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Mechanotransduction in Colonic Smooth Muscle Cells

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Abstract. We evaluated mechanisms which mediate alterations in intracellular biochemical events in response to transient mechanical stimulation of colonic smooth muscle cells. Cultured myocytes from the circular muscle layer of the rabbit distal colon responded to brief focal mechanical deformation of the plasma membrane with a transient increase in intracellular calcium concentration ($[Ca^{2+}]_i$) with peak of 422.7 ± 43.8 nM above an average resting $[Ca^{2+}]_i$ of 104.8 ± 10.9 nM ($n = 57$) followed by both rapid and prolonged recovery phases. The peak $[Ca^{2+}]_i$ increase was reduced by 50% in the absence of extracellular Ca^{2+}, while the prolonged $[Ca^{2+}]_i$ recovery was either abolished or reduced to ≤15% of control values. In contrast, no significant effect of gadolinium chloride (100 μM) or lanthanum chloride (25 μM) on either peak transient or prolonged $[Ca^{2+}]_i$ recovery was observed. Pretreatment of cells with thapsigargin (1 μM) resulted in a 25% reduction of the mechanically induced peak $[Ca^{2+}]_i$ response, while the phospholipase C inhibitor U-73122 had no effect on the $[Ca^{2+}]_i$ transient peak. $[Ca^{2+}]_i$ transients were abolished when cells previously treated with thapsigargin were mechanically stimulated in Ca^{2+}-free solution, or when Ca^{2+} stores were depleted by thapsigargin in Ca^{2+}-free solution. Pretreatment with the microfilament disrupting drug cytochalasin D (10 μM) or microinjection of myocytes with an intracellular saline resulted in complete inhibition of the transient. The effect of cytochalasin D was reversible and did not prevent the $[Ca^{2+}]_i$ increases in response to thapsigargin. These results suggest a communication, which may be mediated by direct mechanical link via actin filaments, between the plasma membrane and an internal Ca^{2+} store.

Key words: Mechanotransduction — Inositol 1,4,5-trisphosphate — IP_3 — Cytoskeleton — Intracellular calcium — Cytochalasin D

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Introduction

Distention of the intestine resulting in stretch of the bowel wall is a natural mechanical stimulus of smooth muscle. While the neurally mediated effects of intestinal distention on contractile activity have been well characterized in the form of the peristaltic reflex, the direct effects of distention on gastrointestinal smooth muscle activation, in the form of muscle mechanotransduction have received little attention. However, it has been proposed that conformational changes in plasma membrane-associated molecules as well as in extracellular matrix and cytoskeleton may function as sensors and transducers for external mechanical forces applied transiently or chronically to cells (Watson, 1991; Vandenburgh, 1992). Proposed mechanotransducers involved in the modulation of intracellular biochemical events in response to application of mechanical forces include phospholipase C (PLC), adenylate cyclase, stretch-sensitive ion channels and the Na^+/H^+ exchanger (Watson, 1991). For example, G protein-independent, mechanically induced activation of adenylate cyclase resulting in increased cyclic AMP levels has been described in mouse lymphoma cells (Watson, 1991). Calcium release from intracellular stores has also been shown to result from a single stretch of cultured lung epithelial cells (Wirtz et al., 1990). Mechanosensitive activation of intracellular signaling mechanisms has been inhibited by interruption of the actin filaments, and less frequently, by disruption of the actin and microtubular cytoskeleton (Watson, 1990).

The effect of prolonged mechanical stimulation on intracellular signaling mechanisms in smooth muscle from the urinary bladder and vasculature (Buck, 1983), and on cardiac muscle has been reported (Komuro et al.,

1991). Multiple intracellular signaling pathways are activated in response to prolonged myocardial stretch, including tyrosine kinases, mitogen-activated protein kinases, protein kinase C, phospholipases A2, C and D (Sadoshima et al., 1993*b*) and immediate early genes (Sadoshima et al., 1993*a*).

The classical mechanism by which mechanosensitive cells are activated in response to acute stretch of the plasma membrane is via opening of stretch-activated ion channels which result either in membrane depolarization secondary to cation influx, or which directly allow for Ca^{2+} influx. Stretch-activated ion channels have been described in a wide variety of cells including amphibian muscle (Brehm, Kulberg & Moodt-Corbett, 1984), avian skeletal muscle (Ghuharay et al., 1984), and endothelial cells (Lansman, Hallam & Rick, 1987). While stretch-activated channels in animal cells usually have greatest permeability for K^+ ions, the channels described in endothelial cells are six times more permeable to Ca^{2+} than to Na^+, and have therefore been implicated as a source for activator Ca^{2+} (Lansman et al., 1987). Endothelial cells have also been shown to respond to mechanical distortion of the plasma membrane with a propagated $[Ca^{2+}]_i$ transient, and this intracellular response has been shown to be dependent on extracellular Ca^{2+} (Demer et al., 1993).

In the current report, we evaluated candidate mechanotransduction mechanisms previously reported in other cell types in colonic smooth muscle cells. In particular, we wanted to test the following hypotheses: (i) Is the $[Ca^{2+}]_i$ increase seen in response to mechanical stimulation mediated by Ca^{2+} release from intracellular stores or by Ca^{2+} influx pathways in the plasma membrane, or by both mechanisms? (ii) Is the mechanotransduction mediated by direct mechanical coupling between the plasma membrane and the Ca^{2+} release site, or does this coupling involve a chemical messenger? Our results suggest that the mechanically induced $[Ca^{2+}]_i$ transient in colonic myocytes involves the release of Ca^{2+} from an intracellular store via interactions between the plasma membrane-actin cytoskeleton and the Ca^{2+} release mechanisms associated with these stores, and/or via a Ca^{2+}-induced Ca^{2+} release mechanism. The partial dependence of influx on extracellular Ca^{2+} is consistent both with a mechanosensitive, and with a store-operated Ca^{2+} pathway in the plasma membrane. Part of these results have previously been reported in abstract form (Ennes, Young & Mayer, 1996).

Materials and Methods

Materials

Hanks Buffered Saline Solution (GIBCO BRL) supplemented with 20 mM HEPES buffer, pH 7.4 was used as normal external solution. Ca^{2+}-free solutions were prepared without $CaCl_2$, and with addition of EGTA (1 mM). Stock solutions of the phospholipase C (PLC) dependent process inhibitor U-73122, and its inactive analogue U-73343 (Calbiochem) were prepared in DMSO (5 mM), and then diluted into external saline (final concentration 10 μM) immediately before use. Incubation times of these compounds in the cell culture were less than 120 sec. Solutions containing the phosphokinase C inhibitor calphostin C (1 μM, Calbiochem) were first light activated in room fluorescent lighting for 1 hr (Bruns et al., 1991). Cells in culture were incubated for 1 hr with the activated solution before the start of the experiment. Salines containing La^{3+} or Gd^{3+} were phosphate free.

Cell Culture

Myocytes from the circular muscle layer of the distal rabbit colon were put into primary culture as previously described (Ennes et al., 1995) by plating onto circular glass coverslips. Cells were studied after 6–9 days in culture.

Immunocytochemistry and Fluorescence Microscopy

Cultured cells were treated with 10 μM cytochalasin D (Calbiochem) for 1 hr and then fixed for 15 min at 4°C with previously chilled methanol. Some cultures were returned to saline for 30, 60, and 120 min before fixation. The cells were then incubated with CGA7, a monoclonal mouse IgG2b antibody that recognizes actin isotypes alpha and gamma of smooth muscle cells (Enzo Diagnostics). The incubation was carried at 37°C (CO_2 incubator) for 30 min using a 1:1 antibody dilution. At the end of the first incubation, cells were then washed three times with 0.1 M Phosphate buffer and incubated for 1 hr at room temperature with goat anti-mouse FITC labeled the second antibody (American Qualex). After a three-step washing the coverslips were carefully mounted on slides with Anti-Fade. Fluorescent images were obtained with Leica Microscope fitted with epifluorescence illumination.

Calcium Measurement

Cells were incubated in external saline containing the calcium indicator dye Fura-2 AM (5 μM, Molecular Probes), for 1 hr at 37°C, followed by rinse in external saline. The coverslips were then mounted in a coverslip chamber (volume 1 ml) and cells allowed to rest for 15 min. The chamber was then mounted on the stage of a Zeiss 100TV inverted microscope, with a 40× objective (Fluar, Zeiss). $[Ca^{2+}]_i$ was measured with a videomicroscopy system (Attofluor Ratio Vision, Atto Instruments) attached to the microscope. Background subtraction, shading correction, and calibration could be performed using the software supplied with the system. Calibration of the Fura-2 fluorescence was accomplished in vitro by the use of a series of buffered Ca^{2+} standards containing magnesium (Molecular Probes) and Fura-2 potassium salt. Ca^{2+} concentrations at points within the cell were measured within regions of interest, or ROIs consisting of pixel arrays (100 points, 8 μm × 6 μm) where all points were averaged together. Concentration and time information from each ROI is stored on computer disk for later construction of concentration profiles for each ROI. All experiments were performed at room temperature (20–23°C).

Mechanical Stimulation

Light, focal, mechanical stimulation was provided by a small (1–3 μm tip diameter) fire-polished glass pipette connected to a micropositioner

(Eppendorf), which transiently (0.5 sec) depressed the muscle cell membrane. The tip of the pipette was initially placed several microns above the surface of the cell and programmed to move in a downward direction for a distance of 2 μm for a duration of 0.5 sec, followed by a return to the start position. If no response was observed, the transient tip deflection was increased by 2 μm increments. After the response, at the end of the run, the tip deflection was observed under transient illumination to visually verify that the membrane depression was slight (no more than 3 μm) and had not visibly damaged the cell.

Microinjection

Microinjection of cells with various compounds was performed with an electronically controlled micropositioner and micropipette pressure injection system (Eppendorf). Micropipettes were filled with standard internal solution (containing in mM: 92 KCl, 20 KOH, 1 $CaCl_2$, 1 $MgCl_2$, 11 EGTA, 20 HEPES pH 7.4) containing 1 mM Texas Red conjugated dextran (10,000 MW). The fluorescent indicator was injected to be able to identify microinjected cells after the 60 min postinjection period. Injection times were 0.5–2.0 sec and injection pressures were 300–500 hPa. Visual inspection of cells showed no evidence of swelling or deformation of injected cells and injected myocytes retain their ability to increase cyclic AMP concentrations in response to forskolin following microinjection of the cyclic AMP Fluorosensor, FlCRhR (AttoInstruments).

Data Analysis and Statistics

Resting $[Ca^{2+}]_i$ levels, and the amplitude of the initial $[Ca^{2+}]_i$ transient and the prolonged $[Ca^{2+}]_i$ increase (increment above resting level) were measured. The amplitude of the prolonged $[Ca^{2+}]_i$ increase was measured at 100 sec following the peak response. Data are reported as mean values ± *SE* throughout the text. Statistical analysis was performed on data following logarithmic transformation using paired Student's *t*-test. Significance was expressed at the $P < 0.05$ level throughout. Paired experiments were performed for most conditions, where pretreatment values from a single cell in normal saline were compared with posttreatment values from the same cell obtained after exposure to an experimental solution. In situations where paired experiments could not be performed, $[Ca^{2+}]_i$ responses to mechanical stimulation during pharmacologic intervention were compared to responses obtained on the same day on cells from parallel or 'sister' cultures. These situations are indicated in the text.

Results

Calcium responses to transient mechanical stimulation were studied in 80 myocytes which had been in culture for 6–9 days. Under control conditions, in normal external saline, 90% of myocytes (51 out of 57 cells) showed a local $[Ca^{2+}]_i$ increase in response to focal mechanical stimulation, followed by intracellular propagation of a $[Ca^{2+}]_i$ transient. As shown in Figs. 1 and 2*a*, with normal extracellular $[Ca^{2+}]$, the $[Ca^{2+}]_i$ response showed an initial peak, occurring within 1 sec of the stimulus, and a biphasic recovery to resting levels, with an initial rapid decay followed by a prolonged, slow recovery to resting $[Ca^{2+}]_i$ (referred to as ''plateau phase'' throughout the text). The mean time period to return to baseline $[Ca^{2+}]_i$

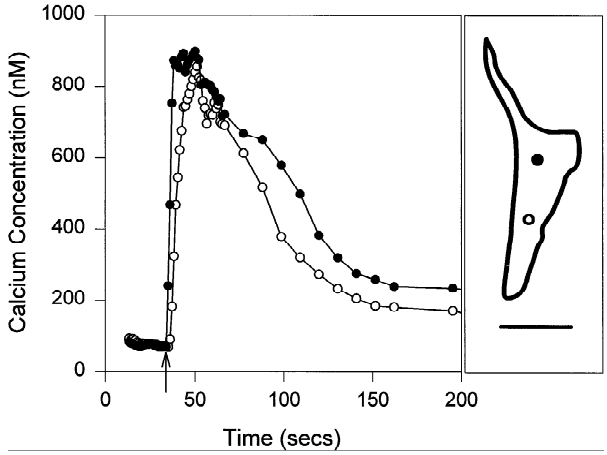

Fig. 1. Mechanically induced $[Ca^{2+}]_i$ transient. Shown is a mechanically induced $[Ca^{2+}]_i$ transient, monitored at 2 regions of interest (stimulation point, marked by the solid circles; distal point, marked by open circles). $[Ca^{2+}]_i$ changes are plotted against time. Stimulation time marked by arrow. A fast rising $[Ca^{2+}]_i$ transient is followed by both a rapid recovery phase, and a prolonged, slowly decaying return of $[Ca^{2+}]_i$ to resting level (''plateau phase''). The $[Ca^{2+}]_i$ transient propagated from the site of stimulation to the distal region of the cell, without significant change in transient amplitude. The inset at the right shows a tracing of the cell outline with indicated regions of interest. Scale Bar 20 μm.

was 271.4 ± 44.8 sec ($n = 40$; range: 50–1216 sec). Mean resting $[Ca^{2+}]_i$ was 104.8 ± 10.9 nM (range: 10 nM to 284 nM), and the mean amplitude of the mechanically induced transient under control conditions was 422.7 ± 43.8 nM (range: 29 nM to 1750 nM; $n = 57$). While the resting $[Ca^{2+}]_i$ values were relatively stable, the amplitude of the $[Ca^{2+}]_i$ transient varied significantly between cells. However, repeated mechanoresponses in a single cell were fairly stable when applied after recovery of $[Ca^{2+}]_i$ to resting levels (Figs. 1 and 2*a*). Under these circumstances, cells could be restimulated up to 3 times without a significant decrease in the amplitude of the $[Ca^{2+}]_i$ transient. In six paired experiments, the mean amplitude during the initial stimulation was 590.7 ± 115.6 nM and the mean amplitude during a second stimulation 10 min later was 527.2 ± 100.8 nM ($n = 6$). In contrast, when cells were restimulated within 30 sec of the initial stimulus, during the early plateau phase of the $[Ca^{2+}]_i$, no significant $[Ca^{2+}]_i$ transient could be induced (Fig. 2*b*). As previously reported (Young, Ennes & Mayer, 1996), cells after 6–9 days in culture showed larger $[Ca^{2+}]_i$ transients than cells in culture for 11–14 days (mean amplitude 114.7 ± 20.9 nM; $n = 20$). Below, we describe the results evaluating the possible involvement of Ca^{2+} influx via stretch-activated plasma membrane channels (i), release from intracellular Ca^{2+} stores (ii) and the involvement of the actin cytoskeleton (iii) in this mechanosensitive increase $[Ca^{2+}]_i$.

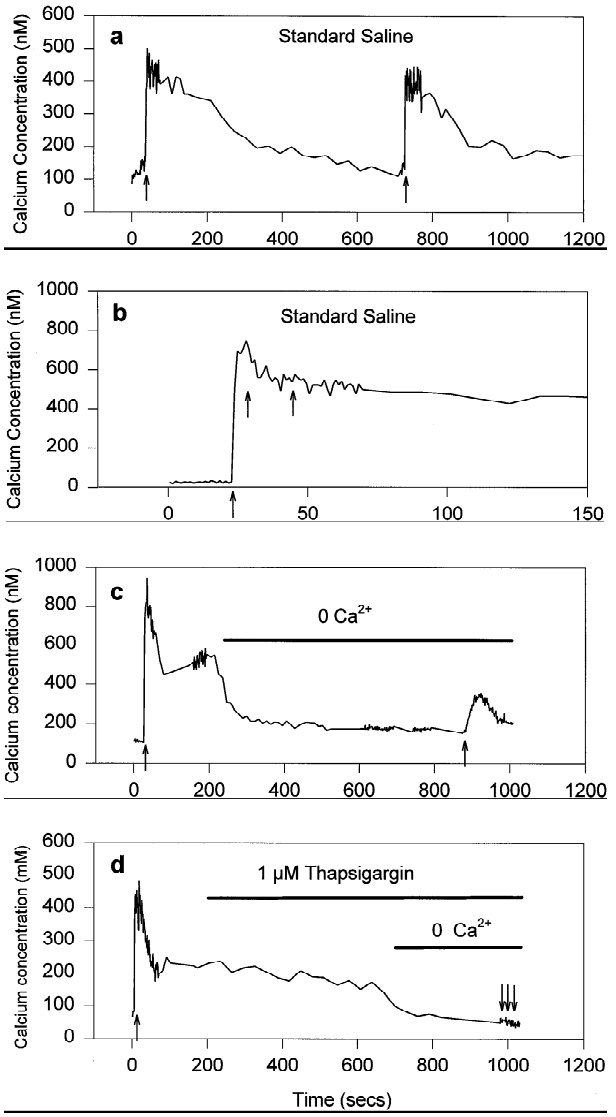

Fig. 2. Dependence of $[Ca^{2+}]_i$ transient on release from intracellular Ca^{2+} stores. Shown are experiments from 4 different cells. (a) Repeated mechanostimulation (at times marked by arrows) in standard saline, resulted in reproducible $[Ca^{2+}]_i$ responses. Similar to the graph shown in Fig. 1, mechanostimulation produced an initial transient increase in $[Ca^{2+}]_i$, followed by a prolonged, slow return to resting $[Ca^{2+}]_i$ levels. After 700 sec, another stimulation produced a second similar mechanoresponse. Differing widths of the trace reflect differing sample rates. (b) When the additional mechanostimulation was applied within 5 sec of the first stimulus, (during the rapid recovery phase), or 30 sec (during the initial plateau phase), no $[Ca^{2+}]_i$ transient could be induced. Note different time scale from Fig. 2*a*. (c) Removal of extracellular Ca^{2+} during the extended plateau phase following the first stimulus resulted in an immediate return of $[Ca^{2+}]_i$ to baseline values. Under these conditions, the peak $[Ca^{2+}]_i$ response to a second stimulus was greatly reduced, and no plateau phase was observed. (d) Following mechanostimulation in normal saline, cells were perfused with external solution containing 1 μM thapsigargin, followed by perfusion with Ca^{2+}-free solution. Similar to Fig. 2*c*, a prompt return of $[Ca^{2+}]_i$ to baseline values were observed in Ca^{2+}-free solution. However, no further $[Ca^{2+}]_i$ transient could be induced.

Contribution of Ca^{2+} Influx through the Plasma Membrane

When measured within 5 min of solution change, the initial amplitude of the mechanically induced $[Ca^{2+}]_i$ transient was significantly reduced to 50% of control value by removal of extracellular Ca^{2+} (pretreatment: 403.8 ± 55.7 nM; post-treatment: 187.8 ± 35.9 nM; $n = 9$), while the resting level was not affected (pretreatment: 220.7 ± 22.1 nM; post-treatment 204.4 ± 18.9; $n = 9$). A representative experiment is shown in Fig. 2*c*. This is in contrast to the response in normal extracellular $[Ca^{2+}]$ where the amplitude of the second $[Ca^{2+}]_i$ transient induced 10 min after the initial stimulation was not different from the initial amplitude (Fig. 2*a*). Addition of 100 μM Gd^{3+}, a blocker of certain stretch-activated cation channels (Yang & Sachs, 1989) neither affected resting levels (pretreatment: 191.6 ± 38.9 nM; post-treatment: 184.3 ± 37.8 nM; $n = 7$) nor the transient amplitude (pretreatment: 619.8 ± 98.4 nM; post-treatment 542.3 ± 94.5 nM; $n = 7$). Similarly, addition of 25 μM La^{3+} did not significantly affect the resting level of $[Ca^{2+}]_i$ (pretreatment: 153.6 ± 32.9 nM; post-treatment: 153.0 ± 24.5 nM) or transient amplitude (pretreatment: 331.2 ± 71.5 nM; post-treatment: 473.3 ± 189.4 nM; $n = 6$), while 100 μM La^{3+}, a concentration that blocks both Ca^{2+} influx and Ca^{2+} efflux across the plasma membrane, increased both the resting $[Ca^{2+}]_i$; (treated: 177.5 ± 6.5 nM; $n = 4$), and the mechanically induced $[Ca^{2+}]_i$ transient (treated: 794.8 ± 318.6; $n = 4$, $p = 0.01$) as compared to 'sister' cultures.

To determine if the mechanically induced $[Ca^{2+}]_i$ increase may have been due to cell wounding, with transient influx of Ca^{2+} through a leak in the plasma membrane, we examined two emission wavelengths (340 nm, 380 nm) of Fura-2 at the point of stimulation during stimulation and recovery. A simple perforation in the plasma membrane should also result in efflux of the fluorescent indicator, resulting in a loss of dye signal. As shown for a representative experiment in Fig. 3, the optical signal at 334 nm wavelength *increased* as a result of stimulation, followed by a *return* of both 334 nm and 380 nm signals to resting intensities after the stimulation, with no apparent loss of dye from the cell. Furthermore, as shown in Fig. 2*b*, multiple stimulations, spaced seconds apart, do not produce additional $[Ca^{2+}]_i$ transients, as might be expected from simple membrane perforation.

Contribution of Release from Intracellular Ca^{2+} Stores

Intracellular Ca^{2+} release in response to agonists in acutely dissociated myocytes from the circular muscle layer of the colon of different species is thought to be mediated largely by release from Ins(1,4,5)P_3-sensitive

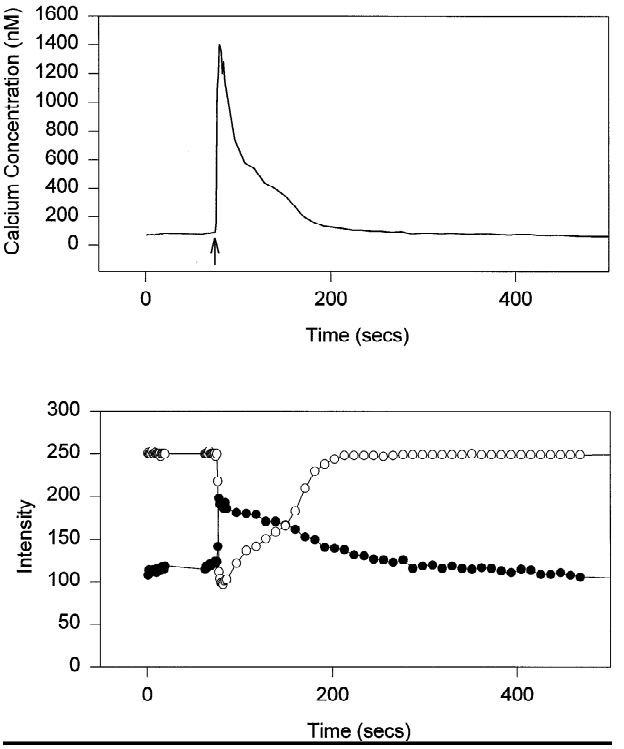

Fig. 3. Evidence against Ca^{2+} influx secondary to cell wounding. No loss of indicator dye signal. *Upper panel.* Characteristic increase in $[Ca^{2+}]_i$ in response to brief focal mechanical stimulation (applied at arrow). $[Ca^{2+}]_i$ returned to baseline values with a biphasic time course within 200 sec. *Lower panel.* Time course of optical signals monitored at 334 nm (solid circles) and 380 nm (open circles) corresponding to the $[Ca^{2+}]_i$ response shown in upper panel. As a result of mechanostimulation, the 334 nm signal increases, then returns to resting intensity level. The 380 nm signal shows an immediate decrease, and then *increases* during the recovery phase back to resting intensity level.

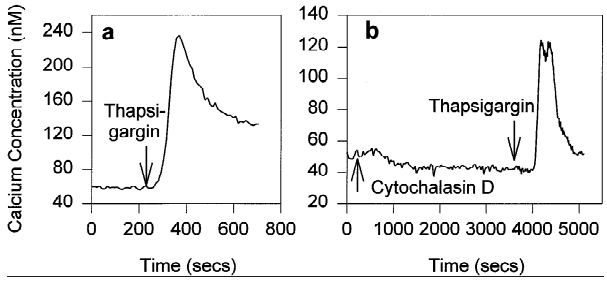

Fig. 4. Effect of cytochalasin D on Ca^{2+} release from thapsigargin-sensitive stores. (a) Thapsigargin (1 mM, start of perfusion marked by arrow) addition resulted in a transient increase in $[Ca^{2+}]_i$ followed by a slow recovery toward resting levels. Data from a single representative cell. (b) Perfusion with 10 μM cytochalasin D over the course of 1 hr did not significantly affect final resting $[Ca^{2+}]_i$ (start of perfusion marked by the arrow). After 1 hr, cytochalasin D was replaced with thapsigargin (1 μM, marked by arrow) and the $[Ca^{2+}]_i$ increased, showing that cytochalasin D also does not prevent $[Ca^{2+}]_i$ mobilization by thapsigargin. Shown are averaged tracings from 12 cells.

Ca^{2+} stores (Murthy, Grider & Makhlouf, 1991). As shown in Fig. 4*a* for a representative myocyte, addition of 1 μM thapsigargin to the bath caused a transient increase in mean resting $[Ca^{2+}]_i$, followed by a slow return toward baseline values within 2–3 min. Eighty two percent (47/57) of cells showed this response to thapsigargin with a mean increase in $[Ca^{2+}]_i$ of 86.5 ± 15.8 nM). Cells were assayed for their mechanosensitivity in the presence of the thapsigargin, 10 min after its addition to the bath. Thapsigargin had no effect on resting levels assessed at 10 min (pretreatment: 184.6 ± 38.5 nM; post-treatment 170.2 ± 33.7 nM; $n = 9$), but resulted in a statistically significant 25% reduction in the mechanically induced $[Ca^{2+}]_i$ transient (pretreatment: 439.8 ± 138.3; post-treatment: 367.6 ± 118.0; $n = 9$, $P = 0.03$). When compared to the control response obtained in cells from 'sister cultures,' treatment with the phosphokinase C inhibitor calphostin C (1 μM) had no significant effect on resting $[Ca^{2+}]_i$ (control: 107.0 ± 17.3 nM ($n = 27$); treatment: 108.3 ± 27.2 nM; $n = 18$), nor on transient amplitude (control: 625.5 ± 180.0 nM; treatment: 524.1 ± 146.7 nM; $n = 8$). Similarly, treatment of cells with the phospholipase C inhibitor U-73122 (10 μM) had no effect on resting $[Ca^{2+}]_i$ (control: 68.8 ± 5.9 nM; treatment: 72.2 ± 16.2 nM; $n = 8$), nor on the amplitude of the $[Ca^{2+}]_i$ transient (control: 100.7 ± 24.4 nM; treatment: 101.1 ± 20.0 nM; $n = 8$).

Refilling of Intracellular Ca^{2+} Stores

In many cell types, depletion of intracellular Ca^{2+} stores by agonists or thapsigargin results in Ca^{2+} influx through the plasma membrane (Putney, 1990). To determine if the prolonged $[Ca^{2+}]_i$ increase following transient mechanical stimulation was due to such capacitative Ca^{2+} entry (Putney, 1990), we evaluated the dependence of the $[Ca^{2+}]$ plateau on extracellular Ca^{2+} and its sensitivity to La^{3+}. As shown in Fig. 2*c*, removal of extracellular Ca^{2+} resulted in an immediate return of $[Ca^{2+}]_i$ to baseline values. In contrast, no significant effect of La^{3+} (25 μM) on the amplitude of the prolonged $[Ca^{2+}]_i$ response was seen ($n = 4$).

To further characterize the partial dependence of $[Ca^{2+}]_i$ transient amplitude on both extracellular Ca^{2+} and thapsigargin-sensitive stores, we first depleted Ca^{2+} stores with thapsigargin followed by stimulation of cells in Ca^{2+} free medium. A representative experiment is shown in Fig. 2*d.* In contrast to the partial inhibition of the transient by removal of extracellular Ca^{2+} alone (Fig. 2*c*), the mechanically induced $[Ca^{2+}]_i$ transient was completely abolished by prior depletion of intracellular stores.

Application of 10 mM caffeine to the extracellular solution caused a slight transient increase in resting $[Ca^{2+}]_i$ in only 2 out of 21 cells. Caffeine treatment of

cells had no significant effect on the amplitude of the stimulated transient ($n = 6$). Application of 50 μM ryanodine resulted in a small transient increase in $[Ca^{2+}]_i$ levels in 3 out of 18 cells, without any significant effect on the transient amplitude.

Application of the phospholipase A_2 inhibitor $AACOCF_3$ did not significantly affect resting $[Ca^{2+}]_i$ (pretreatment: 155.2 ± 2 nM; post-treatment 143.2 ± 25.5 nM; $n = 6$), nor the amplitude of the $[Ca^{2+}]_i$ transient (pretreatment: 410.3 ± 119.8 nM; post-treatment 449.8 ± 128.6 nM; $n = 6$).

Involvement of Cytoskeleton in Mechanotransduction

In a variety of cell types, disruption of the cytoskeleton abolishes mechanosensitive stimulation of intracellular signaling molecules (Watson, 1990; Vandenburgh, 1992). To determine the role of cytoskeletal and/or cytoplasmic structural networks in the potential mechanical coupling of the plasma membrane and Ca^{2+} compartments in mechanotransduction of colonic myocytes, we evaluated the effects of rapid transient cell volume expansion, and of the microtubule disrupting drug colchicine and the microfilament disrupting agent cytochalasin D on mechanically induced $[Ca^{2+}]_i$ transients.

Microinjection of myocytes with intracellular saline containing Texas red conjugated dextran resulted in a complete inhibition of mechanosensitivity when stimulated 60 min later without any effect on resting $[Ca^{2+}]_i$ ($n = 8$). Cell integrity was demonstrated by the ability of cells to take up Fura-2 AM and to retain Fura-2.

Preincubation with colchicine (20 μM for 2 hr) had no effect on resting $[Ca^{2+}]_i$ (127.8 ± 19.4 nM; $n = 4$) or the amplitude of the mechanically induced $[Ca^{2+}]_i$ transient (122.5 ± 33.8 nM; $n = 4$). Occasionally, $[Ca^{2+}]_i$ oscillations resulted after a single mechanostimulation, a behavior not observed in normal saline. Preincubation with cytochalasin D (10 μM for 1 hr) did not change resting $[Ca^{2+}]_i$ (117.4 ± 11.4 nM), but in contrast to colchicine completely abolished mechanically induced $[Ca^{2+}]_i$ transients in 72% of cells ($n = 14$). Following washout of cytochalasin D and reincubation in normal saline, cells regained their mechanosensitivity. The time course of recovery of mechanosensitivity is shown in Fig. 5. The mean amplitude of the $[Ca^{2+}]_i$ transient after 60-min recovery in normal saline was 93% of the amplitude under control conditions. In one cell, $[Ca^{2+}]_i$ oscillations resulted after a single mechanostimulation.

To determine the integrity of intracellular Ca^{2+} stores following treatment with cytochalasin D, we evaluated the effect of the compound on thapsigargin-induced $[Ca^{2+}]_i$ changes. As shown in Fig. 4*b*, as the average response from 12 cells, neither the amplitude nor the slow decline in thapsigargin-induced $[Ca^{2+}]_i$ increase were blocked by incubation of cells with cytochalasin D.

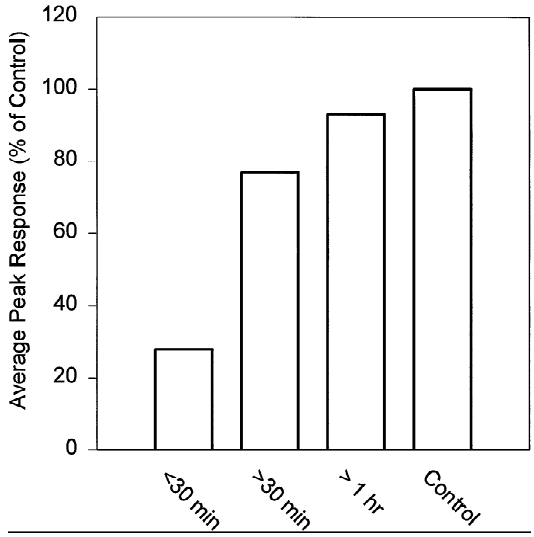

Fig. 5. Effect of cytochalasin D on mechanosensitivity. Cells were treated with cytochalasin D (20 μM, 1 hr) then returned to normal saline. Within 30 min of recovery time in normal saline, the cells regained 28% of their responsivity, and after 60 min, the cells had recovered 93% of the mechanoresponse (total of 14 cells).

To confirm that cytochalasin D will disrupt microfilaments in colonic smooth muscle cells, we performed immunocytochemical staining for smooth muscle-specific actin under control conditions, in the presence of cytochalasin D, and following washout and reincubation in cytochalasin D-free control solution. Cytochalasin D disrupted actin filaments, while return of the cells to normal saline allowed the filaments to regain their structure, in a time course similar to recovery of mechanosensitivity as shown in Fig. 6.

Discussion

We have demonstrated that brief focal mechanical deformation of the plasma membrane of colonic myocytes from the circular muscle layer results in a $[Ca^{2+}]_i$ transient which propagates across the cell. As $[Ca^{2+}]_i$ transients could be induced repeatedly in the same cell, were not associated with leakage of the fluorescent indicator, and were only partially dependent on extracellular $[Ca^{2+}]$, our findings suggest that the mechanically induced $[Ca^{2+}]_i$ response is not due to cell wounding, but is related to both Ca^{2+} influx and release of Ca^{2+} from an intracellular compartment.

Mechanically induced Ca^{2+} Influx through Plasma Membrane

In different cell types, mechanically induced $[Ca^{2+}]_i$ transients involve both influx of extracellular Ca^{2+} and release of Ca^{2+} from intracellular stores (Charles et al.,

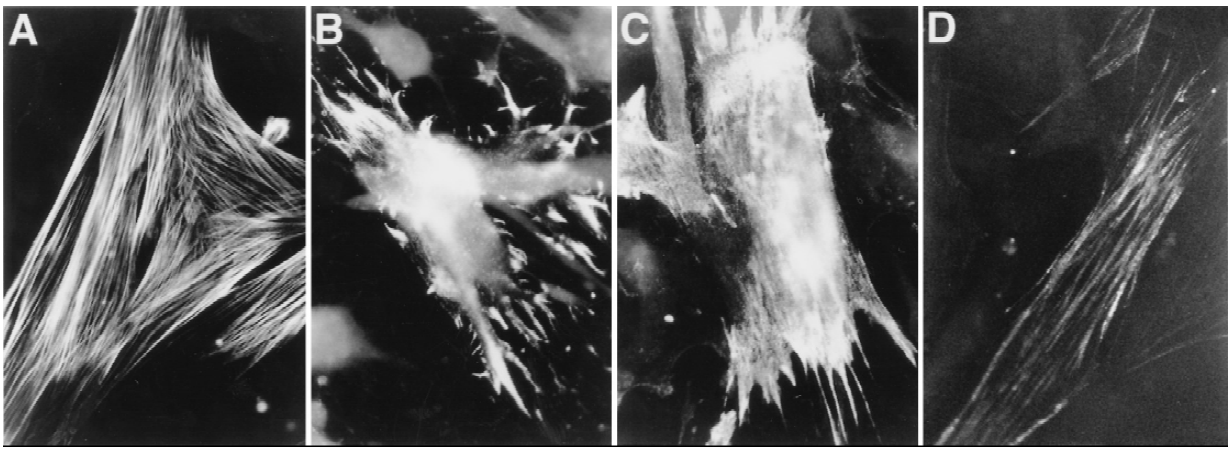

Fig. 6. Effect of cytochalasin D on actin filaments. Shown are myocytes stained with antibody to smooth muscle specific actin. (*A*) Untreated cells show actin organized in filaments throughout the cell. (*B*) Immediately after treatment with 10 μM cytochalasin D for 1 hr, cells show complete disruption of actin filamental structure. (*C*) 30 min after return to normal saline, actin filamental structure starts to return. (*D*) after 2 hr in normal saline, there is a more complete return of actin filamental structure (500×).

Table 1. Chemical sensitivity of the mechanoresponse: paired experiments

Treatment	Control		Treated				
	Rest	Amplitude	Rest	Amplitude	n	*P* Rest	*P* Amplitude
Ca^{2+}-free saline	220.7 ± 22.1	403.8 ± 55.7	204.4 ± 18.9	187.8 ± 35.9	9	NS	0.003
Thapsigargin	158.3 ± 28.3	532.3 ± 82.2	146.5 ± 26.4	270.0 ± 75.3	9	NS	0.02
100 mM Gd^{3+}	191.6 ± 38.9	619.8 ± 98.4	184.3 ± 37.8	542.3 ± 94.5	7	NS	NS
25 μM La^{3+}	153.6 ± 32.9	331.2 ± 71.5	153.0 ± 24.5	473.3 ± 189.4	6	NS	NS
10 μM $AACOCF_3$	155.2 ± 29.9	410.3 ± 119.8	143.2 ± 25.5	449.8 ± 128.6	6	NS	NS

Summary of data from paired experiments (pretreatment and posttreatment) on single cells. Comparisons were made on resting $[Ca^{2+}]_i$ and amplitude of the mechanically induced transient. For each treatment, *n* reports the number of cell pairs, and *P* the results of a *T*-test on both resting (first entry), and amplitude (second entry) values.

1991; Sanderson, Charles & Dirksen, 1990; Demer et al., 1993). Influx of activator Ca^{2+} in response to stretch can occur either directly through stretch-activated ion channels (Bibby & McCulloch, 1994; Yang et al., 1989) with a high permeability for Ca^{2+} (Lansman et al., 1987), or through voltage-sensitive Ca^{2+} channels, which are activated by membrane depolarization associated with the opening of stretch-activated cation channels. For example, in vascular smooth muscle, stretch-induced contractions are partially dependent on Ca^{2+} influx through dihydropyridine-sensitive Ca^{2+} channels (Nakayama et al., 1993). In the current study, the amplitude of the initial $[Ca^{2+}]_i$ transient was partially dependent on extracellular Ca^{2+}, since removal of extracellular Ca^{2+} reduced the amplitude by 50%. The observation that neither La^{3+} nor Gd^{3+} reduced the amplitude of the transient, rules out the involvement of voltage-sensitive Ca^{2+} channels (such as the L type Ca^{2+} channels previously described in these cells) or common stretch-activated channel. However, influx may have occurred through a Ca^{2+} pathway not sensitive to these blockers, such as a Ca^{2+}-permeable cation channel. We have recently shown that $[Ca^{2+}]_i$ increase in DRG somas induced by the same mechanical stimulus as used in the current study is mediated by a stretch-activated cation channel (Raybould et al., 1996). However, in contrast to the current study, the pathway in DRG neurons is Gd^{3+}-sensitive.

G protein-mediated PLA_2 activation has recently been suggested as a mechanism to increase Ca^{2+} influx through the plasma membrane in guinea pig intestinal smooth muscle from the longitudinal layer (Murthy et al., 1995). Our findings that $AACOCF_3$ had no effect on the mechanically induced $[Ca^{2+}]_i$ transient argues against such a mechanism in mechanotransduction in colonic cells of the circular layer.

Release of Ca^{2+} from Intracellular Stores

Mechanically Induced Ins(1,4,5)P_3 Production

A series of studies have identified stimulus-induced $Ins(1,4,5)P_3$ production as a sufficient mechanism to ex-

plain the initial $[Ca^{2+}]_i$ transient in response to activation of plasma membrane receptors (Murthy et al., 1991), as well as in response to mechanical stimulation of the plasma membrane (Hansen et al., 1995; Sanderson et al., 1990). For example, Sanderson and coworkers have provided strong evidence for mechanically stimulated $Ins(1,4,5)P_3$ production as the mechanism for Ca^{2+} wave propagation in rabbit airway epithelial cells (Hansen et al., 1995). In rat glial cells (Charles et al., 1991) and bovine aortic endothelial cells (Demer et al., 1993), mechanical stimulation in Ca^{2+}-free medium no longer causes a $[Ca^{2+}]_i$ increase in the stimulated cell, while a Ca^{2+} wave is seen in adjacent cells, suggesting production of a messenger substance in the stimulated cell followed by diffusion into adjacent cells. While the role of G-protein coupled activation of PLC is firmly established as the mechanism for receptor-stimulated $Ins(1,4,5)P_3$ production, the mechanism(s) involved in mechanically induced $Ins(1,4,5)P_3$ production are not known. It has been suggested that in analogy to conformational changes in response to receptor-agonist interactions, mechanical deformation of the plasma membrane, possibly translated to target proteins via cytoskeletal structures may result in similar conformational changes resulting in PLC activation (Sanderson et al., 1994). This mechanism has been referred to as mechanosensitive catalysis (Watson, 1990). However, the PLC inhibitor U-73122, in a concentration which has previously been shown to inhibit PLC activity in airway epithelial cells (Hansen et al., 1995) and which prevents the $[Ca^{2+}]_i$ increase in response to substance P in colonic myocytes (*unpublished observations*) did not affect the amplitude of the $[Ca^{2+}]_i$ transient, arguing against such mechanosensitive enzyme activation as the singular cause of $[Ca^{2+}]_i$ increase in the *stimulated* smooth muscle cell. However, in a semiconfluent culture of these same muscle cells, U-73122 will block the *inter*cellular propagation of the $[Ca^{2+}]_i$ wave (Young et al., 1996) as it also does in rabbit airway cells (Hansen et al., 1995). Thus, in contrast to some other cell types, our findings make it unlikely that the mechanotransduction in the *stimulated* colonic smooth muscle is singularly dependent on *intra*cellular Ca^{2+} release via mechanically stimulated $Ins(1,4,5)P_3$ production, while propagation of the *inter*cellular wave is singularly dependent on $Ins(1,4,5)P_3$ production

Release from Thapsigargin-sensitive Stores

In contrast to other cell types, in the current study we found only a small contribution of Ca^{2+} from thapsigargin-sensitive $Ins(1,4,5)P_3$-releasable Ca^{2+} stores in the mechanically induced $[Ca^{2+}]_i$ transient in colonic myocytes. It was not possible to determine the effect of direct $Ins(1,4,5)P_3$ receptor inhibition with intracellular heparin injection, since the sudden cell pressure/volume change induced by microinjection of control solution abolished the mechanically induced $[Ca^{2+}]_i$ response. However, the depletion of $Ins(1,4,5)P_3$-sensitive intracellular stores with the Ca^{2+}-ATPase inhibitor thapsigargin resulted only in a 25% reduction of the $[Ca^{2+}]_i$ transient. The thapsigargin protocol used in the current study is sufficient to completely deplete $Ins(1,4,5)P_3$-sensitive $[Ca^{2+}]_i$ stores since we have previously shown (Young et al., 1996) that the same protocol abolished the *inter*cellular propagation of $[Ca^{2+}]_i$ transients, a spatial phenomenon thought to be dependent on release of Ca^{2+} from $Ins(1,4,5)P_3$-sensitive stores. A study in the permeabilized smooth muscle cell line DDT_1MF-2 demonstrated that thapsigargin inhibited total Ca^{2+} uptake into internal pools by 75%, all of which were $Ins(1,4,5)P_3$-sensitive (Bian et al., 1991). Even though thapsigargin-resistant Ca^{2+} pumps on $Ins(1,4,5)P_3$-sensitive Ca^{2+} stores have been reported in cells exposed chronically to thapsigargin (Waldron, Short & Gill, 1995), such mechanisms are unlikely to play an important role in the current study. In contrast to the $[Ca^{2+}]_i$ response observed in response to thapsigargin, no significant $[Ca^{2+}]_i$ increase was seen with caffeine or ryanodine. These findings are consistent with reports by Kummerle et al., indicating that ryanodine-sensitive stores do not play a significant role in intracellular Ca^{2+} release in myocytes from the circular muscle layer of the guinea pig intestine (Kummerle, Murthy & Makhlouf, 1994).

Our findings do not rule out the additional involvement of Ca^{2+} release from other intracellular compartments, such as thapsigargin-insensitive stores, mitochondrial Ca^{2+} stores, Ca^{2+} bound to buffers on the inner leaflet of the plasma membrane (Post & Langer, 1992; Peskoff, Post & Langer, 1992), or Ca^{2+} trapped within caveolae of the plasma membrane. Caveolae have been well characterized in smooth muscle, where they may form multilobulated structures, which open into the extracellular space. The basal lamina which covers the cell surface passes over the necks of caveolae without penetrating them (15). Caveolae occupy approximately 50% of the cell surface and have close association with the subplasmalemmal SR. If the integrity of the caveolae is maintained by cytoskeletal structures, the observed effects with cytochalasin may be related to a reversible disappearance of these invaginations of the plasma membrane.

In summary, our findings are most consistent with a small component of Ca^{2+} release from thapsigargin-sensitive stores which may either have been triggered by the mechanically induced $[Ca^{2+}]_i$ influx through the plasma membrane (Putney, 1990) or by a direct mechanical coupling of the Ca^{2+} release channel on these stores with the plasma membrane. The observation that depletion of thapsigargin-sensitive stores in the absence of

extracellular Ca^{2+} completely abolished the $[Ca^{2+}]_i$ transient, while either treatment alone produced only a partial reduction, is consistent with both concepts.

The Plateau Phase of the Mechanoresponse

Removal of extracellular Ca^{2+} did not affect resting $[Ca^{2+}]_i$, but abolished the plateau phase of the mechanically induced $[Ca^{2+}]_i$ transient in 57% of cells, and reduced the amplitude in the remaining cells to $\leqslant$15% of control values. In addition, the start of the plateau phase is frequently observed as an increase separated from the decline of the initial transient (Fig. 2*c*, and *d*). Application of a second mechanical stimulation during the plateau phase, given within 30 sec of the first stimulus, does not produce a mechanically induced $[Ca^{2+}]_i$ transient, presumably due to incomplete refilling of the mechanosensitive store. The fact that neither La^{3+} nor Gd^{3+} had a significant effect on the plateau phase suggests that it is not due to Ca^{2+}-selective influx pathway, such as I_{CRAC} (Clapham, 1996). These characteristics are suggestive of a store-operated channel (SOC, Clapham, 1996). While the time course of store-operated Ca^{2+} influx during receptor-mediated cell activation is determined by receptor-ligand interactions, the mechanism underlying the prolonged Ca^{2+} influx following the brief mechanical stimulus is not known.

Involvement of Actin Cytoskeleton in Ca^{2+} Release

The findings that the Ca^{2+} release mechanism is reversibly inhibited by disruption of actin filaments (but not affected by inhibition of $Ins(1,4,5)P_3$ production) is consistent with two hypotheses: that the plasma membrane is directly coupled to an intracellular Ca^{2+} compartment via actin fibers, or that the actin cytoskeleton plays a role in the activation of the plasma membrane Ca^{2+} pathway.

According to the first hypothesis, deformation of the plasma membrane would result in the activation of a stretch-activated mechanism associated with an intracellular Ca^{2+} compartment. For example, actin filaments have been reported to be involved in stretch sensitivity of fibroblasts (Arora et al., 1994). Rossier, Bird & Patney, 1991 have reported that $Ins(1,4,5)P_3$ receptor-containing vesicles from rat liver may be attached to the plasma membrane through cytoskeletal elements such as actin. Similarly, a complex consisting of $Ins(1,4,5)P_3$ receptor and ankyrin has been isolated from a lymphoma cell line where it has been demonstrated that binding of ankyrin to the $Ins(1,4,5)P_3$ receptor in light density vesicles significantly inhibited $Ins(1,4,5)P_3$ binding and $Ins(1,4,5)P_3$ stimulated internal Ca^{2+} release (Bourguignon et al., 1993). The fact that $[Ca^{2+}]_i$ oscillations were induced in some colonic smooth muscle cells treated with cytochalasin D, is consistent with a disinhibition of Ca^{2+} release induced by basal $Ins(1,4,5)P_3$ production. An analogous actin cytoskeleton-dependent disinhibition has been described for adenylate cyclase in lymphoma cells (Watson, 1990). The observation that a sudden increase in cell pressure/volume induced by microinjection of intracellular solution resulted in a complete inhibition of the mechanically induced Ca^{2+} response, while the integrity of the plasma membrane remained intact is consistent with a disruption of the cytoskeletal elements responsible for the stretch sensitivity.

In summary, our findings suggest the involvement of several mechanisms in colonic smooth muscle cells by which a transient mechanical stimulus is transduced into a change in $[Ca^{2+}]_i$. The observations are most consistent with a combination of (a) Ca^{2+} release from a thapsigargin-sensitive intracellular compartment which is either directly mechanically coupled to the plasma membrane, or is activated via Ca^{2+}-induced Ca^{2+} release; (b) refilling of this compartment by influx of extracellular Ca^{2+} via a store-operated Ca^{2+} pathway, and (c) a direct mechanosensitive Ca^{2+} influx through a cation-channel in the plasma membrane. These findings extend previous observations in different cell types of actin cytoskeleton-mediated mechanically induced modulation of intracellular signaling events.

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