by thapsigargin. The initial resting $[Ca^{2+}]_i$ was 47 nM. Following 5 minutes in the presence of 1 μ M thapsigargin the cell $[Ca^{2+}]_i$ slowly rises to a peak of 151 nM and stabilized to 87 nM. Neither prolonged incubation periods nor increased thapsigargin concentration (100 μ M) affected cell $[Ca^{2+}]_i$ further.

Panel B. Effect of pretreatment with 1 μ M thapsigargin on VSMC $[Ca^{2+}]_i$ rise in response to 10 mM caffeine. Following a typical transient and tonic rise in $[Ca^{2+}]_i$ in response to 10 mM caffeine, the subsequent wash (with 1 μ M thapsigargin included) for ten minutes, followed by challenge with 10 mM caffeine + 1 μ M thapsigargin, had no significant effect on either the transient or tonic response. (n = 4).



was variation in response to 1 μ M thapsigargin over time during the first 15 minutes of treatment. However, after 15 minutes of monitoring, the increased $[Ca^{2+}]_i$ stabilized to 67 ± 5 nM ($p = 0.02$, $n = 4$). Neither prolonged incubation (1 hour), nor increased thapsigargin concentration (100 μ M) further increased $[Ca^{2+}]_i$. Cell lengths gradually decreased in the presence of thapsigargin. After 20 minutes in 1 μ M thapsigargin cell lengths decreased from 142 ± 14 μ m to 126 ± 11 μ m (89 ± 8 %, $n = 8$). Pretreatment with 1 μ M thapsigargin had no significant effect ($n = 4$) on either transient or tonic $[Ca^{2+}]_i$ increases in response to 10 mM caffeine (fig. 24B).

Part III. Physiological Relevance of the Caffeine-Sensitive Ca^{2+} Store in VSMCs

Figure 25 shows the effect of 10 mM caffeine pretreatment on 1 μ M noradrenaline-induced $[Ca^{2+}]_i$ increases in vascular smooth muscle cells. A representative trace (panel A) shows that application of 10 mM caffeine transiently increases $[Ca^{2+}]_i$ to 529 nM followed by a tonic $[Ca^{2+}]_i$ of 111 nM. After five minutes, 1 μ M noradrenaline was added on top of caffeine. No additional $[Ca^{2+}]_i$ increase was observed. After washout, 1 μ M noradrenaline induced a transient $[Ca^{2+}]_i$ increase of 442 nM and a tonic $[Ca^{2+}]_i$ increase of 118 nM. Results of four cells (fig. 25B) indicate pretreatment with 10 mM caffeine results in abolishment of the 1 μ M noradrenaline-induced $[Ca^{2+}]_i$ increase ($p = 0.01$).

Figure 26 shows the effect of 10 μ M noradrenaline pretreatment on 10 mM caffeine-induced $[Ca^{2+}]_i$ increases in vascular smooth muscle cells. A sample trace (panel A) shows that application of 10 μ M noradrenaline transiently increased $[Ca^{2+}]_i$

Figure 25. Trace and graph showing effect of pretreatment with caffeine on VSMC response to noradrenaline

Panel A. Sample trace of $[Ca^{2+}]_i$ in response to 1 μ M noradrenaline with and without pretreatment with 10 mM caffeine. The first trace shows the resultant transient (529 nM) and tonic (111 nM) increase in $[Ca^{2+}]_i$ upon addition of caffeine. The second trace indicates that in the continued presence of caffeine, 1 μ M noradrenaline has no additional effect on $[Ca^{2+}]_i$. The final trace shows the control response to 1 μ M noradrenaline following VSMC washing. This gave a transient $[Ca^{2+}]_i$ increase of 442 nM followed by a tonic $[Ca^{2+}]_i$ increase of 118 nM.

Panel B. Effect of pretreatment with 10 mM caffeine on VSMC $[Ca^{2+}]_i$ rise in response to 1 μ M noradrenaline. Results of four cells indicate pretreatment with caffeine results in abolishment of the noradrenaline-induced $[Ca^{2+}]_i$ increase ($p = 0.01$). Following washout, application of 1 μ M noradrenaline induced a transient and tonic $[Ca^{2+}]_i$ increase of 304 ± 67 nM and 48 ± 16 nM respectively.

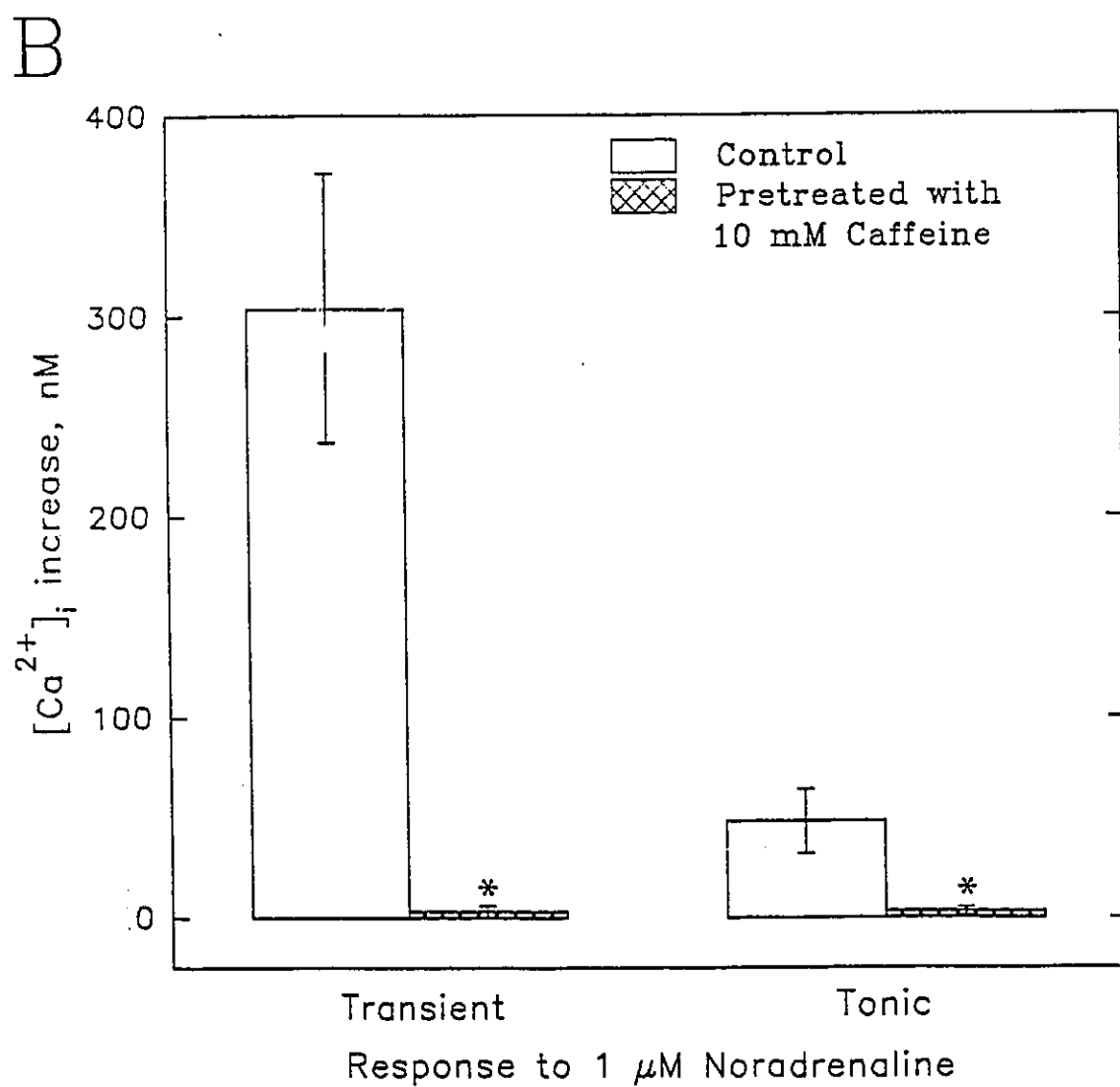
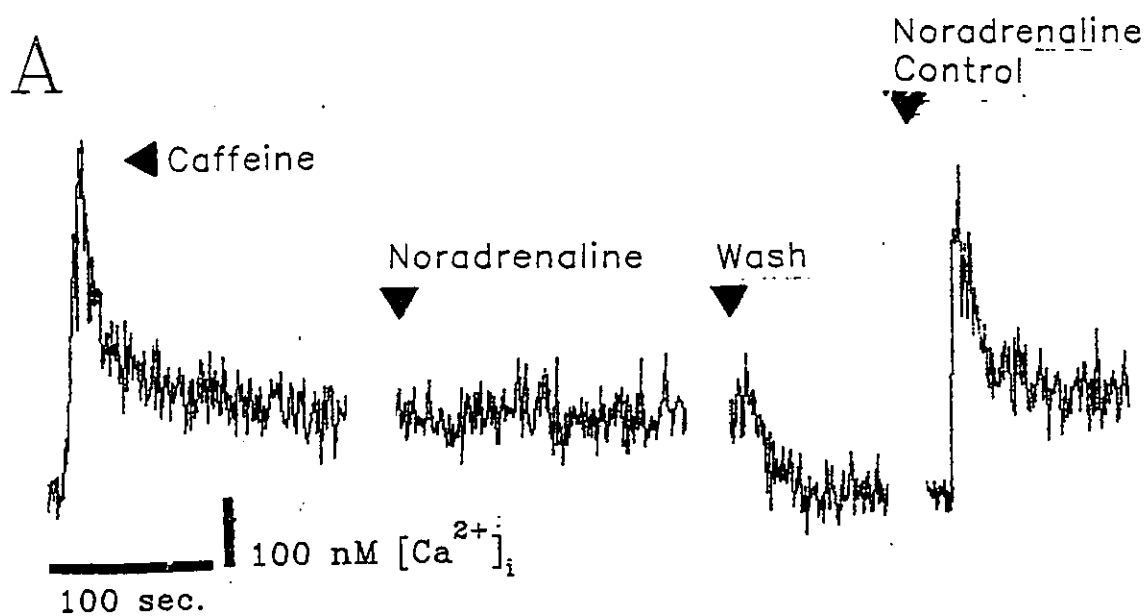
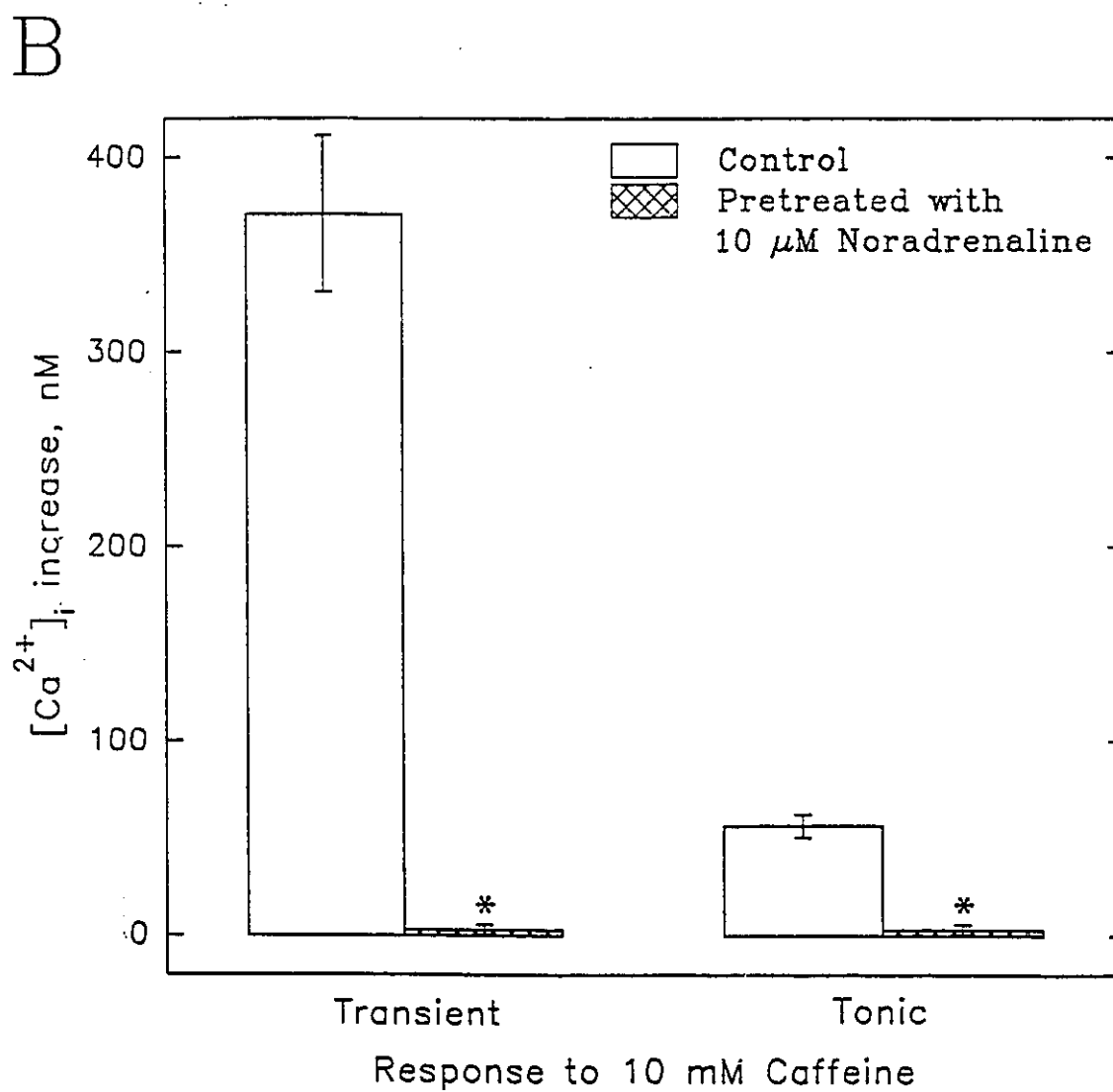
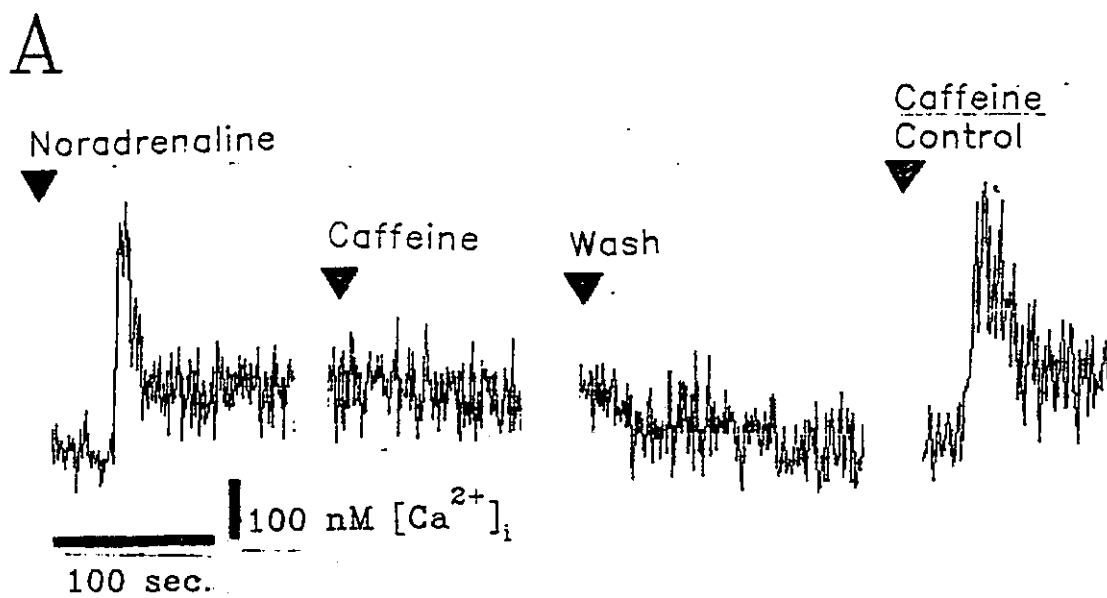


Figure 26. Trace and graph showing effect of pretreatment with noradrenaline on VSMC response to caffeine

Panel A. Sample trace showing $[Ca^{2+}]_i$ response to 10 mM caffeine with and without pretreatment with 10 μ M noradrenaline. The first trace shows the resultant transient (368 nM) and tonic (111 nM) increase in $[Ca^{2+}]_i$ following addition of 10 μ M noradrenaline. The second trace shows that in the continued presence of 10 μ M noradrenaline, caffeine has no additional effect on $[Ca^{2+}]_i$. The final trace shows the control response to caffeine following VSMC washing. This gave a transient $[Ca^{2+}]_i$ increase of 387 nM followed by a tonic $[Ca^{2+}]_i$ increase of 123 nM.

Panel B. Effect of pretreatment with 10 μ M noradrenaline on VSMC $[Ca^{2+}]_i$ rise in response to 10 mM caffeine. Results of four cells indicate pretreatment with 10 μ M noradrenaline results in abolishment of the caffeine-induced $[Ca^{2+}]_i$ increase ($p = 0.001$). Following washout, application of caffeine induced a transient and tonic $[Ca^{2+}]_i$ increases of 371 ± 40 nM and 57 ± 6 nM respectively.



to 368 nM followed by a maintained tonic $[Ca^{2+}]_i$ of 111 nM. After 5 minutes, caffeine was added on top of 10 μ M noradrenaline. No additional $[Ca^{2+}]_i$ response was observed. After washout, caffeine induced a transient $[Ca^{2+}]_i$ increase of 387 nM and a tonic $[Ca^{2+}]_i$ increase of 123 nM. $[Ca^{2+}]_i$ returned to basal levels (56 - 64 nM) following washout of both caffeine and noradrenaline. Results of four cells (fig. 26B) indicate pretreatment with 10 μ M noradrenaline results in abolishment of the caffeine-induced $[Ca^{2+}]_i$ increase ($p = 0.001$).

The $[Ca^{2+}]_i$ responses to 10 μ M noradrenaline and 10 mM caffeine were similar. 10 μ M noradrenaline elicited a transient $[Ca^{2+}]_i$ increase to 382 ± 42 nM followed by a tonic $[Ca^{2+}]_i$ increase of 100 ± 16 nM. In the same cells, 10 mM caffeine resulted in a transient $[Ca^{2+}]_i$ increase of 408 ± 38 nM and a tonic $[Ca^{2+}]_i$ increase to 93 ± 12 nM ($n = 4$). Thus there was no significant difference between maximal caffeine and maximal noradrenaline VSMC $[Ca^{2+}]_i$ response in terms of either the transient or tonic $[Ca^{2+}]_i$ ($n = 4$). $[Ca^{2+}]_i$ returned to basal levels (37 ± 3 nM, $n = 4$) following washout of both caffeine and noradrenaline. Following application of 10 μ M noradrenaline, the cells shortened to 36 ± 8 % of their original length (119 ± 6 μ m to 42 ± 9 μ m, $n = 4$).

DISCUSSION

(A) Single Cell Measurement of $[Ca^{2+}]_i$ in Vascular Smooth Muscle.

There are a number of advantages in measuring $[Ca^{2+}]_i$ at the single cell level (Thomas & Delaville, 1991). Changes in cellular $[Ca^{2+}]_i$ in a population of cells in response to stimulation is often heterologous. Complex spatial-temporal fluctuations in $[Ca^{2+}]_i$ may appear in one cell but its neighbouring cells may manifest an entirely different $[Ca^{2+}]_i$ response. The ability to correlate $[Ca^{2+}]_i$ in reference to a particular cell, which otherwise would be "averaged out" in a cell population, therefore represents an important advance. Concurrent changes attributable to a single cell such as length, membrane potential, or other intracellular ion concentrations (Na^+ , Mg^{2+}) can be measured in the same single cell. Extracellular influences such as nerves and endothelium are eliminated in the single cell preparation, although this deviation from normal physiology must be kept in mind. Removal of the surrounding extracellular matrix of dense connective tissue diminishes diffusion-related barriers. However, its removal eliminates the normal passive resistance to reductions in VSMC size, especially length, following contraction. The use of single cells in conjunction with digital scanning microscopy allows the mapping of $[Ca^{2+}]_i$ activity at the sub-cellular level. The use of single cells also allows application of patch-clamp techniques to simultaneously record ion channel activities and $[Ca^{2+}]_i$.

There are however disadvantages to the use of single cells to monitor $[Ca^{2+}]_i$. Cell isolation procedures, such as the use of enzymes, may compromise membrane integrity. This may cause leakage of extracellular Ca^{2+} into the cytoplasm, or damage

surface proteins such as receptors, important in normal cell physiology. The larger physical sample size of tissue preparations allows the concurrent measurement of smooth muscle tissue tension development. Although individual smooth muscle cells have been tied to a force transducer and tension development measured, this is a difficult process (Warshaw *et al.*, 1986). The use of tissue preparations e.g. containing nerves and/or endothelium, affords a more physiologically comparable environment in which to measure $[Ca^{2+}]_i$. Use of tissue preparations may however interfere with correct fluorescent dye loading. The AM ester form of these dyes is hydrophobic and will preferentially partition into connective tissue and fat found in the extracellular space of the tissue preparation. Although highly fluorescent, the non-hydrolysed ester does not produce a $[Ca^{2+}]$ -dependent signal. Thus lower intracellular dye concentration combined with a relatively high non- $[Ca^{2+}]$ responsive extracellular fluorescence may lead to errors in determination of $[Ca^{2+}]_i$.

(B) Fura-2 Study of Ca^{2+} in Vascular Smooth Muscle

The fura-2 technique has been applied to study Ca^{2+} in freshly isolated VSMCs from the rat tail artery. One potential problem with fura-2 and other Ca^{2+} -binding probes is the possibility of buffering $[Ca^{2+}]_i$. This may occur if the intracellular concentration of the dye is too high. The possibility of such a buffering effect interfering with excitation-contraction coupling in VSMCs was tested in our study. The contraction responses to 10^{-9} - 10^{-5} M noradrenaline in fura-2 loaded cells were not significantly different from those in control cells. Studies on rat aortic smooth muscle have indicated

that a high (1.2 mM) concentration of cytosolic fura-2 is required to affect $[Ca^{2+}]_i$ and the rate of contraction (Bruschi *et al.*, 1988). This is greatly in excess of the 100-200 μ M concentration found under typical loading conditions (Uto *et al.*, 1991, Williams & Fay, 1990).

The present study examined the effect of caffeine on Ca^{2+} mobilization and cell contraction. As was noted from previous studies, single VSMCs do not relax after shortening, particularly under strong stimulation conditions (van Dijk & Laird, 1984; Driska & Porter, 1986). The change in cell morphology from a relaxed to a contracted state could potentially affect the Ca^{2+} fluorescent signal. With contraction, the fura-2 concentration inside the cell may be altered due to changes in cell volume. It was estimated that in single SMCs from the stomach of *Bufo marinus*, a 50 % shortening of cell length would result in about a 15 % decrease in cell volume (Fay & Delise, 1973). In our studies comparing the Ca^{2+} signal on cells before and after caffeine-induced contraction, we observed no significant changes in the resting $[Ca^{2+}]_i$ or in the increase induced by caffeine. Thus, $[Ca^{2+}]_i$ may be measured in VSMCs irrespective of their contractile state. The use of the fura-2 ratio method to determine $[Ca^{2+}]_i$ can be credited for reducing the impact of the cell shortening since the fluorescent signal ratio is independent of both dye concentration and light path length (Grynkiewicz *et al.*, 1985).

(C) Resting $[Ca^{2+}]_i$ in single VSMCs

In freshly isolated single VSMCs, the reported resting $[Ca^{2+}]_i$ is in the range of 84 to 120 nM (Katsuyama *et al.*, 1991; Baró *et al.*, 1993; Li *et al.*, 1993). Similar

resting $[Ca^{2+}]_i$ ranging from 40 to 120 nM have been estimated in cultured vascular cells (Simpson & Ashley, 1989; Erdbrügger *et al.*, 1993) and in intact vascular preparations (Itoh *et al.*, 1992; Jensen *et al.*, 1993). These values are close to our estimation of a resting level of 44 nM in cells from the rat tail artery. The relaxed state of these cells is in accordance with their low resting $[Ca^{2+}]_i$, well below the 100 - 180 nM threshold for contraction as determined from chemically skinned VSM tissues (Filo *et al.*, 1965; Itoh *et al.*, 1992).

Our studies have shown that resting cellular $[Ca^{2+}]_i$ from the rat tail artery is influenced by the extracellular $[Ca^{2+}]$. Thus removal of external Ca^{2+} resulted in reduction of $[Ca^{2+}]_i$ to half (21 nM) the normal resting $[Ca^{2+}]_i$ level. Similar reduction was observed in single cells from the porcine coronary artery (Sumimoto & Kuriyama, 1986) and in intact rat aortic strips (Watanabe *et al.*, 1992). The resting $[Ca^{2+}]_i$ was not affected by either nifedipine, TMB-8 or ryanodine.

(D) Caffeine-induced Ca^{2+} Release in Vascular Smooth Muscle

The Ca^{2+} store specific to the actions of caffeine is recognized as originating from the SR (Endo *et al.*, 1977; van Breemen & Saida, 1989; Somlyo, 1985). The present study employing rat tail artery VSMCs shows that the increase in $[Ca^{2+}]_i$ induced by caffeine is concentration dependent. Lower concentrations of caffeine elicited only a transient $[Ca^{2+}]_i$ increase that decayed back to resting levels within a relatively short period (2-3 min.), in spite of the continued presence of caffeine. Although our experiments were not specifically designed to study kinetics of the $[Ca^{2+}]_i$ response, it

was evident that the time to reach peak response is shortened with increasing concentrations of caffeine. Higher caffeine concentrations additionally produced a smaller but lasting (> 20 min.) secondary tonic $[Ca^{2+}]_i$ increase. This tonic response immediately followed the initial transient $[Ca^{2+}]_i$ rise. A similar two component (transient + tonic) increase in $[Ca^{2+}]_i$ has been reported in some other VSMC preparations following stimulation with caffeine (Watanabe *et al.*, 1992) and noradrenaline (Pacaud *et al.*, 1992; Ito *et al.*, 1993). In other VSMC preparations, only a transient component was evoked by caffeine and noradrenaline (Itoh *et al.*, 1992; Baró *et al.*, 1993).

In guinea pig coronary myocytes, the decay of $[Ca^{2+}]_i$ in the transient response could be forestalled by the addition of La^{3+} , indicating La^{3+} -sensitive Ca^{2+} extrusion is responsible for the transient response (Ganitkevich & Isenberg, 1993). However, La^{3+} is known to block both the plasma membrane Ca^{2+} -ATPase and Na^+ - Ca^{2+} exchange. It was subsequently determined that the Ca^{2+} -ATPase was mainly responsible for the Ca^{2+} extrusion since removal of Na^+ from the extracellular medium did not modify the transient response to caffeine.

The transient $[Ca^{2+}]_i$ increase in response to 10 mM caffeine averaged 392 nM in our study. This is similar to results obtained from other single VSMC preparations (Baró *et al.*, 1993) or from intact vascular strips (Itoh *et al.*, 1992). In addition, we observed that the transient $[Ca^{2+}]_i$ response to caffeine has a fairly wide distribution, ranging from 200-815 nM. This may in part be due to the different size of the Ca^{2+} store in different cells. The heterogeneity in the caffeine response is corroborated by a

study on cultured rat aortic cells showing that caffeine increased $[Ca^{2+}]_i$ substantially in only one third of the cell population. The remaining cells showed no or only very weak response (Shin *et al.*, 1991). The tonic $[Ca^{2+}]_i$ increase observed with our VSMC preparation occurred when the applied caffeine concentration exceeded 0.6 mM. This is similar to findings in rat aortic strips (Watanabe *et al.*, 1992). In addition, there was a positive correlation between the magnitude of the transient and tonic $[Ca^{2+}]_i$ responses in the rat tail artery VSMC preparation.

(E) Ca^{2+} -dependence of the Caffeine Response

In rat tail artery VSMCs, removal of external Ca^{2+} did not significantly affect the initial transient $[Ca^{2+}]_i$ response resulting from the first application of caffeine. This is similar to observations in other VSMC preparations (Katsuyama *et al.*, 1991; Itoh *et al.*, 1992; Watanabe *et al.*, 1992). However, second and subsequent caffeine challenges in the absence of extracellular Ca^{2+} failed to elicit any transient $[Ca^{2+}]_i$ component. This finding is in agreement with the suggestion that the transient response is due to release of Ca^{2+} from an intracellular store. With the first caffeine challenge in Ca^{2+} -free solution, there was still Ca^{2+} remaining in the intracellular store to be released. However, there would be no Ca^{2+} left in the depleted store to be released by subsequent caffeine challenges. This experiment also suggests that external Ca^{2+} is required to replenish the intracellular Ca^{2+} store, as was demonstrated by the recovery of the transient response after Ca^{2+} was reintroduced to the external medium.

Unlike the transient increase, the tonic response was abolished immediately upon removal of external Ca^{2+} . Similar findings were reported in rat aortic muscle strips (Watanabe *et al.*, 1992). Therefore, the tonic response is most likely to be due to influx of external Ca^{2+} . However, the influx is not mediated by L-type Ca^{2+} channels as nifedipine has no effect on the tonic rise in Ca^{2+} . Similar sustained Ca^{2+} influx via yet unknown pathways has been observed in other cell types, including endothelial cells (Schilling & Elliot, 1992).

Ca^{2+} entry may be related to emptying of intracellular Ca^{2+} stores. It has been demonstrated that depleting the agonist-sensitive intracellular store enhances Ca^{2+} influx through a verapamil-insensitive pathway in VSMCs (Missiaen *et al.*, 1990). One model that links depletion of internal Ca^{2+} stores with Ca^{2+} influx is the capacitive entry pathway (Putney, 1990). This model proposes a continual replenishment of the intracellular store via influx of extracellular Ca^{2+} , with the rate of influx being dependent of the amount of Ca^{2+} present in the intracellular store. Our finding of a good correlation between the size of the tonic $[\text{Ca}^{2+}]_i$ component and the preceding transient $[\text{Ca}^{2+}]_i$ rise is consistent with this interpretation. There is evidence to suggest that Ca^{2+} entry is mediated by the release of a yet unidentified " Ca^{2+} -influx factor" from intracellular organelles upon Ca^{2+} depletion from intracellular stores (Randriamampita & Tsien, 1993; Parekh *et al.*, 1993).

(F) Effect of Ryanodine on Caffeine-Induced $[Ca^{2+}]_i$ Responses

The effects of agents known to influence Ca^{2+} mobilization on the caffeine associated $[Ca^{2+}]_i$ response were examined in the present study. Caffeine-induced Ca^{2+} release is mediated by Ca^{2+} release channels situated in the SR. Studies on aortic Ca^{2+} release channels reconstituted in a lipid bilayer showed that 2 mM caffeine increased the open probability of these channels by tenfold (Watanabe *et al.*, 1992). Ryanodine at nano- to micromolar concentrations induced a long-lived open state in these channels (Herrmann-Frank *et al.*, 1991). Ryanodine acts on smooth muscle cells by producing a slow increase in Ca^{2+} efflux, as demonstrated in ^{45}Ca studies of the rabbit aorta (Hwang & van Breemen, 1987). This increased Ca^{2+} efflux was accompanied by a decrease in net cellular Ca^{2+} content. It was suggested that ryanodine released Ca^{2+} slowly from the SR into the cytoplasm and was subsequently extruded (with little or no accumulation inside the cell) by the surface membrane transport systems. Thus at concentrations used for pharmacological studies ($\sim \mu M$ range), ryanodine's inhibition of vascular contraction to caffeine may be attributed to leakage through the opened Ca^{2+} release channels, thereby effecting depletion of stored Ca^{2+} (Ashida *et al.*, 1988; Katsuyama *et al.*, 1991). Our study on the rat tail artery showed that ryanodine by itself did not produce a significant change in either the resting $[Ca^{2+}]_i$ or did it cause contraction. As expected, it did significantly inhibit both the $[Ca^{2+}]_i$ and contraction response associated with the action of caffeine. These observations are in accordance with other studies using intact vascular preparations (Itoh *et al.*, 1992).

A similar situation occurred with the cumulative addition of caffeine. In this case the maximum response to caffeine was observed at 2 mM. The decline in the $[Ca^{2+}]_i$ response at higher doses is consistent with discharge of a large proportion of stored Ca^{2+} . Thus only the remaining Ca^{2+} was available to be released by 10 mM caffeine, producing a significantly attenuated $[Ca^{2+}]_i$ response.

(G) Effect of Ca^{2+} Antagonists

Nifedipine blocks specifically voltage-dependent L-type Ca^{2+} channels situated within the plasma membrane. In the present study 1 μ M nifedipine had no significant effect on the $[Ca^{2+}]_i$ or contraction response to caffeine. Thus the resting $[Ca^{2+}]_i$ and the caffeine-induced Ca^{2+} increase are not modulated by external Ca^{2+} through L-type Ca^{2+} channels. However, studies have indicated that caffeine is capable of inducing membrane depolarization in some vascular preparations. In guinea pig mesenteric artery, a 10 mV depolarization was observed with 5 mM caffeine (Itoh *et al.*, 1981). Caffeine may induce membrane depolarization through activation of Ca^{2+} -activated Cl^- channels by the released Ca^{2+} (Byrne & Large, 1988). In rat portal vein, full activation of these Cl^- channels occurred at 600 nM $[Ca^{2+}]_i$ (Pacaud *et al.*, 1992). Ca^{2+} released by caffeine can also induce membrane depolarization by activating a cation current with a reversal potential of +14 mV (Wang *et al.*, 1993).

In contrast to nifedipine, TMB-8 was originally classified as an intracellular Ca^{2+} antagonist (Chiou & Malagodi, 1975). Recent studies indicate that it has multiple actions, including inhibition of Ca^{2+} and other membrane currents (Himmel & Ravens, 1990)

and on the VSM contractile system (Ishihara & Karaki, 1991). In the present study, we examined only the effect of TMB-8 at a concentration of 10 μ M. Neither the transient nor the tonic $[Ca^{2+}]_i$ was depressed in response to caffeine. In the rabbit aorta, very high concentrations of TMB-8 (> 300 μ M) were required to produce a noticeable effect on caffeine-induced contraction and transient increase in $[Ca^{2+}]_i$ (Ishihara & Karaki, 1991). This concentration was much higher than that necessary to block completely the responses to K^+ and noradrenaline.

(H) Effect of Thapsigargin

The naturally occurring sesquiterpene lactone thapsigargin is known to induce increases in $[Ca^{2+}]_i$ independent of production of inositol phosphates. The increase in $[Ca^{2+}]_i$ is due a specific inhibition of ATP-dependent Ca^{2+} transport into the SR as well as the release of sequestered Ca^{2+} from internal stores (Thastrup *et al.*, 1990). A similar mode of action of thapsigargin on VSMC has been confirmed (Xuan *et al.*, 1992).

Results of our study are consistent with the actions of thapsigargin on VSMCs, namely a gradual but sustained increase in $[Ca^{2+}]_i$. Maintenance of the sustained $[Ca^{2+}]_i$ increase is supported by continual entry of extracellular Ca^{2+} through yet unidentified pathways (Xuan *et al.*, 1992). We observed no significant effect of thapsigargin on the caffeine-induced Ca^{2+} increase. This may be due to ineffective depletion of Ca^{2+} from the SR by thapsigargin in this particular VSMC preparation. It is now recognized that there may be more than one form of SR Ca^{2+} -ATPase and that thapsigargin is specific only for the 110 kDa form (Papp *et al.*, 1991). Studies on the

rat mesenteric artery also showed that thapsigargin did not inhibit the contraction to caffeine or serotonin (Shima & Blaustein, 1992).

There may be variations in the response to thapsigargin in different vascular preparations. For example, contraction to thapsigargin in the dog mesenteric artery, but not in rat aorta, is dependent on Ca^{2+} influx through L-type Ca^{2+} channels (Low *et al.*, 1991). There are also differences amongst laboratories, even with the same vascular preparation. Thus thapsigargin was reported to have either no effect on the basal tension (Shima & Blaustein, 1992) or to cause contraction (Mikkelsen *et al.*, 1988; Low *et al.*, 1992) in rat aorta.

(I) Caffeine-Induced Ca^{2+} Increase and Contraction

It is well established that contraction is triggered by Ca^{2+} (Hartshorne & Kawamura, 1992). In the present study shortening is correlated with peak transient $[\text{Ca}^{2+}]_i$ increase induced by caffeine. The range in $[\text{Ca}^{2+}]_i$ for the contraction is very narrow, with half-maximal shortening occurring at 189 nM and the maximum reached at 326 nM. In intact rat aortic strips (Itoh *et al.*, 1992; Watanabe *et al.*, 1992) and other smooth muscle preparations (Boland *et al.*, 1992), tension development to noradrenaline also coincides with transient $[\text{Ca}^{2+}]_i$ increases within a narrow range.

The contraction response to caffeine is more complex in an intact muscle strip than single cells which can only contract but not relax. In intact muscle strips, caffeine elicits only a transient contraction, even in the presence of a tonic increase in Ca^{2+} (van der Bent & Beny, 1991; Watanabe *et al.*, 1992). Furthermore, caffeine causes relaxation and

inhibits the sustained increase in Ca^{2+} -, in K^{+} -, and in noradrenaline-precontracted cell preparations (Watanabe *et al.*, 1992). Caffeine reduces K^{+} - and noradrenaline-stimulated Ca^{2+} influx, as demonstrated in ^{45}Ca flux studies (Leijten & van Breemen, 1984). In porcine coronary artery, the relaxation to caffeine is accompanied by membrane hyperpolarization (van der Bent & Beny, 1991). In studies with skinned preparations, caffeine appears to have no direct effect on the contractile apparatus in guinea pig mesenteric artery (Itoh *et al.*, 1981) or porcine coronary artery (Itoh *et al.*, 1982). However in intact preparations there are indications that caffeine may decrease the sensitivity of the contractile elements to Ca^{2+} (Ahn *et al.*, 1988; Watanabe *et al.*, 1992; van der Bent & Beny, 1991). In chicken gizzard smooth muscle, caffeine has been shown to directly inhibit MLCK (Ozaki *et al.*, 1990). The inhibitory effect of caffeine can be mimicked to a certain extent by the lipid soluble dibutyl derivative of cAMP. This would indicate that increases in cAMP resulting from inhibition of phosphodiesterase may contribute to the action of caffeine (Watanabe *et al.*, 1992).

(J) Physiological Role of the Caffeine-Sensitive Ca^{2+} Store

Similar to caffeine, noradrenaline induced a biphasic increase in $[\text{Ca}^{2+}]_i$ levels. The initial transient increase is due to release of internal Ca^{2+} while the tonic rise is due to influx of external Ca^{2+} (Pacaud *et al.*, 1992). In the rat tail artery VSMC, the increase in both the transient and the tonic components to maximum stimulation by caffeine and noradrenaline are identical. An interesting observation is that the cells contracted more to noradrenaline (to 36 % of resting cell length) than to caffeine (to 44 %

of resting cell length) despite the fact that both agents elicited a similar rise in Ca^{2+} . This difference in contraction may be related to a decrease in sensitivity of the contractile elements to caffeine (Ahn *et al.*, 1988; Watanabe *et al.*, 1992) and/or an increase in sensitivity to noradrenaline (Itoh *et al.*, 1992).

That the caffeine-sensitive Ca^{2+} store participates in physiological function has been repeatedly demonstrated. The present and other ^{45}Ca -flux studies (Leijten & van Breemen, 1984) suggest that caffeine and noradrenaline have complete access to the same Ca^{2+} stores and Ca^{2+} can be depleted from these stores either by caffeine or noradrenaline. No additional Ca^{2+} can be released even when challenged by high concentrations of the other stimulant. Other than depleting the Ca^{2+} stores, it should be noted that caffeine may also directly inhibit noradrenaline-triggered Ca^{2+} release (Ahn *et al.*, 1988).

In contrast to the present study, noradrenaline was found to only partially deplete the caffeine-sensitive Ca^{2+} store in the rabbit mesenteric artery (Saida & van Breemen, 1984; Itoh *et al.*, 1992). Thus the extent of overlap between the noradrenaline- and caffeine-sensitive Ca^{2+} stores may vary depending on the preparation.

(K) Future Experiments

Throughout the study, single VSMC oscillations were occasionally noted in response to a variety of stimuli. Differences were noted in frequency, amplitude and duration of the oscillatory response. It would be interesting to further characterize the nature of these $[\text{Ca}^{2+}]_i$ oscillations under a variety of conditions such as the possible

dependence on agonist concentration, membrane voltage, or extracellular Ca^{2+} . Additional studies should also extend the present observations seen with noradrenaline. For example the effects of the agents used in the present study e.g. ryanodine, TMB-8, nifedipine, thapsigargin, removal of extracellular Ca^{2+} , on noradrenaline-induced $[\text{Ca}^{2+}]_i$ signals should be investigated. Their actions on the noradrenaline response could similarly be correlated with transient and tonic $[\text{Ca}^{2+}]_i$ changes and corresponding VSMC length. The importance of both Na^+ - Ca^{2+} exchange and the Na^+ - K^+ -ATPase in the maintenance of $[\text{Ca}^{2+}]_i$ in single VSMCs should also be investigated further. For example, the effect of the Ca^{2+} ionophore 4-Br-A23187 on $[\text{Ca}^{2+}]_i$ in the presence of monensin and ouabain could be observed in our VSMC preparation.

The possibility also arises of combining the present fura-2 epifluorescence technique with the whole cell electrode recording technique. This would allow concurrent measurement of fluorescence-based $[\text{Ca}^{2+}]_i$ and cell length changes in conjunction with electrophysiological properties such as cell voltage and/or current. Attachment of a micropipette to single VSMCs may allow direct fura-2 loading of the pentapotassium salt. In addition, this technique might allow the direct infusion of agents through the micropipette directly into the cytoplasm. This would greatly facilitate the study of the effects of second messengers such as InsP_3 , cAMP, or cGMP without the need for membrane-permeable analogues.

CONCLUSIONS

The fura-2 fluorescent technique has been applied to measure $[Ca^{2+}]_i$ in single vascular smooth muscle cells as well as intact vascular strips. However, correlation of $[Ca^{2+}]_i$ changes with contraction has been done previously only on vascular strips. In the present study, we showed that it is possible to correlate $[Ca^{2+}]_i$ changes with cell shortening at the single cell level.

One problem with single cell studies is that the cell morphology changes with stimulation, and therefore may affect Ca^{2+} response. Despite the fact that many Ca^{2+} signalling studies with single cells have been reported in the literature, there have been no attempts to systematically examine if the contraction of these cells affects the Ca^{2+} response. In the present study, we showed that the Ca^{2+} responses to caffeine are reproducible with repeated challenges, regardless of the contractile state of the cell.

The fact that the Ca^{2+} signals are reproducible upon repeated challenges allows meaningful pharmacological studies on the same cell. This is important as there can be great heterogeneity in the Ca^{2+} signal response amongst cells. On this basis, we have shown that some agents known to affect Ca^{2+} regulation such as TMB-8, nifedipine and thapsigargin have no significant effects on the caffeine-induced Ca^{2+} responses. We have also shown that caffeine and noradrenaline are equally effective in releasing intracellular Ca^{2+} and inducing a tonic increase in $[Ca^{2+}]_i$.

In the present study we have demonstrated that isolated single cells from the rat tail artery can be used for pharmacological studies of Ca^{2+} signalling with the fura-2 technique. The single cell preparation may be further exploited by combining

measurement of ion channel activities using patch clamp recording with the simultaneous monitoring of $[Ca^{2+}]_i$ with fura-2.

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