Relationship between force and Ca^{2+} concentration in smooth muscle as revealed by measurements on single cells

(excitation–contraction coupling/contraction/fura-2/fluorescence/calcium)

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ABSTRACT The role of Ca^{2+} in regulating smooth muscle contraction was investigated by measuring isometric force and $[\text{Ca}^{2+}]$ simultaneously in individual single smooth-muscle cells. $[\text{Ca}^{2+}]$ was measured with fura-2 and a high time-resolution dual-wavelength digital microfluorimeter, and force was measured with an ultrasensitive force transducer attached to a probe around which was tied one end of the cell. Both $[\text{Ca}^{2+}]$ and force increase after maximal electrical stimulus, with $[\text{Ca}^{2+}]$ increasing considerably before the first detectable increase in force. Force development exhibited maximal sensitivity to $[\text{Ca}^{2+}]$ between 150 and 500 nM Ca^{2+} . This Ca^{2+} sensitivity can account for the fact that many physiological stimuli produce full contraction even though such stimuli only increase Ca^{2+} to 600–800 nM. When Ca^{2+} was induced to increase rapidly, the relation between $[\text{Ca}^{2+}]$ and force exhibited hysteresis. During the onset of contraction, force at a given $[\text{Ca}^{2+}]$ was lower than during the muscle's return to rest, thus suggesting the existence of a slow step(s) linking Ca^{2+} and force development in smooth muscle. The direction of this hysteresis reversed during contractions in which Ca^{2+} increased slowly, suggesting that the contractile process becomes desensitized to $[\text{Ca}^{2+}]$ with time. These relations between calcium and force in intact single smooth-muscle cells differ in many respects from the relation found previously in chemically permeabilized multicellular preparations of smooth muscle.

Calcium is considered the key regulator of contraction of smooth muscle (1, 2), although details of this regulation remain somewhat unclear. The relationship between force and $[\text{Ca}^{2+}]$ has been studied in skinned smooth-muscle strips (3–11) and in smooth muscles that were loaded with aequorin by temporarily permeabilizing the tissue (12). The sensitivity of force to $[\text{Ca}^{2+}]$ found in chemically permeabilized (skinned) preparations by various investigators varies by almost two orders of magnitude, and several studies showed that the sensitivity can be altered by calmodulin addition (5, 6, 13) or by varying either inorganic phosphate (11) or Mg^{2+} concentration (10). Thus, the loss of important regulatory proteins or ions during permeabilization of the preparation may obfuscate the relationship seen between $[\text{Ca}^{2+}]$ and force. In addition, multicellular preparations introduce even more uncertainties because the complex structural connections, diffusion gradients, and inherent cell differences make difficult the extrapolation of results from tissue to single cell.

To determine the relationship between $[\text{Ca}^{2+}]$ and force in smooth muscle in a manner that avoids these uncertainties, we devised methods to measure both intracellular $[\text{Ca}^{2+}]$ and isometric force simultaneously in single intact smooth-muscle cells. Results reveal that $<1 \mu\text{M}$ calcium is needed for full contractile activation and $[\text{Ca}^{2+}]$ of ≈ 250 nM produces half-maximal force. The threshold for force development was

≈ 150 nM, just slightly greater than the $[\text{Ca}^{2+}]$ of the resting cell. Results also show that a slow step in the activation pathway linking $[\text{Ca}^{2+}]$ and force limits the rate of force development. This slow step, not the rate of $[\text{Ca}^{2+}]$ change *per se*, accounts for the several hundred-msec delay between stimulation and onset of active-force production characteristic of smooth muscle. Finally, our results show that the contractile process exhibits decreasing sensitivity to calcium during slow contraction–relaxation cycles.

METHODS

Smooth muscle cells were isolated from the stomach of the toad *Bufo marinus* as described (14). Briefly, the procedure involves incubating thin slices of stomach wall first in a dilute solution of collagenase and trypsin and then in a solution of collagenase alone. Cells released from the tissue in this latter solution are collected by mechanically separating the tissue from the cell suspension. Soybean trypsin inhibitor was added to all cell suspensions to suppress any remaining trypsin activity.

Cells were loaded with the calcium-sensitive dye fura-2 (15) by incubating them in a solution of the acetoxymethyl ester form of the dye, fura-2/AM (Molecular Probes, Eugene, OR). Cells were incubated in this solution at 31°C for 60 min and then returned to room temperature. The amount of fura-2/AM added to the cell suspension was adjusted according to cell density at the ratio of 1.25×10^{-9} mol of fura-2/AM per 10^6 cells. These loading conditions were found to produce intracellular fura-2 concentrations of 30–100 μM as estimated by comparing total fluorescence to that from droplets of fura-2 solutions in oil.

A dilute suspension of cells that had been loaded with fura-2 was placed on a cover-slip chamber on the stage of an inverted microscope, and the cells were allowed to settle. A single cell was then attached at both ends to two special glass microprobes, each of which had a single anionic-exchange-resin particle cemented to its tip. After attachment of cell to probe, a small section of the muscle cell was wrapped at each end by micromanipulation around the attached probe. The procedure was basically that described by Warshaw and Fay (16). One probe was attached to a micromanipulator, allowing fine adjustment of cell length, and the other probe was attached to a Cambridge Technology model 430 ultrasensitive force transducer (Cambridge, MA). This device has a compliance of $<1.0 \mu\text{m}$ per 0.1 mN and a frequency response of >200 Hz, allowing changes in force as small as 10 nN to be resolved. The cell was stimulated to contract by passing a 0.1-msec-duration, 25- to 100-V pulse between a pair of platinum wires (diameter, 100 μm), insulated except at their ends, which were positioned to within 100 μm on each side of the cellular long axis. Throughout these procedures

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the cover-slip chamber was filled with bicarbonate-buffered amphibian physiological saline (14) that contained 1.8 mM Ca^{2+} . A humidified stream of 95% N_2 /5% CO_2 was directed over the open top of the chamber to maintain pH and to lower bath O_2 content, thereby minimizing photobleaching of fura-2 (17). Neither decrease of P_{O_2} in the medium from this procedure nor fura-2 at an estimated intracellular concentration of 30–100 μM altered the contractile response of the smooth-muscle cells, as the delay between stimulation and onset of force development and the maximal rate of rise of force after maximal electrical stimulation did not differ significantly between cells exposed to $\approx 20\%$ O_2 and cells exposed to 95% N_2 /5% CO_2 plus fura-2. [Average delay to onset of force development and maximum dF/dt in unloaded, normally oxygenated cells were 201 ± 42 msec ($n = 11$; from ref. 18) and 0.50 ± 0.15 $\mu\text{N}/\text{sec}$ ($n = 6$; S.Y. and F.S.F., unpublished observations), respectively, and in our hypoxic, fura-2-loaded cells the respective values were 265 ± 98 msec ($n = 10$) and 0.62 ± 0.09 $\mu\text{N}/\text{sec}$ ($n = 9$).]

Our dual-wavelength microspectrofluorimeter (DMF) system for exciting and detecting fura-2 fluorescence is diagrammed in Fig. 1. The setup consists of a modified Zeiss IM-35 inverted microscope with a specially designed excitation light path and a photomultiplier tube (PMT) operating as a photon-counting device. Most control functions and data acquisition were accomplished via a DEC model PDP 11/73 microcomputer. Excitation wavelength was set by a filter wheel of our design (manufactured by Omega Optical, Brattleboro, VT), which was mounted to the shaft of a rapidly spinning motor and in these experiments was typically operated at 170 revolutions per sec, allowing determination of $[\text{Ca}^{2+}]$ at 6-msec intervals. The filter wheel has two transmissive sectors, one that passes primarily 340-nm light and the other that passes primarily 380-nm light (each with a band pass of ≈ 10 nm at 1/2 peak transmission). The excitation light is then diverted through the objective (Nikon UVF 40 $\times$) to the cell by a dichroic mirror. The fluorescent image passes through a mask placed at an intermediate-image plane that blocks the image except for the central cell region; this greatly reduces background light intensity. The emission light then passes through an emission filter (centered at 500 nm; band pass, 60 nm) and is directed to the photocathode of the PMT (Thorn EMI type 9813B). The PMT output was monitored by photon-counting circuitry (E.G. & G. Ortec, Oak

Ridge, TN), which was interfaced to the DEC PDP 11/73 computer.

Fig. 2A shows a bright-field image of a fura-2-loaded cell mounted on the microprobes, and Fig. 2B shows a 500-nm fluorescence image of the same cell when illuminated with 380-nm light. When data was being collected, the intermediate-image plane mask would pass only light from the central part of the cell to the photomultiplier tube. In addition, when cell data was being collected, the field-stop diaphragm in the excitation-light path was used to narrow excitation-beam width so that the highly fluorescent anion-exchange beads were not illuminated. Measurements of cellular fluorescence were corrected for background fluorescence determined for both 340- and 380-nm excitation at the end of each run by collecting fluorescence data with the cell out of the viewing field. The 340- and 380-nm fluorescence values were not corrected for cell autofluorescence, which was typically $<2\%$ of the signal seen in fura-2-loaded cells. $[\text{Ca}^{2+}]$ was calculated by the ratio method (15) by the following equation:

$$[\text{Ca}^{2+}] = K_d \times B \times [(R - R_{\min}) / (R_{\max} - R)],$$

where K_d is the dissociation constant of fura-2 for Ca^{2+} [determined *in vivo* to be 200 nM by Williams *et al.* (19)], R is the fluorescence ratio at 340/380, $R_{\min}$ and $R_{\max}$ are the values of R for fura-2-free acid in a glass chamber placed on the microscope without Ca^{2+} and with saturating $[\text{Ca}^{2+}]$, respectively, and B is the ratio of the 380-nm-fluorescence signals of fura-2 acid without Ca^{2+} to that with saturating $[\text{Ca}^{2+}]$.

RESULTS

Fig. 3 illustrates the typical change in force and calcium of a single smooth-muscle cell after submaximal electrical stimulation. $[\text{Ca}^{2+}]$ increases from its resting value of about 140 nM to a peak of 210 nM, and force increases to 1.05 μN . $[\text{Ca}^{2+}]$ increases slowly in this cell, and the increase in force appears to follow it closely. However, after maximal stimulation, the calcium concentration increases much more rapidly. Under these conditions, the increase in calcium clearly precedes the elevation in force (Fig. 4) with a delay time that averaged 265 (± 95 SD) msec in eight cells. This delay time far exceeds the several-msec delay between elevation of

FIG. 1. Schematic of our hardware setup for measuring force and $[\text{Ca}^{2+}]$ in a single smooth-muscle cell. (A) 75-W Xenon-arc lamp. (B) collimating lens. (C) four-sector filter wheel (two small blank sectors and two transmissive sectors). (D) field stop diaphragm. (F) dichroic mirror. (G) Nikon UVF 40 $\times$ objective. (I) intermediate-image plane mask. (J) emission filter. (K) force transducer. (L) three-axis micromanipulator. PMT, photomultiplier tube. (Inset) Two transmissive sectors of the filter wheel pass the indicated wavelengths of light.

FIG. 2. Photomicrographs of a single smooth-muscle cell loaded with fura-2 and mounted for force measurement. (A) Bright-field illumination; (B) 500-nm fluorescence image of same cell (380 nm, excitation λ). Both images are the average of 128 video frames from a silicon intensified target camera. (550 $\times$.)

calcium and rise in force seen in striated muscle (20) and resembles the delay between imposition of an electrical stimulus and onset of shortening seen in untethered single smooth-muscle cells loaded with fura-2 (21) and the onset of force in non-fura-2-loaded cells (18).

Fig. 5 illustrates the dependence of the rate of increase in force (dF/dt) on the rate of increase in calcium ($d[Ca^{2+}]/dt$) during the first 2 sec after stimulation. dF/dt increases with increasing $d[Ca^{2+}]/dt$ to a $d[Ca^{2+}]/dt$ of ≈ 1000 nM/sec, beyond which dF/dt seems to plateau. This relationship suggests that the rate of calcium increase can limit the rate of force production when the former rate is below, but not above, 1000 nM/sec. The plateau level of dF/dt of ≈ 0.6 μ N/sec could reflect a limitation on the rate of force development inherent to the contractile apparatus *per se*, or the plateau could represent a more complicated function that also reflects the maximum rate for reactions linking Ca^{2+} to contractile-system activation. To decide between these possibilities, we compared dF/dt after maximal activation in non-fura-2-loaded cells with dF/dt after a step shortening (sufficient to reduce force to zero) that was imposed when the contractile machinery was fully activated and force had peaked. dF/dt after step shortening was found to be ≈ 1.5 times initial dF/dt . This finding shows that the plateau value of the initial dF/dt experiments is not the maximal rate at which a completely activated contractile apparatus can develop force. Thus, another process must limit the initial development of force when $d[Ca^{2+}]/dt$ is high.

FIG. 3. Record of isometric force and $[Ca^{2+}]$ from a single smooth-muscle cell loaded with fura-2 after a brief electrical stimulus. Arrow, time of 0.1-msec electrical stimulus to cell.

FIG. 4. Calcium and force records from a single fura-2-loaded smooth-muscle cell showing delay between increase in cytosolic $[Ca^{2+}]$ and onset of force development. Arrow, time of 0.1-msec electrical stimulus to cell. The first apparent drop in force is an electrical artifact caused by our event marker and represents neither mechanical perturbation nor force change. Delay was calculated as the time interval from initiation of stimulus to the point at which force increased above its baseline value.

Insights into the calcium sensitivity of the activation process in smooth muscle cannot be obtained from the $[Ca^{2+}]$ -force relationship during onset of force development when $d[Ca^{2+}]/dt$ is high because force may not reflect the extent of activation due to the relative slowness of the processes linking $[Ca^{2+}]$ with force. Although elevating $[Ca^{2+}]$ precisely to constant steady-state values and determining the $[Ca^{2+}]$ dependence of force would have been desirable, this maneuver was not experimentally feasible. However, it was possible to submaximally stimulate some cells such that force increased slowly and then peaked or stabilized for several seconds. At instants where $dF/dt = 0$ (and where $d[Ca^{2+}]/dt$ was less than ≈ 200 nM/sec), the reactions linking calcium and contractile activation would be predicted to be approximately at equilibrium. Measurements of $[Ca^{2+}]$ and force at such times could indicate the calcium sensitivity of the activation process in smooth-muscle cells. Fig. 6 shows data from 15 contractions of 11 cells in which we obtained such measurements. Threshold for the elevation of force appeared to be $[Ca^{2+}]$ of ≈ 125 –150 nM, and maximal force was obtained at ≈ 500 –600 nM. Generally similar results are obtained from the continuous relationship between $[Ca^{2+}]$ and force during onset of force production after submaximal stimuli, when $d[Ca^{2+}]/dt$ is so low that activation is assumed to keep pace with $[Ca^{2+}]$.

FIG. 5. Relationship between rate of increase of calcium concentration ($d[Ca^{2+}]/dt$) and the rate of increase in force (dF/dt). Error bars represent SD for $d[Ca^{2+}]/dt$ dimension and SEM for dF/dt dimension. Rate of calcium increase was the absolute increase in $[Ca^{2+}]$ within the first 0.5 sec after stimulation; dF/dt was the largest value measured in the interval between 1–2 sec after stimulation.

FIG. 6. The relationship between force and calcium. Cell records were chosen where force and $[Ca^{2+}]$ both peaked or remained stable for several seconds at the same time. ■, Individual measurements (12 observations in 8 cells); X, average resting Ca^{2+} in these cells before stimulation ($103 \text{ nM} \pm 11 \text{ SEM}$; $n = 11$); △, average force ($1.69 \mu\text{N} \pm 0.48 \text{ SEM}$) of three cells in which Ca^{2+} exceeded 2000 nM.

The data points shown in Fig. 6 were gathered within ≈ 15 sec after the stimulus and thus represent the $[Ca^{2+}]$ -force relationship only within this period. However, evidence exists that this sensitivity of force to $[Ca^{2+}]$ declines later in a contraction. Fig. 7 shows a plot of force versus $[Ca^{2+}]$ throughout the time course of two different calcium transients, one where the increase in $[Ca^{2+}]$ was rapid (peak of 530 nM in 4.6 sec) and one where the increase was slow (peak of 260 nM in 26 sec). The cell in which calcium increased rapidly shows a type of hysteresis that would be expected when a relatively slow step (e.g., myosin phosphorylation) exists between the $[Ca^{2+}]$ increase and force production, indicating that force at first cannot keep pace with the rapid increase in $[Ca^{2+}]$. However, as the rate of calcium increase slows, the force- $[Ca^{2+}]$ function would be predicted to collapse to the quasi-steady-state relationship of Fig. 6. In fact, in all five cells in which $d[Ca^{2+}]/dt$ was slow ($< 200 \text{ nM/sec}$) and where $[Ca^{2+}]$ eventually returned to near resting levels, hysteresis was seen (Fig. 7, Slow trace), which was opposite to that seen in cells where the calcium increased more rapidly. That is, the contractile process appears to be more sensitive to $[Ca^{2+}]$ during onset of force development than later in the contraction. In one cell in which we elicited three reversible contractions within a short period, this

FIG. 7. The two types of force-calcium hysteresis seen in single smooth-muscle cells. —■— (labeled Slow), Force- $[Ca^{2+}]$ relation over the period of a contraction in which increase in $[Ca^{2+}]$ occurred slowly (26 sec to peak); - -□- - (labeled Fast), similar plot from another cell in which increase in $[Ca^{2+}]$ was much faster (4.6 sec to peak).

reverse hysteresis was most pronounced during the first contraction, diminished in the second contraction, and completely missing in the third contraction. Calcium sensitivity during the initial increase in force declined from the first to third contraction. This observation could be explained by a "desensitization"-causing process that has a relatively slow onset and persists over time even into full relaxation. This hysteresis is probably not an artifact from the kinetics of Ca^{2+} binding to fura-2 because the dye equilibrates to a step change in $[Ca^{2+}]$ with a half-time of < 10 msec (22). Thus, fura-2 should be essentially at equilibrium during these slow contractions.

DISCUSSION

Results from these simultaneous measurements of isometric force and $[Ca^{2+}]$ in a single smooth-muscle cell provide more information about the role of Ca^{2+} in controlling smooth-muscle contraction. Our results agree with other studies on smooth muscle revealing a delay between electrical stimulation and the onset of contractile activation (18, 21, 23)—a delay much longer than that seen in striated muscles (19). In addition, our results revealed that $[Ca^{2+}]$ can increase to $> 250 \text{ nM}$ during this latent period for force development. Therefore, the delay in force development cannot be explained as being due to delay in the onset of Ca^{2+} release into the cytoplasm. Furthermore, the delay cannot be explained by the existence of a $[Ca^{2+}]$ threshold for activation, as the steady-state relation of $[Ca^{2+}]$ and force (Fig. 6) reveals that the contractile process should be almost 50% activated by a change in $[Ca^{2+}]$ to 250 nM. The delay seen between stimulus and onset of force development is unlikely to be caused by any lag introduced by a step in the smooth-muscle cross-bridge cycle between attachment and cycling of cross-bridges: stiffness, which reflects cross-bridge attachment, exhibits a similarly long delay before increasing along with force after stimulation of single smooth-muscle cells (18, 23). Furthermore, recent experiments using caged ATP in skinned strips of smooth muscle revealed virtually no delay between uncaging of ATP and the onset of force development in strips in which myosin light chains were thiophosphorylated before the ATP "jump" (24)—a finding showing the lack of a long latency in the cross-bridge cycle *per se*. The uniquely long delay between stimulation and onset of force development must, therefore, reside in the step(s) linking $[Ca^{2+}]$ to activation. Quite possibly the delay resides in a step(s) linking Ca^{2+} and light chain phosphorylation because a delay of several-hundred msec is seen between stimulation and the first significant increase in myosin-light chain phosphorylation in another smooth muscle preparation which also exhibits a several-hundred-msec delay in the onset of active-force development (25).

After the delay, force increases at a rate that depends on the rate of $[Ca^{2+}]$ elevation to a rate of $\approx 1000 \text{ nM/sec}$, beyond which this rate plateaued. This apparent upper limit on force-development rate does not appear to reflect limits imposed by the contractile machinery *per se*, because dF/dt after a rapid 10% decrease in cell length in fully activated cells was considerably greater than the upper limit on dF/dt during activation. Thus, the dF/dt after maximal activation must be significantly limited by the speed of events linking $[Ca^{2+}]$ with force development and not limited by the speed at which a fully activated-contraction system can develop force or by the rate of $[Ca^{2+}]$ elevation.

The quasi-steady-state dependence of force on $[Ca^{2+}]$ that we saw at the single cell level resembles in some respects that recently reported by De Feo and Morgan (12) in multicellular preparations. Results on both single cells and tissue reveal that half-maximal force is produced at $\approx 250 \text{ nM}$ Ca^{2+} with depolarizing stimuli. The results with aequorin on the mul-

ticellular preparations suggest, however, that the relationship between Ca^{2+} and force is considerably steeper than seen for the single cell. It is unclear whether this difference reflects differences in methods (single cell versus multicellular tissue; fura-2 versus aequorin) or simply reflects differences between smooth muscles (ferret aorta and portal vein at 35°C versus toad stomach muscle at 25°C).

The $[\text{Ca}^{2+}]$ sensitivity of force reported here for single intact cells is much greater than the Ca^{2+} sensitivity reported for chemically permeabilized (skinned) preparations of the same cell type (26, 27). The difference is presumably due to the loss or rearrangement of contractile proteins during skinning. Our results on intact cells resolve an apparent paradox that developed when Williams *et al.* (19) reported that maximal stimulation of single smooth-muscle cells by K^+ depolarization or the muscarinic agonist carbamylcholine causes $[\text{Ca}^{2+}]$ to increase to no more than 600–800 nM, a $[\text{Ca}^{2+}]$ change, which compared with data from skinned cells, suggested that $[\text{Ca}^{2+}]$ could not account for the ability of these stimuli to induce maximal contraction. By contrast, the $[\text{Ca}^{2+}]$ -force relation in these intact cells can account for the ability of these stimuli to fully activate contractile activity.

Finally, our results reveal that the relationship between force and calcium is not fixed, but rather is probably modulated by other mechanisms. We have demonstrated here a shift in the coupling of the Ca^{2+} signal and force production that occurs in a time-dependent manner after activation. This decline in the calcium sensitivity is opposite to what would be expected from a "latch" bridge effect proposed by Dillon *et al.* (28) and which has been seen in skinned arterial strips (7) and in intact arterial strips (12). Our results give no direct information about the type of mechanism(s) that might cause this desensitization. The desensitization could result from an alteration of the sensitivity of the myosin-light chain phosphorylation process to calcium or, alternatively, could result from some other process that affects cross-bridge dynamics. Our observations would suggest that the desensitization process is slow, taking many seconds to develop, and can persist for some time after cessation of contraction and the return of the $[\text{Ca}^{2+}]$ to resting level. The function of this desensitization process is unclear but might relate to the type of mechanical activity normally engaged in by these gastric cells. In contrast to arterial cells, gastric cells normally contract rhythmically, and the ability of the tissue composed of these cells to function properly depends on relaxation after contraction; this desensitization could assist that relaxation process.

Our evidence thus suggests desensitization of the contractile process to Ca^{2+} after stimulation in smooth muscle; ample precedent exists for desensitization developing in other cellular systems where negative feedback uncouples the response from the activating stimulus (29, 30). Further work is required to determine the events underlying the desensitization process observed in this smooth muscle.

The current work has directly viewed the relationship between $[\text{Ca}^{2+}]$ and force in single smooth-muscle cells. Such findings have, in turn, enabled us to assess what step(s) in the activation process most probably produces the long delay between stimulation and onset of active-force development. The force- $[\text{Ca}^{2+}]$ relationship outlined here reconciles this role of $[\text{Ca}^{2+}]$ in excitation-contraction coupling in smooth muscle with the rather limited range over which $[\text{Ca}^{2+}]$ changes with physiological stimuli. Finally, the pres-

ent studies reveal an as-yet-unknown process that alters contractile sensitivity. Further studies that use these general approaches may give insight into these and other processes underlying excitation-contraction coupling in smooth muscle.

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