Ca^{2+} movement in smooth muscle cells studied with one- and two-dimensional diffusion models

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ABSTRACT Although many of the processes involved in the regulation of Ca^{2+} in smooth muscle have been studied separately, it is still not well known how they are integrated into an overall regulatory system. To examine this question and to study the time course and spatial distribution of Ca^{2+} in cells after activation, one- and two-dimensional diffusion models of the cell that included the major processes thought to be involved in Ca regulation were developed. The models included terms describing Ca influx, buffering, plasma membrane extrusion, and release and reuptake by the sarcoplasmic reticulum. When possible these processes were described with known parameters. Simulations with the models indicated that the sarcoplasmic reticulum Ca pump is probably primarily responsible for the removal of cytoplasmic Ca^{2+} after cell activation. The plasma membrane Ca-ATPase and Na/Ca exchange appeared more likely to be involved in the long term regulation of Ca^{2+}. Pumping processes in general had little influence on the rate of rise of Ca transients. The models also showed that spatial inhomogeneities in Ca^{2+} probably occur in cells during the spread of the Ca signal following activation and during the subsequent return of Ca^{2+} to its resting level.

INTRODUCTION

It has been well established that Ca^{2+} plays an important role as a second messenger in most, if not all, normally functioning cells. At higher concentrations, Ca^{2+} is also important in the initiation of processes that ultimately lead to the segregation and death of damaged or diseased cells. Intracellular [Ca^{2+}] is normally maintained at very low levels and rises transiently during signaling processes as a result of influx from the extracellular space and/or release from storage sites within the cytoplasm. For signaling to be effective, mechanisms must be in place for the maintenance of low resting Ca^{2+} levels in the face of a concentration gradient of several orders of magnitude between the Ca sources (the extracellular medium and intracellular storage sites) and the cytoplasm. It is also necessary that cells be capable of removing, buffering and sequestering Ca^{2+} once intracellular levels rise.

In all muscle types, a rise in intracellular [Ca^{2+}] is one of the essential steps that link membrane stimulation to the activation of the contractile proteins. In smooth muscle, although the role of Ca^{2+} in excitation contraction coupling is well documented, less is known about the mechanisms involved in the maintenance of resting Ca levels and in the release and reuptake of Ca^{2+} during contraction. Both extracellular influx and release of Ca^{2+} from intracellular storage sites is thought to occur during stimulation but the relative importance of these two sources may be different in different smooth muscle cell types and in the same cell type when stimulated in different ways. Resting [Ca^{2+}] in smooth muscle has been measured and found to be ~150 nM (Williams et al., 1985); extracellular [Ca^{2+}] is normally ~2 mM. A number of pumping processes, including a plasma membrane Ca-ATPase, Na/Ca exchange and a sarcoplasmic reticulum (SR) Ca-ATPase, may contribute to the maintenance of resting Ca levels in the face of inward leakage through the plasma membrane and out of intracellular storage sites. Influx of Ca^{2+} through plasma membrane Ca channels, release of Ca^{2+} from the SR, Ca buffering mechanisms, and the Ca removal processes noted above all contribute, to various degrees, to the determination of the magnitude and kinetics of the Ca transient that turns on the contractile process in smooth muscle. Because of this, it is important for an understanding of smooth muscle contraction to understand not only these Ca regulatory processes themselves but also the extent to which each contributes to the rising and falling phases of the Ca signal. Because the Ca signal is generated from discrete locations in cells, such as the SR and the plasma membrane, it is likely that spatially localized mechanisms feed back on and regulate this signal. Thus, in addition to an understanding of the more global, temporal aspects of the Ca signal, it is also essential that the spatial properties of the signal be explored.

One of our goals, in the work presented here, was to develop a mathematical model of Ca diffusion and the Ca regulatory processes in smooth muscle to gain an understanding of how these processes may work to-

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gether to control the temporal and spatial distribution of Ca^{2+} in cells following Ca influx from the extracellular medium or release from intracellular storage sites. When possible, we modeled processes using parameters available from the literature. However, because many of the Ca handling mechanisms in smooth muscle have not yet been fully defined, we were not able to do this in all cases. When precise information was not available, we modeled mechanisms in a general qualitative manner and then applied constraints to the model based on experimental evidence to obtain a more quantitative fit to observed results. A second goal of our work was to use the model to examine the ability of current experimental methods to differentiate between different proposed Ca handling mechanisms. This can suggest ways to improve current methods, can point to other experimental approaches that might be tried and can help define those parameters that have a significant influence on Ca handling and, consequently, are most worthy of further experimental study.

Our results indicate that the SR Ca-ATPase is probably the primary mechanism by which Ca^{2+} is removed from the cytoplasm of smooth muscle cells following stimulation. However, Ca pumping processes in general have little influence on the rate of rise of the Ca transient. Our work also shows that the relatively slow rate of Ca diffusion and high degree of Ca buffering in cells could give rise to significant spatial inhomogeneities in the distribution of Ca^{2+}. The magnitude and extent of these inhomogeneities will be dependent upon the distribution of Ca sources and pumping sites within cells.

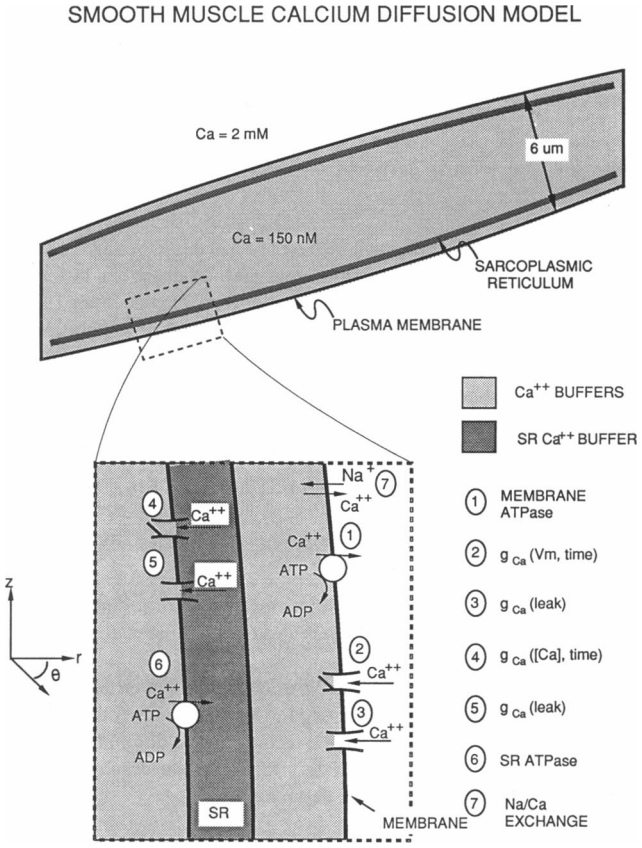

FIGURE 1 Model of the smooth muscle cell used for the simulations. The portion of the cell modeled was assumed to be cylindrical and to have a circular cross section with a diameter of 6 um. The SR was assumed to comprise ~6% of the cell volume and to be located near the surface membrane in the one dimensional simulations. In some of the two-dimensional simulations, the SR was assumed to be distributed throughout the cell. The individual elements of the model and the parameters used in the simulations are described in methods and elsewhere throughout the text. The Na/Ca exchanger shown in the figure was not explicitly modeled but its potential contribution to Ca handling is discussed in the text. The diffusion equations were described in terms of a cylindrical coordinate system (r, θ, z). The z axis of the system was oriented along the long axis of the cell and the r axis was oriented in the radial direction in the cell.

METHODS

Smooth muscle cell model: General description

For the simulations that are reported here the smooth muscle cell was assumed to be cylindrical with a diameter of 6 um (Fig. 1). The dimensions of the model cell were chosen to be consistent with those of single smooth muscle cells isolated from the stomach of the toad *Bufo marinus* because these cells are currently the best characterized single cell preparation. At rest, only a Ca leak channel in the surface membrane that was compensated for by a Ca pump was assumed to be open. When the membrane permeability increased upon stimulation, Ca^{2+} moved by diffusion through water filled pores 75 Å (membrane thickness) in length. At the beginning of the simulations intracellular [Ca^{2+}] was taken as 150 nM and extracellular [Ca^{2+}] as 2 mM. The extracellular Ca-pool was infinite in extent, well stirred and was not altered by the Ca^{2+} taken into the cell. The sarcoplasmic reticulum of smooth muscle was modeled as a source (SR release) and sink (SR pump) of Ca^{2+} located at various positions within the cell. Ca^{2+} release from the SR began when intracellular Ca^{2+} in the immediate vicinity of the SR rose to a predetermined switch level (Ca-induced Ca-release). Release was modeled (as described below) as due to an initial rapid exponential increase in SR permeability followed by a slower exponential decline in permeability. The SR Ca pump velocity was assumed to be dependent on intracellular [Ca^{2+}] in the immediate vicinity of the SR and to follow the kinetics described below.

Diffusion equations

Two diffusion equations were solved numerically for the simulations. In the first case, a one-dimensional equation was solved to describe Ca^{2+} diffusion in the smooth muscle cell in the radial direction only. In the second case, diffusion was also allowed to occur along the long axis of the cell (z axis) so that Ca^{2+} movement away from localized regions of high Ca^{2+} could be modeled. In the equations presented below Ca designates [Ca^{2+}].

For the one dimensional problem, diffusion was described by the equation:

$$\frac{\partial Ca}{\partial t} = \frac{1}{r}\frac{\partial}{\partial r}\left(rD\frac{\partial Ca}{\partial r}\right) + F(Ca, t, r), \quad (1)$$

where the first term is derived from Fick's laws of diffusion for a cylindrical system in which only radial diffusion occurs and $F(Ca, t, r)$ includes other concentration, time and position dependent sources and sinks within the cell that will be described in detail below. To solve this equation, the explicit finite difference method described by Crank (1975) was used. Briefly, the cell was divided into 30 concentric annuli each 0.1 um in thickness. The Ca^{2+} diffusion term for the jth annulus can be written as:

$$\frac{\Delta Ca}{\Delta t} = \frac{D}{(\Delta r)^2}[(Ca_{j+1} - 2Ca_j + Ca_{j-1}) + (Ca_{j+1} - Ca_{j-1})/2(j-1)] \quad \text{for } j > 1, \quad (2)$$

$$\frac{\Delta Ca}{\Delta t} = \frac{4D}{(\Delta r)^2}(Ca_2 - Ca_1) \quad j = 1 \text{ (center of model cell)}. \quad (3)$$

This equation was applied to diffusion through the intracellular medium and a similar equation was used for diffusion through the plasma membrane.

In the two-dimensional model, diffusion was assumed to occur in both the radial and z directions (along the long axis of the cell; see Fig. 1). In this case, the diffusion equation included an additional term for z axis Ca^{2+} movement and the term describing the sources and sinks of Ca^{2+} in the cell was also z position dependent.

$$\frac{\partial Ca}{\partial t} = \frac{1}{r}\frac{\partial}{\partial r}\left(rD\frac{\partial Ca}{\partial r}\right) + \frac{\partial}{\partial z}\left(D\frac{\partial Ca}{\partial z}\right) + F(Ca, t, r, z). \quad (4)$$

The numerical solution (Eqs. 2 and 3) was modified as described by Crank (1975). For the numerical integration, 0.1 um length elements (equal in size to the thickness of the radial annuli) were used. Because the cell continued along the z axis beyond the region modeled, for the numerical integration, diffusion was assumed to continue without reflection one length element beyond this region. This assumption meant that there were no sources or sinks of Ca^{2+} immediately beyond the area of the cell that was modeled. This constraint produced no apparent discontinuities in $[Ca^{2+}]$ at the ends of the modeled area.

In the membrane, Ca^{2+} was assumed to move by simple diffusion through water filled pores. If the membrane has a permeability of $P_{Ca}(t)$ and a thickness of 75 Å, the diffusion of Ca^{2+} through the membrane is equivalent to its diffusion through a fluid with diffusion coefficient

$$D_{membrane} = P_{Ca}(t) \times 75\text{Å}, \quad (5)$$

(see, for example, Woodbury, 1965).

Inside a cell the mobility of Ca^{2+} is less than that in water because of the physical barrier imposed by the cytoskeleton and organelles and because Ca^{2+} binds to buffers and pumps within the intracellular space. If one examines electron micrographs of smooth muscle cells in longitudinal or cross-section (see Kargacin and Fay, 1987, for example), it is apparent that a significant portion of the intracellular space is taken up by myofilaments and other structures. To account for this restricted intracellular diffusion space in the models, the diffusion coefficient for Ca^{2+} within the cell was assumed to be 4×10^{-6} cm^2/s $\sim 1/2$ that in water (7×10^{-6} cm^2/s; see Kushmeric and Podolsky, 1969). To arrive at this value for the intracellular diffusion coefficient, the amount of space per unit cross-sectional area occupied by intracellular structures in smooth muscle cells was estimated from electron micrographs. A conservative estimate obtained in this way suggested that perhaps 40–50% of the intracellular space in smooth muscle cells is occupied by myofilaments, mitochondria, SR, and other structures and consequently unavailable as free diffusion space. Our estimate of a cytoplasmic diffusion coefficient is consistent with values used by Backx et al. (1989) and Sala and Hernandez-Cruz (1990).

The boundaries between the extracellular space and the membrane and the intracellular space and the membrane where the diffusion coefficient changed were treated as described by Crank (1975) for diffusion through composite media.

Membrane permeability change

To model the time dependent opening and closing of surface membrane Ca channels during smooth muscle activation, the permeability of the membrane was transiently increased as described by the following equation:

$$P = P_0(1 - \exp^{-t/T_{on}})(\exp^{-t/T_{off}}) \quad (6)$$

This equation is similar to those used by Cannell and Allen (1984) and Backx et al. (1989) and describes an exponential rise and fall in Ca-permeability. The permeability constant, P_0, was adjusted so that the maximum permeability reached during the transient (6×10^{-6} cm/s) was approximately equal to that calculated by Walsh and Singer (1980) from electrical recordings made from isolated smooth muscle cells from the toad stomach. The time constants T_{on} and T_{off} were adjusted so that the time course of the permeability transient matched the current records made by Becker et al. (1989) from voltage clamped toad stomach smooth muscle cells. The assumption that the time course of the Ca current is proportional to the time course of the permeability change is consistent with the current form of the Goldman-Hodgkin-Katz constant field equation (see Hille, 1984) provided that the electro-chemical driving force for Ca^{2+} does not change significantly during the Ca influx. This would be true for a smooth muscle cell under voltage clamp bathed in a well stirred medium with $[Ca^{2+}]_{out} \gg [Ca^{2+}]_{in}$.

Plasma membrane Ca-pump and leak

In addition to a transient change in plasma membrane permeability to Ca^{2+}, a plasma membrane extrusion mechanism (Ca-pump) and a Ca leak were also incorporated into the model. The equation

$$\frac{\Delta Ca}{\Delta t} = \frac{V_{max}(Ca)^n}{K_{PM} + (Ca)^n} \quad (7)$$

was used to describe the Ca^{2+} movement by the plasma membrane pump. The maximum pump velocity (V_{max}) expected for smooth muscle cells (3.2×10^{-13} mol/cm^{2}s), K_{PM} (200 nM) and Hill coefficient ($n = 1$) used in the simulations were taken from the work reported by Lucchesi et al. (1988; see also Carafoli, 1987). To convert the pump velocity into concentration units for the simulations, the surface area per unit length (A) and volume per unit length (Va) of the annulus immediately under the membrane were estimated from the dimensions of the model cell.

The inward leak through the plasma membrane was described by the equation

$$\frac{\Delta Ca}{\Delta t} = K_{leakPM}(Ca_{extracellular} - Ca) \quad (8)$$

in which K_{leakPM} was adjusted so that Ca-influx at rest through the leak balanced resting pump efflux determined from Eq. 7.

Sacroplasmic reticulum release and pumping

Although questions about the location, extent and the kinetics of Ca^{2+} pumping and release of the smooth muscle SR are currently of much experimental interest, they remain, as yet, largely unresolved. The presence of an SR in smooth muscle is suggested by both structural and physiological evidence (Devine et al., 1972; Itoh, et al., 1981; Kargacin and Detwiler, 1985; Iino, 1989). Biochemical evidence suggests that the Ca pumping and release processes may be similar in many respects to the more completely described mechanisms of cardiac and skeletal muscle (Eggermont et al., 1988; Wuytack et al., 1989). In modeling the SR in smooth muscle, known constants were incorporated when possible and other parameters were adjusted to match observed data. The SR in the modeled smooth muscle cells was assumed to comprise ~6% of the cell volume based on the results of Devine et al. (1972). The SR acted like a source that could release Ca^{2+} into the cytoplasmic element in which it was located. As described below, simulations were done with the model to examine the effects of the location of the SR in smooth muscle cells on the spatial and temporal distribution of Ca^{2+}. Release of Ca^{2+} from the SR was modeled as due to Ca-induced Ca-release. When intracellular Ca^{2+} at the location of the SR rose to a preset level, Ca^{2+} release from the SR into the cytoplasm started and was approximated by the equation:

$$\frac{\Delta Ca}{\Delta t} = K_{SR}(Ca_{SR} - Ca), \quad (9)$$

which is similar to the equations used by Cannell and Allen (1984) and Backx et al. (1989) for models of skeletal and cardiac muscle respectively. The magnitude of the coefficient K_{SR} was time dependent and described by the equation

$$K_{SR} = K_0(1 - \exp^{-t/T_{onSR}})(\exp^{-t/T_{offSR}}). \quad (10)$$

The time constants, T_{onSR} and T_{offSR}, were assumed to be equal to those of the surface membrane Ca channel as described above. K_0 was adjusted to vary the extent of SR release in different simulations.

Uptake of Ca^{2+} by the smooth muscle SR Ca-pump was assumed to be similar to that of skeletal and cardiac muscle. Uptake velocity was assumed to be concentration dependent and described by the Hill equation

$$\frac{\Delta Ca}{\Delta t} = \frac{V_{maxSR}(Ca)^n}{K_1 + (Ca)^n}. \quad (11)$$

Where V_{maxSR} is the maximum uptake velocity, K_1 the $[Ca^{2+}]^2$ at half maximal velocity and n the Hill coefficient. V_{maxSR} was varied in the simulations as described below. The Hill coefficient was taken as 2 and K_1 as 0.048 μM^2 from the values describing SR Ca uptake in skeletal muscle (see Kargacin et al., 1988). A Ca leak through the SR membrane similar to that described above for the surface membrane was also incorporated into the model and adjusted to match the SR pumping rate at rest so that no net movement across the SR membrane occurred at rest.

Based on estimates of the Ca gradient that can exist across SR membranes (Schatzmann, 1989), the initial SR $[Ca^{2+}]_{free}$ was taken as 1.5 mM. SR Ca buffering or binding sites were not explicitly modeled, although the SR was allowed to continue to take up Ca^{2+} during simulations after the free $[Ca^{2+}]_{SR}$ reached 1.5 mM. The absence of an SR Ca buffer in the model meant that the free concentration dropped more rapidly after SR release in the simulations than would be expected in real cells, however, the concentration difference between the SR (>1 mM) and the cytoplasm (<1 μM) remained high during the simulated Ca transient and it is unlikely that a significant error was introduced by the simplifications that were made.

Ca buffering

A number of potential buffering systems for intracellular Ca have been proposed (Robertson et al., 1981; Bond et al., 1984; Sala and Hernandez-Cruz, 1990). In the interest of simplicity and because relatively little is known about Ca buffering in smooth muscle, we chose to lump all Ca buffers in our model into a single immovable buffer. The possible existence of movable buffers in cells and the facilitated diffusion of Ca^{2+} that might result from Ca binding to these buffers will be discussed later. To model Ca buffering, we incorporated a rate equation for the buffering system in the model,

$$\frac{\Delta Ca}{\Delta t} = -K_{on}([buffer]_{free})(Ca) + K_{off}[Ca]_{bound\ to\ buffer} \quad (12)$$

The on and off rate constants, K_{on} and K_{off} (10^8/M-s and 10^2/s, respectively), were chosen to fall within the general range of values described in the literature for cytoplasmic Ca buffers (see Robertson et al., 1981). The total buffer concentration in the cell was altered for some simulations but was chosen to be 230 μM in most cases similar to the value for total buffering reported by others (Bond et al., 1984). With a K_d of 10^{-6} M this meant that the free buffer concentration at rest was 200 μM. With these parameters intracellular $[Ca^{2+}]$ in the modeled cells was buffered to the extent that the ratio of bound to free Ca was ~90:1 when intracellular $[Ca^{2+}]$ reached 0.8 μM during a Ca transient. This ratio is in general agreement with estimates by Becker et al. (1989) for smooth muscle cells.

Simulations with the one- and two-dimensional models were run on either a Silicon Graphics GTX or Personal Iris computer (Silicon Graphics, Mountain View, CA).

RESULTS AND DISCUSSION

Characteristics of the modeled Ca transient

Fig. 2 shows the time course of the rise and fall of average intracellular $[Ca^{2+}]$ in the model cell compared to the free $[Ca^{2+}]$ measured by Becker et al. (1989) from a fura-2 loaded isolated smooth muscle cell. The parameters used in the simulations will be discussed below. The excellent agreement between the actual Ca trace and that derived from the simulation indicated that the model, as constructed, could provide a good general description of the Ca transient in isolated cells. Based on this result, it was then of interest to examine, in greater detail, the various Ca regulatory processes that were incorporated into the model to better understand how each contributed to the spatial and temporal characteristics of the Ca transient.

SR Ca release and its effects on the rising phase of the Ca transient

The mechanism by which Ca^{2+} is released from the SR in smooth muscle is not well understood. A number of the

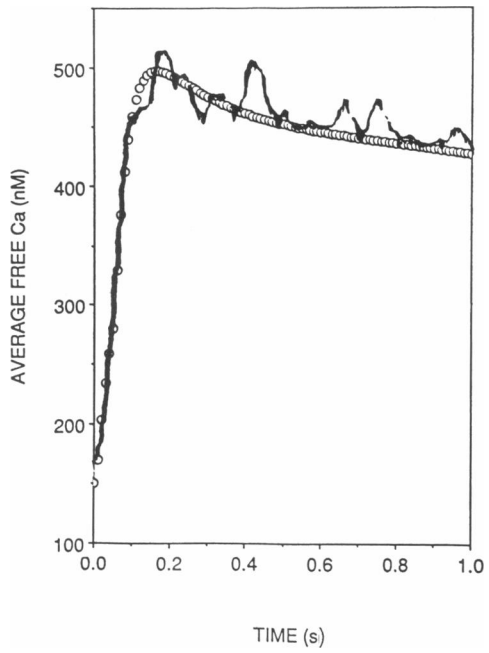

FIGURE 2 Rise of intracellular [Ca^{2+}] following cell stimulation predicted by the model and obtained experimentally. The parameters used in model will be discussed throughout the text. The experimental trace was obtained by P. L. Becker, J. V. Walsh, and F. S. Fay (University of Massachusetts Medical School, unpublished results) and is the Ca transient (measured with fura-2) obtained from an electrically stimulated isolated smooth muscle cell.

possibilities that have been proposed, however, can be classified into two broad categories: (*a*) release involving a second messenger (eg., Ca induced Ca release; IP_3 induced Ca release); (*b*) release directly coupled to membrane potential changes perhaps electrically or mechanically. Coupling through a second messenger could involve a multiplicity of diffusion, binding, and channel opening events and would, as a consequence, be expected to be slower than a direct coupling mechanism. It is also possible that, in response to some stimuli, the influx of extracellular Ca^{2+} is the only source of Ca^{2+} for contraction. To determine if it was possible to distinguish these mechanisms by the rate of rise of intracellular [Ca^{2+}] in the model after stimulation, the simulations described below were run. The SR space was assumed to be located within a 0.1-μm thick annulus located in the cell 0.2 μm away from the plasma membrane, similar to the diagram in Fig. 1. The total intracellular Ca buffer concentration was set at 230 μM. When the SR was included in simulations, release from the SR and influx from the extracellular space were adjusted so that both contributed approximately equally to the total Ca signal. The Ca channel in the SR was assumed to have the same opening and closing kinetics as that of the plasma membrane channel but to be controlled by either intracellular [Ca^{2+}] (Ca-induced Ca-release) or to be electrically or mechanically coupled to the surface membrane (direct coupling). For Ca-induced Ca-release, Ca^{2+} acted on a simple switch, as described in methods, that opened to allow Ca efflux when the [Ca^{2+}] at the SR reached a preset level, [switch]. For direct coupling, SR release was started at the same time that the Ca^{2+} permeability of the surface membrane began to increase. To see if a diffusionally delayed release process could be detected in the rising phase of the Ca transient, [switch] was varied and the time course of the resulting transient was compared to the time course expected for direct coupling.

Fig. 3 *A* shows the initial 100 ms of the Ca transient in the model cell at three different [switch] (300 nM,

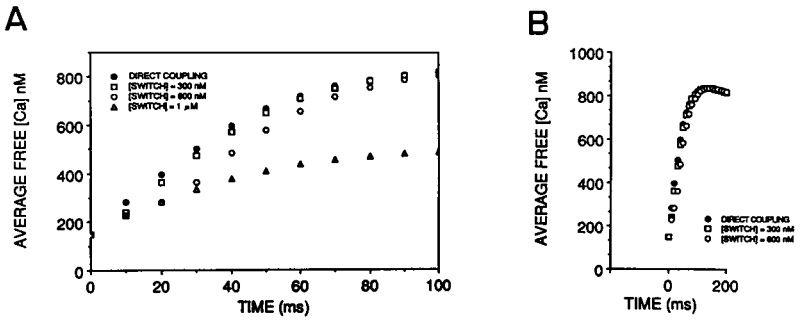

FIGURE 3 Effects of [switch] on the rise of the Ca transient in the model cell. (*A*) The concentration of Ca^{2+} necessary to induce Ca release from the SR was set at three different levels (300 nM, 600 nM, 1 μM; *open symbols*). The closed symbols show the Ca rise assuming that SR release began at the same time that Ca influx through the membrane began (direct coupling). (*B*) The direct coupling curve from (*A*) and those obtained with [switch] of 300 nM and 600 nM are replotted at lower time resolution to approximate the rate of Ca rise that one might expect to see in experimental records.

600nM, and 1 μM) and with direct coupling (*closed circles*). As can be seen, as [switch] increased, a small but noticeable delay in the rise of $[Ca^{2+}]$ occurred until at a [switch] of 1 μM uptake of Ca^{2+} by the SR and plasma membrane pumps prevented Ca^{2+} from reaching a high enough concentration to trigger release during the period of the simulation. The latter result supports the conclusion of Iino (1989) that a Ca sensitivity of > 1 μM for Ca-induced Ca release would be too low for such a mechanism to effectively couple membrane excitation and SR release in smooth muscle. If release involves a second messenger other than Ca^{2+} the delay seen in Fig. 3*A* could be greater or less depending upon the kinetics of the second messenger mechanism and the diffusion coefficient of the messenger molecule. One would also expect the delay to be less if, as suggested by the work of Devine et al. (1972), some elements of the SR are within 10–20 nm of the surface membrane. Although not shown in Fig. 3*A*, there was virtually no difference between the direct coupling curve and curves obtained from simulations in which only Ca influx occurred (no SR release). For the latter simulations, the Ca permeability of the plasma membrane was adjusted so that the average intracellular $[Ca^{2+}]$ rose to the same level as that shown in Fig. 3*A*.

Although it is possible in the simulations shown in Fig. 3*A* to differentiate diffusionally delayed SR Ca release from more rapid release due to direct coupling or from Ca influx alone, the differences in the traces are very slight. When plotted on a slower time scale (Fig. 3*B*) it seems unlikely that an integrated Ca signal by itself, such as that obtained from a fura-2 loaded cell monitored with a photomultiplier, could be used to reliably distinguish among the possible mechanisms for SR release or to tell whether release occurred at all.

In the simulations described above, SR release and Ca influx were assumed to contribute approximately equally to the Ca transient. If the primary source of Ca^{2+} for contraction was the SR and a small Ca influx served only to trigger release one would expect a more pronounced delay before the onset of the rapid rising phase of the transient. In actual experiments, the relatively long delay expected between cell stimulation and the rise of the Ca transient might be apparent in a fura-2 signal. The results of Becker et al. (1989) in which the Ca transient measured in fura-2 loaded cells and the integral of the Ca current from voltage clamp records were compared and found to follow roughly the same time course are consistent with the absence of a pronounced delay between Ca influx and SR release or with Ca influx as the sole source for contractile Ca^{2+}.

Mechanisms of Ca removal following cell stimulation

For Ca^{2+} to be an effective second messenger, it is necessary that cells have mechanisms to precisely regulate its intracellular concentration and to remove it after a signal has arrived at appropriate sites in the cell and its effects on cell function have taken place. In smooth muscle, a number of processes have been proposed that may contribute to either the extrusion or sequestration of intracellular Ca^{2+} following contractile stimulation. As described in methods and illustrated in Fig. 1 two mechanisms for lowering intracellular $[Ca^{2+}]$ were incorporated into our model, a SR Ca-ATPase and a plasma membrane Ca-ATPase. The results of Lucchesi et al. (1988) indicate that the $[Ca^{2+}]$ for half maximal stimulation of the plasma membrane pump is 200 nM and that the maximum pump velocity is 3.2×10^{-13} mol-Ca/s-cm^2 cell surface area. This maximum velocity, when converted into concentration units (as described in methods) and incorporated into Eq. 7, describing the pump, reduced intracellular free $[Ca^{2+}]$ at the rate shown by the closed symbols in Fig. 4. This rate of change in $[Ca^{2+}]$ was not alone sufficient to account for the rate of decline measured by Becker et al. (1989) with fura-2 as shown by the dashed line in Fig. 4. The rate for the interval between 500 ms and 1 s predicted by the model was, in fact, only 8% of the observed rate.

If the pump density on the smooth muscle cell is

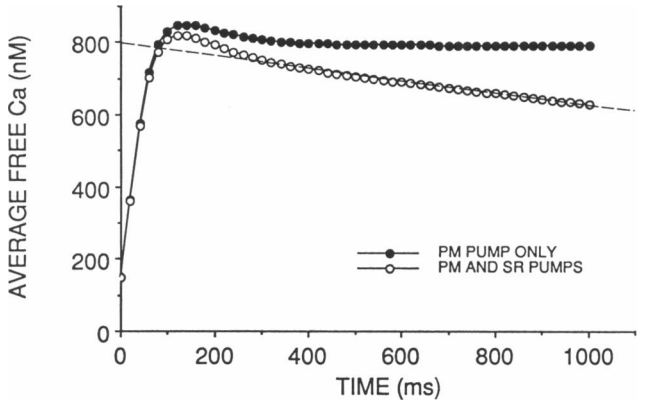

FIGURE 4 Rise and fall of $[Ca^{2+}]$ in the model cell. Average intracellular free $[Ca^{2+}]$ is plotted versus time. (*Closed symbols*) Ca transient expected with the plasma membrane Ca-ATPase acting alone to remove Ca from the cytoplasm following influx and SR release. Open symbols: Ca transient expected with the plasma membrane and SR Ca-ATPases both acting to remove Ca^{2+}. (*Dashed line*) rate of free Ca^{2+} decline (~ 140 nM/s) expected at an intracellular free $[Ca^{2+}]$ of 0.8 μM from the experiments of Becker et al. (1989). Note that, at the level of time resolution shown, there was only a slight difference in the initial rate of rise of the transient in the two simulations.

greater than that found by Lucchesi et al. (1988) and if the presence of caveoli increases the surface membrane of the smooth muscle cell (from capacitance measurements, Langton et al. [1989] suggest that the actual surface membrane of a smooth muscle cell may be twice that calculated from the geometry of the cell), the rate of decline of $[Ca^{2+}]$ would be faster (perhaps by a factor of two or three) than that shown by the closed symbols in the figure. It is also likely, however, that the SR pump and other Ca extrusion mechanisms contribute significantly to the Ca decline. In skeletal and cardiac muscle, the Ca-ATPase of the SR comprises a significant percentage of the total SR membrane protein (Carafoli, 1987). The plasma membrane Ca-ATPase is a much less abundant protein. Similarly, in smooth muscle, it has been suggested (see discussion by Eggermont et al. 1988, for example) that SR pumping may be of primary importance for the immediate lowering of intracellular $[Ca^{2+}]$ after stimulation. As shown in Fig. 4 by the open symbols, Ca pumping at the plasma membrane and at the SR at a rate of $\sim 3 \times 10^{-12}$ mol-Ca/s-cm^2 of SR surface area (assuming the dimensions of the SR in the model cell) lowered intracellular free $[Ca^{2+}]$ at the rate expected from the fura-2 experiments of Becker et al. (1989). The higher SR pump rate could be achieved if the SR pump had a higher Vmax and/or if there was a high density of pump sites on the SR membrane. Because the volume of the SR is limited, however, the capacity of the SR store would have to be increased by the presence of SR buffers. The Ca binding proteins calsequesterin and calreticulin have been shown to be associated with the SR in smooth muscle (Wuytack et al., 1987; and Fliegel et al., 1989*a*,*b*) and could provide the SR space with a significantly higher holding capacity for Ca^{2+}.

Another Ca extrusion mechanism that might contribute significantly to Ca removal is Na/Ca exchange. Although the Na/Ca exchange mechanism has been the subject of much interest (see the review by Lagnado and McNaughton, 1990), its precise mode of operation is still not well understood nor is it known to what extent it contributes to the removal of Ca^{2+} in cells following a transient elevation. In rod outer segments, Schnetkamp (1986) has calculated that the exchanger could lower total $[Ca^{2+}]$ by more than 0.5 mM/s. In cardiac cells also, Na/Ca exchange may be an important mechanism for Ca regulation. Using vesicles derived from the same source (toad stomach smooth muscle) as those used by Lucchesi et al. (1988), Matlib (M.A. Matlib, University of Cincinnati, personal communication) has estimated a Vmax of 16 nmoles/min/mg protein and a K_m of 7.1 μM for Ca^{2+} for the Na/Ca exchanger. Compared to the results of Lucchesi et al. (1988) for the plasma membrane Ca-ATPase, these results suggest that at most the exchanger could contribute to Ca removal to an extent equal to that of the plasma membrane Ca-ATPase although it operates at a higher K_m. As we will show below, a fairly high $[Ca^{2+}]$ may be found near the plasma membrane during early stages of a Ca transient and thus Na/Ca exchange could contribute significantly to Ca^{2+} removal during an excitatory stimulus: however, given the effects of membrane depolarization on the exchanger (Yasui and Kimura, 1990; Lagnado and McNaughton, 1990), the possibility that Na/Ca exchange acts to counter the rise in $[Ca^{2+}]$ following depolarization seems less likely. Instead it is more likely that Na/Ca exchange may be more important in the long term regulation of intracellular Ca^{2+} than in the early response to transient increases. Further experimental work to resolve these possibilities should help clarify the role of this extrusion mechanism in Ca homeostasis.

In Fig. 4, the average $[Ca^{2+}]$ in the cell declined more rapidly during the first 200 ms following the rise of the transient than it did during the last 600 m of the trace. The reason for this biphasic decline requires an analysis of the spatial distribution of Ca^{2+} in the model cell and is discussed in the next section.

Spatial distribution of Ca^{2+}: one-dimensional model

It is important for an understanding of the role of Ca^{2+} in the contractile process in smooth muscle to know not only the temporal characteristics and the mechanisms responsible for the Ca transient but also how Ca^{2+} is spatially distributed within cells during the transient. The presence of a Ca gradient between the periphery and the center of a cell resulting from a diffusion barrier near the surface membrane, for example, might have important influences on Ca dependent ionic channels, pumps and other Ca sensitive cellular processes in the membrane region. For Ca^{2+} to be an effective intracellular signal, it is also essential that its sources, sites of action and storage locations be arranged spatially in the cell to facilitate its precise regulation. If there are inhomogeneities in the Ca distribution in cells following stimulation, one might expect the processes controlled by Ca^{2+} and/or those involved in its regulation to be localized to those regions in which Ca^{2+} rises to its highest levels. In the simulations shown to this point (Figs. 2–4), average intracellular $[Ca^{2+}]$ in the model cell was computed at various times following the onset of Ca^{2+} entry or release from the SR. Such temporal information is similar, for example, to that which one could obtain experimentally with monitoring devices that integrate a Ca dependent fluorescence signal from a cell. In the simulations described below, both spatial and temporal information obtained from the model about

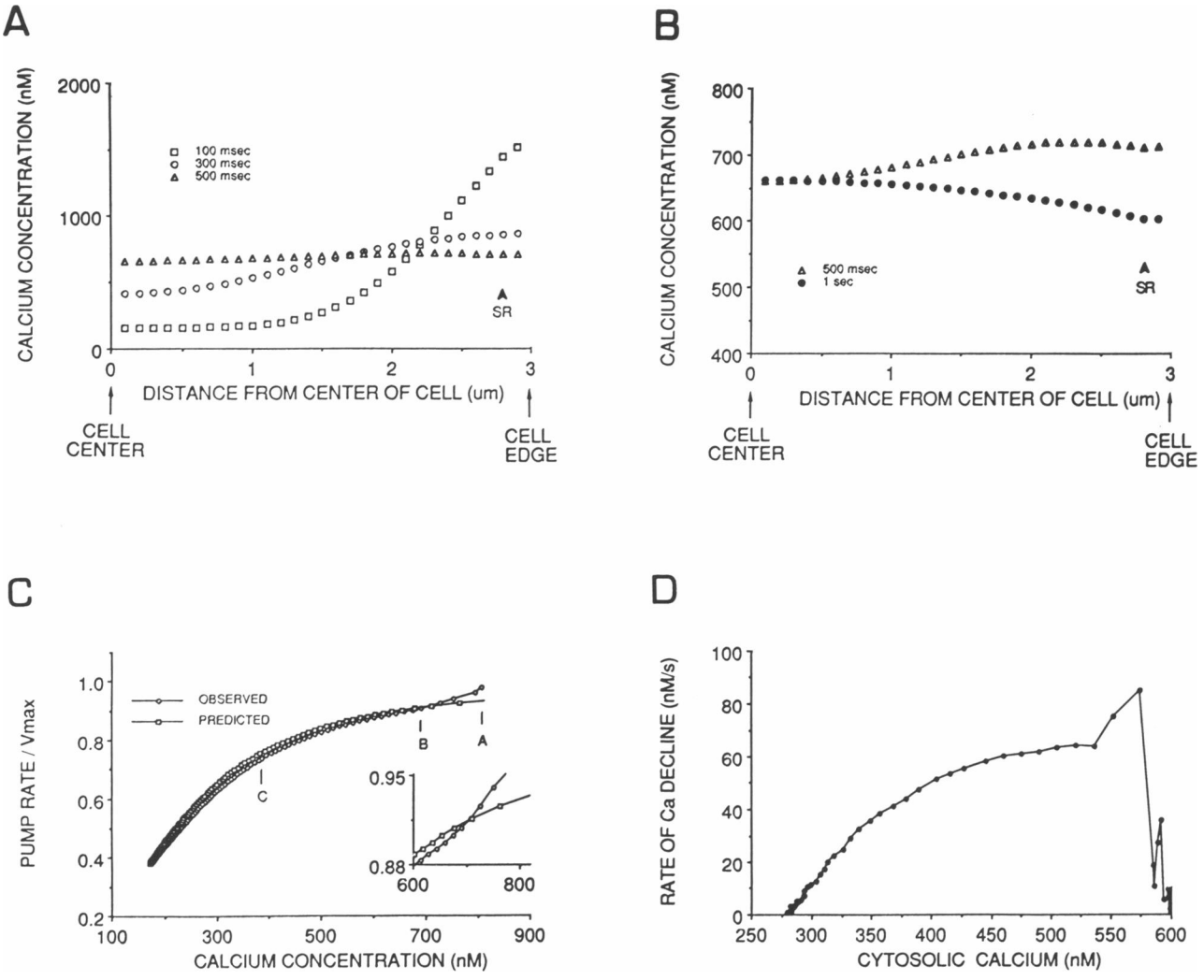

FIGURE 5 (*A* and *B*) Radial distribution of Ca^{2+} in the model cell following stimulation. (*A*) Gradient of Ca^{2+} in the model cell at three different times following stimulation. (*B*) Continuation of the record in (*A*) at longer times. note: the change in scale. (*C* and *D*) Effect of the Ca gradient within smooth muscle cells on Ca pumping rate. (*C*) Relative pump rate ($dCa/dt/V_{max}$) at different average cellular Ca concentrations. (*Open circles*) the pump rate observed from the model cell. (*Open squares*) the pump rate at the SR expected at different Ca concentrations from Eq. 11. The time points corresponding to the points *A*, *B*, and *C* indicated on the curve were 100 ms, 500 ms and 3 s respectively. Inset: region between 600 and 800 nM Ca^{2+} shown on an expanded scale. (*D*) Ca^{2+} decline versus intracellular Ca concentration measured with fura-2 from a voltage clamped toad stomach smooth muscle cell following electrical stimulation.

intracellular Ca^{2+} and the implications of spatial inhomogeneities in Ca^{2+} are described and discussed.

In Fig. 5 *A*, the radial distribution of Ca^{2+} in the model smooth muscle is shown at three times following Ca influx and release from the SR. At 100 ms, there was a steep gradient of Ca^{2+} between the cell surface and the center of the cell. This gradient, which was still visible after 300 ms, decreased with time as Ca influx and release from the SR turned off and as Ca^{2+} diffused throughout the cytoplasm. As Ca^{2+} was pumped out of the cytoplasm by the SR and plasma membrane pumps, $[Ca^{2+}]$ in the vicinity of the pumps was reduced and a slight gradient developed from the center of the cell to the surface. This can be seen in Fig. 5 *B* which is a continuation of the simulation of Fig. 5 *A*. At these later times, diffusion of Ca^{2+} through the cytoplasm and release from the Ca buffers in the cell were better able to keep up with the pump processes and prevented the development of as steep a gradient in $[Ca^{2+}]$ in the cell as was present during net Ca influx (note the change of scale in Fig. 5 *B*). A number of investigators have suggested the possibility that gradients of Ca^{2+}, similar to those predicted by the model in Fig. 5 *A*, develop in cells following influx and/or release from intracellular

stores near the surface membrane (see the recent issue of Cell Calcium edited by Williams and Fay, 1990). The contribution of the SR to such gradients in smooth muscle cells would be dependent upon the coupling of SR release to extracellular influx as well as the location of the SR in the cell. If the SR was closer to the surface membrane, as was discussed earlier, the initial Ca gradient in the cell could be even steeper than that shown in Fig. 5 *A*. Such gradients would also be steeper and maintained for a longer period of time if the diffusion space for Ca^{2+} near the membrane was limited. This might be true in smooth muscle cells in which the physical presence of the SR and caveoli could limit the diffusion space. When, in the model cell, the intracellular diffusion coefficient near the surface membrane (within the first 0.4 μm inside the cell surface) was set at ¼ that in the remainder of the intracellular space, the $[Ca^{2+}]$ near the surface membrane was initially much higher than that shown in Fig. 5 *A*. At 100 ms the concentration in the first annulus inside the cell was >2.5 μM. As time went on, the effect of the restricted space became much less apparent and at 500 ms the gradient was quite similar to that shown in Fig. 5 *B*.

One possible consequence of a gradient of Ca^{2+} similar to that illustrated in Fig. 5 is that membrane Ca pumps and SR pumps located near the surface would pump faster at early times following Ca influx than might be expected from estimates of the average cytoplasmic $[Ca^{2+}]$. The effect of this can be seen in the Ca transient in Fig. 4 where average $[Ca^{2+}]$ declined more rapidly following the peak of the transient than at later times. If the rate of Ca pumping is plotted vs average $[Ca^{2+}]$ for the model cell, a marked variation at high concentrations can be seen in what otherwise looks like a smooth curve illustrative of a saturable removal process (*circles* in Fig. 5 *C*). If, on the other hand, the rate of Ca removal is plotted vs $[Ca^{2+}]$ near the SR pumps, the expected hyperbolic relationship is apparent (*squares* in Fig. 5 *C*). The latter curve is the same as that obtained by directly plotting Eq. 11 assuming Ca^{2+} is uniformly distributed throughout the cytoplasm (i.e., $[Ca^{2+}]$ at every point = average $[Ca^{2+}]$ in the cell). A further illustration of the cause of the apparently anomalous behavior of the pump at high $[Ca^{2+}]$ could be seen upon examination of the spatial distribution of Ca^{2+} in the cell at points *A*, *B* and *C* in Fig. 5 *C*. When the observed pump rate exceeded the expected rate (point *A*), there was a steep gradient of Ca^{2+} in the cell with high $[Ca^{2+}]$ near the membrane similar to that seen at 100 ms in Fig. 5 *A*. At the crossover point (*B*) of the curves, the average $[Ca^{2+}]$ in the cell was equal to SR $[Ca^{2+}]$ and there was little apparent gradient in the cell. At point *C* when the actual pump rate was lower than that expected from the average $[Ca^{2+}]$ a reverse gradient existed in the cell (similar to that shown by the *closed circles* in Fig. 5 *B*).

The apparently anomalous behavior of the Ca removal process as seen in Fig. 5 *C*, has also been observed in experimental records. Fig. 5 *D* shows a plot of dCa/dt vs $[Ca^{2+}]$ derived from the fura-2 signal from an isolated smooth muscle cell (P. L. Becker, University of Massachusetts Medical School, unpublished). The experimental results are qualitatively similar to those predicted by the model (Fig. 5 *C*) and are consistent with the existence of a transient, local, high concentration of Ca^{2+} in cells near the sites of Ca pumping that initially stimulates Ca removal until reuptake and diffusion processes reduce its magnitude. In the trace shown in Fig. 5 *D*, the deviation from the expected hyperbolic relationship is greater than that predicted by the model (Fig. 5 *C*). This could be due to noise in the fura-2 signal from which the experimental curve was derived or might be an indication that there are other factors involved in the removal of $[Ca^{2+}]$ in smooth muscle cells when intracellular concentration is high.

Spatial distribution of Ca^{2+}: two dimensional model

The one-dimensional diffusion model considered thus far assumes that movement of Ca^{2+} in the cylinder representing the smooth muscle cell is radial everywhere. This model allowed us to examine some of the temporal characteristics of Ca transients and to predict that there may be spatial inhomogeneities in $[Ca^{2+}]$ across the cell at early times following influx and SR release. There are limitations imposed by a one-dimensional model, however, that make it difficult to explore other factors that might influence the spatial distribution of $[Ca^{2+}]$ in cells. It has been suggested from the examination of electron micrographs, for example, that the SR in smooth muscle cells is found throughout the cytoplasm rather than confined to an area near the surface membrane as shown in the model in Fig. 1 (see Devine et al., 1972). Consistent with this, is the work of Etter et al. (1990) that indicates that the SR Ca-ATPase in the smooth muscle cells used in our model is distributed at sites throughout the cytoplasm. If the release sites are distributed in this way as well, this could markedly influence the way in which Ca^{2+} moves through the cell following stimulation. To further explore possible spatial and temporal characteristics of Ca diffusion in cells, we developed a two-dimensional diffusion model.

The gradient of Ca^{2+} within the model cell described in Fig. 6 is shown again as a two-dimensional Ca-image in Fig. 6 (*Model 1*). The images represent a 3 μm long cell section. The SR is located 0.2 μm from the plasma membrane as shown in Model 1 (upper figure). The

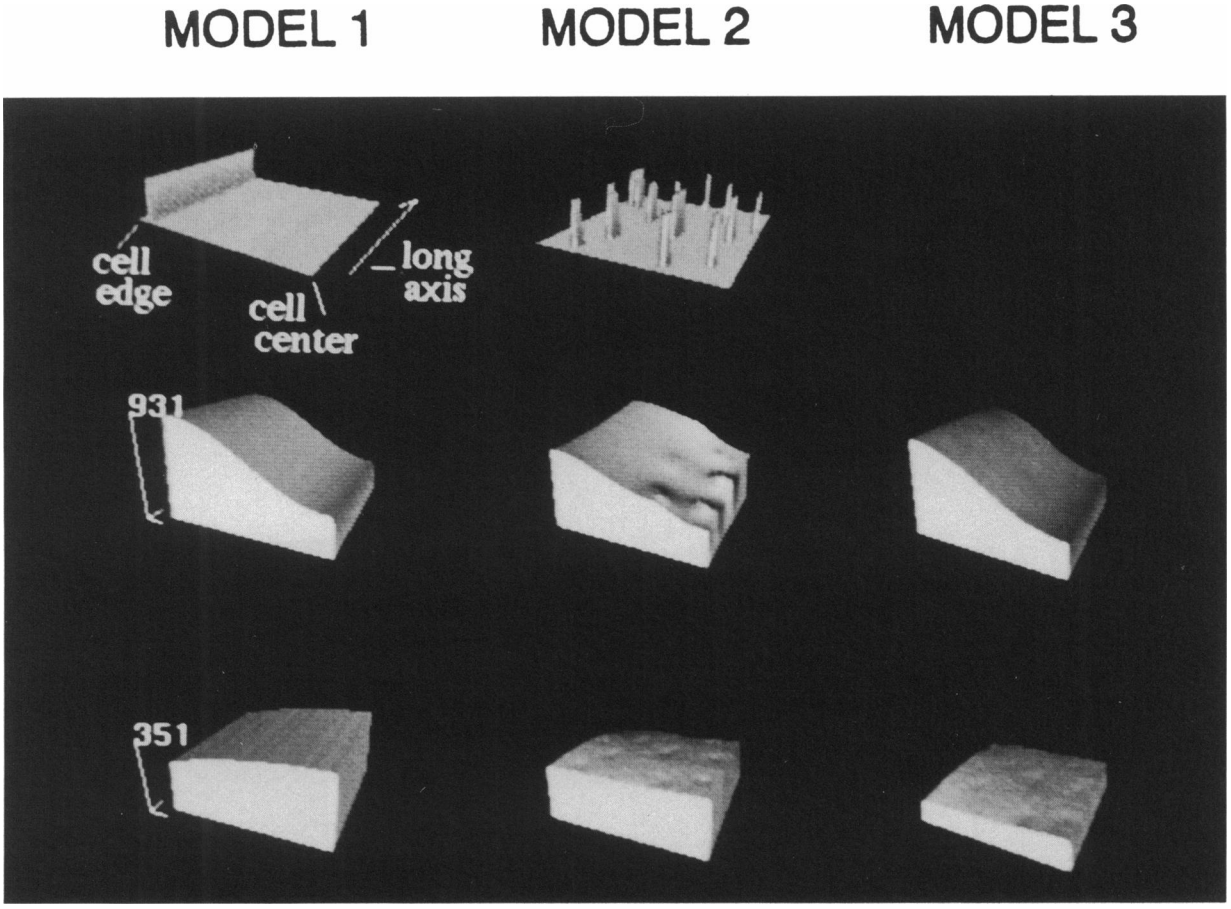

FIGURE 6 Effect of SR distribution on $[Ca^{2+}]$ gradients. Upper row of figures: SR distribution is shown for two of the three models used. In the first case (*Model 1*), the SR (*raised area*) was located only along the membrane. In the second case (*Model 2*) the SR was distributed throughout the cell. The model for the third set of simulations (*Model 3*) was a combination of the other two (see text). (*Middle row* of figures) Ca distribution within the cell for the three model cells 260 ms after influx began. The bar on the left figure represents 931 nM $[Ca^{2+}]$. Note the local areas of high $[Ca^{2+}]$ in the middle figure (*Model 2*) that result from the release from SR elements near the cell center. (*Bottom row* of figures) Ca distribution within the model cells 4 s after influx began. The bar on the left figure represents 351 nM $[Ca^{2+}]$. The slight depressions visible in the middle and right figures (*Model 2* and *Model 3*) show local areas of low $[Ca^{2+}]$ around the SR pump sites.

middle column of images (*Model 2*) show a model cell similar to the one on the left but with the SR located at sites throughout the cytoplasm. The lower two figures for Model 1 and Model 2 show the Ca distribution in the cells at two different times following the initiation of Ca influx through the surface membrane. It can be seen from comparison of the two models, that the presence of SR elements throughout the cell led initially to a more uniform overall $[Ca^{2+}]$ in the cell and to a more rapid decline of the transient. Another possibility is that the Ca release sites of the SR may be separated from the pump sites (Etter et al., 1990). We have also modeled the cell so that the release sites were near the surface membrane (as in *Model 1*) to facilitate the transduction of signals between the extracellular environment and the SR. The pump sites were located throughout the cell (as in *Model 2*). Simulations with this model (*Model 3,* Fig. 6), showed little difference in the rise of cell Ca^{2+} when compared with Model 1 but a much more rapid decline in Ca^{2+} than was seen with either of the other two models. The possibilities predicted by these models should be testable once high time and spatial resolution images of Ca^{2+} in smooth muscle cells become available experimentally. Release of Ca^{2+} from more centrally located SR elements as shown in Model 2 of Fig. 6 might also be detected in such images.

Although membrane depolarization and the entry of extracellular Ca^{2+} can trigger the release of Ca^{2+} from intracellular storage sites in smooth muscle, there is also ample evidence that some stimuli may cause SR release without an accompanying membrane depolarization or significant entry of extracellular Ca^{2+} (Devine et al.,

1972; Kargacin and Detwiler, 1985). The latter might occur where nerve terminals innervate discrete membrane sites and cause a highly localized intracellular Ca release. We have examined one possible mechanism by which an extracellular signal that leads to release of Ca^{2+} from a localized region of the SR might trigger SR release (via Ca-induced Ca-release) from other portions of the smooth muscle cell. The cell was modeled with two SR elements physically separated from one another by a gap as shown in Fig. 7*A*. With a 0.2-μm gap between the SR elements and 230 μM total intracellular [buffer], Ca^{2+} released from the lower SR element did not induce release from the upper element during a 1 s simulation. In reality, not all intracellular Ca buffers are immobile as has been assumed in the model. Recent modeling work by Sala and Hernandez-Cruz (1990) has explored the possible effects of movable buffers on the diffusion of Ca^{2+} through cells and suggests that such buffers might actually facilitate diffusion. This possibility is of course dependent upon the kinetics of Ca binding to the buffer as well as the mobility of the buffer-Ca complex. As an approximation to the effects of a mobile buffer in our model, we lowered the fixed buffer concentration so that Ca^{2+} could move more rapidly through the cell. With a 0.2-μm gap between the SR elements and 115 μM total intracellular [buffer], Ca^{2+} was re-

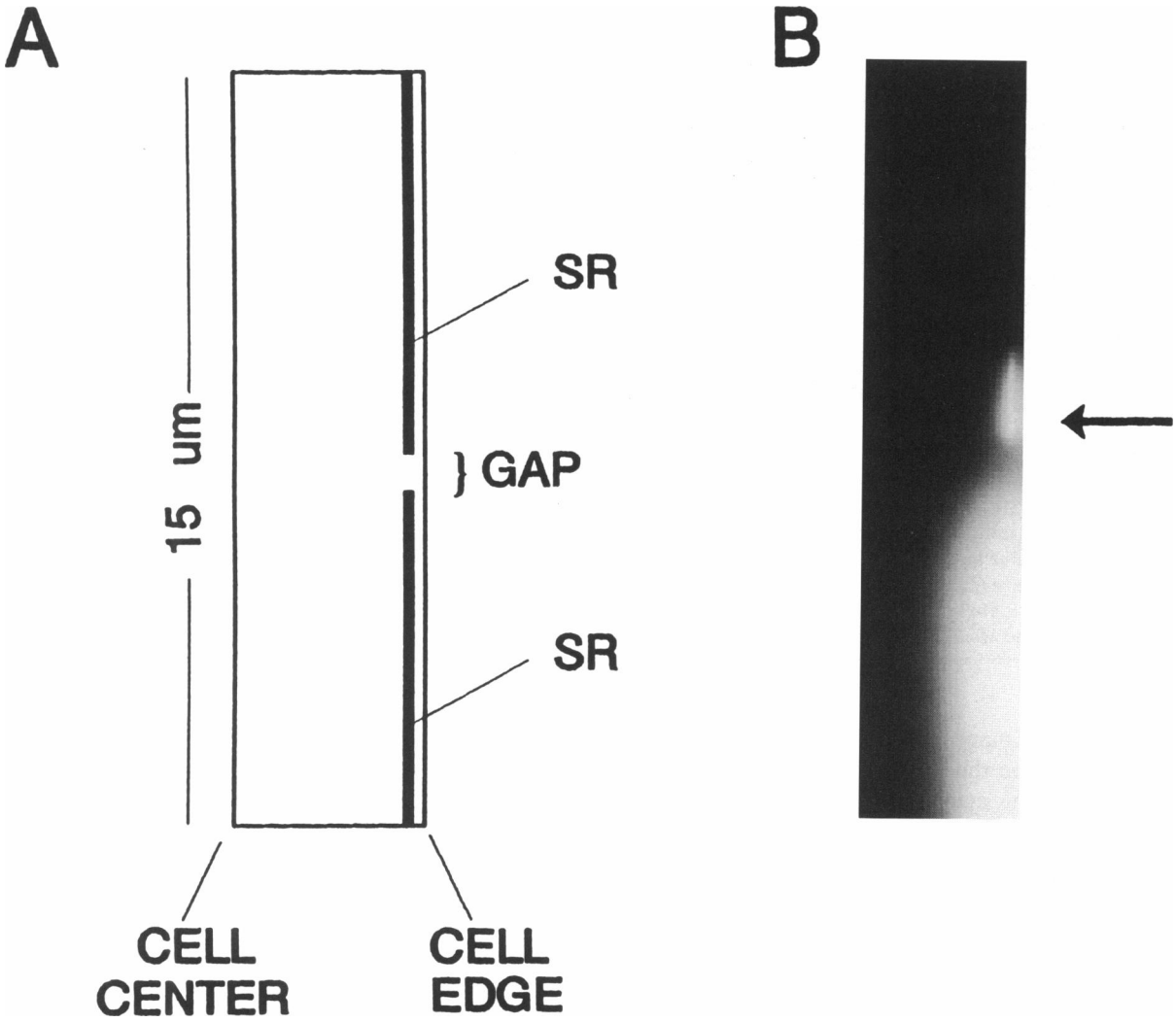

FIGURE 7 Spread of an intracellular Ca signal from one SR element to another. Cell center is on the left in each panel; cell surface on the right; cell radius = 3 μm; length of segment modeled = 15 μm. (*A*) model cell showing two SR elements (*dark bars*) separated by a 1-μm gap. (*B*) results of a simulation with $[buffer]_{TOTAL}$ = 115 μM. The beginning of Ca release from the upper most SR element induced by Ca^{2+} from the lower SR element can be seen (*arrow*). With time, the area of Ca release spread upward along the second SR element toward the top of the panel. In this simulation there was a 1-μm gap between SR elements and submaximal Ca influx along the entire cell (see text).

leased from the upper most SR element <70 ms after release was initiated in the lower most element. When the gap was increased to 0.4 μm, it took over 100 ms for the second element to be triggered. With a gap of 1 μm, release from the second SR element was not seen during a 1 s simulation. Although the presence of a moveable buffer (Sala and Hernandez-Cruz, 1990) might reduce the diffusion time for Ca^{2+} within the cell, the simulations suggest, in general, that, because of the relatively low diffusion coefficient for Ca^{2+} within smooth muscle cells, for there to be a margin of safety for the transmission of the Ca signal, it would be advantageous for cells to have a more densely spaced SR. Cells might also utilize a process other than the diffusion of Ca^{2+} itself from one SR element to another to spread a localized SR signal to other parts of the cytoplasm. We have illustrated one way this could happen in Fig. 7 *B*. In this simulation, with the gap between the SR elements still equal to 1 μm, a submaximal influx of extracellular Ca^{2+} was allowed to occur through the cell membrane along the length of the cell. In this case, Ca release from the second SR element was greatly facilitated by the small extracellular influx and release occurred after 10 ms. An extracellular influx such as this could be triggered by the binding of neurotransmitters to surface receptors. It is also possible that a less well buffered second messenger could be used by the cell to spread the signal for SR release throughout the cytoplasm.

SUMMARY

Comparison of the results of the simulations presented here and the experimental results of others that were discussed in the text, indicates that the Ca regulatory processes that were included in the model could be integrated together to give a good qualitative and quantitative description of the time course and magnitude of the Ca transient in smooth muscle cells. From our results, it seems unlikely that any of the Ca extrusion or sequestration processes that were modeled have a significant influence on the rising phase of the Ca transient in smooth muscle cells. Our results suggest that the plasma membrane Ca-ATPase and Na/Ca exchange are not likely to play a major role in restoring $[Ca^{2+}]$ to rest following a brief depolarization but rather are primarily concerned with the maintenance of resting Ca levels in smooth muscle cells. Other pumping processes, most likely those associated with the SR, appear to be necessary for the removal of Ca^{2+} from the cytoplasm following contractile stimulation.

Our results indicate that the way in which Ca release and/or pump sites are distributed within the intracellular space can significantly influence the overall Ca distribution in cells and the rate of reuptake of Ca^{2+} from the cytoplasm following stimulation. This suggests that even in cells as small as smooth muscle cells it may be important for the release and reuptake sites and the sites of action of Ca^{2+} to be maintained in spatial register. It is also possible that differences in the time course and spatial distribution of cytoplasmic $[Ca^{2+}]$ that result from different stimuli could be important determinants of the overall response of the cell to the stimuli.

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