Stretch-induced Ca\textsuperscript{2+} release via an IP\textsubscript{3}-insensitive Ca\textsuperscript{2+} channel

MADHUMITA JENA MOHANTY AND XINGLI LI
Department of Medicine, Wayne State University School of Medicine and
John D. Dingell Veterans Affairs Medical Center, Detroit, Michigan 48201

Received 7 February 2002; accepted in final form 9 April 2002

Mohanty, Madhumita Jena, and Xingli Li. Stretch-induced Ca\textsuperscript{2+} release via an IP\textsubscript{3}-insensitive Ca\textsuperscript{2+} channel. Am J Physiol Cell Physiol 283: C456–C462, 2002.—Various mechanical stimuli increase the intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) in vascular smooth muscle cells (VSMC). A part of the increase in [Ca\textsuperscript{2+}]\textsubscript{i} is due to the release of Ca\textsuperscript{2+} from intracellular stores. We have investigated the effect of mechanical stimulation produced by cyclical stretch on the release of Ca\textsuperscript{2+} from the intracellular stores. Permeabilized VSMC loaded with \textsuperscript{45}Ca\textsuperscript{2+} were subjected to 7.5% average (15% maximal) cyclical stretch. This resulted in an increase in \textsuperscript{45}Ca\textsuperscript{2+} efflux in response to cyclical stretch. However, 10 \textmu M lanthanum, 10 \textmu M gadolinium, and 10 \textmu M cytochalasin D but not 10 \textmu M nocodazole inhibited the increase in \textsuperscript{45}Ca\textsuperscript{2+} efflux. This supports the existence of a novel stretch-sensitive intracellular Ca\textsuperscript{2+} store in VSMC that is distinct from the IP\textsubscript{3}-, ryanodine-, and NAADP-sensitive stores.


MATERIALS AND METHODS

VASCULAR SMOOTH MUSCLE CELLS (VSMC) in vivo are exposed to various mechanical stimuli such as cyclical stretch due to the pulsatile nature of the blood flow, shear stress during turbulent blood flow, or mechanical strain during balloon angioplasty. Mechanical stimulation of VSMC results in an increase in intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) (2, 21, 29). This increase in [Ca\textsuperscript{2+}]\textsubscript{i} is at least partly due to the release of Ca\textsuperscript{2+} from the intracellular stores (3, 16, 15, 17, 29). Ca\textsuperscript{2+} is released from the intracellular stores via two known channels in VSMC: one sensitive to inositol 1,4,5-trisphosphate (IP\textsubscript{3}) and the other sensitive to ryanodine (11). The sensitivity of ryanodine channels varies greatly in different types of VSMC but is generally low. Ryanodine receptors are not present in all smooth muscle cell types, and their presence or absence is influenced by cell growth and differentiation status. Some smooth muscle cells like myometrium and A7r5 cells do not express ryanodine receptors (20). A third channel, a nicotinic acid adenine dinucleotide phosphate (NAADP)-sensitive Ca\textsuperscript{2+} channel, has been identified in sea urchin eggs, pancreatic acinar cells, and neuronal cells but has not yet been identified in VSMC (5).

The release of Ca\textsuperscript{2+} from the intracellular stores in response to mechanical stimulation may occur via any of the three known channels of Ca\textsuperscript{2+} release or via a channel that is distinct from these known channels. There is considerable evidence that the cytoskeleton interacts directly with the plasma membrane as well as the intracellular stores (12, 31). Therefore, mechanical stimulation of the plasma membrane could be directly transmitted to the intracellular stores via the cytoskeleton without a biochemical mediator, resulting in a release of Ca\textsuperscript{2+} from the intracellular stores. We have investigated whether mechanical stimulation of VSMC produced by cyclical stretch results in a release of Ca\textsuperscript{2+} from the intracellular stores via the IP\textsubscript{3}, ryanodine, or NAADP channels or from a channel distinct from these known channels of Ca\textsuperscript{2+} release.

Address for reprint requests and other correspondence: M. J. Mohanty, HPB, Suite 908, 4160 John R Road, Detroit, MI 48201 (E-mail: jenam@intmed.wayne.edu).
Results are expressed as rate constant (fraction of remaining 45Ca2+ percentage. This movement is translated to the cultured cells, resulting in strain. The use of Bioflex plates in conjunction with loading posts produces uniform radial strain over 85% of the culture surface. The Flexercell strain unit computer software allowed us to assign the desired maximal percentage of elongation of the culture plate membrane. It also continuously displays the actual percentage of elongation of the culture plate membrane, as well as the waveform data during stretching and, thus, quantitates the actual magnitude of the degree of stretch. When the Bioflex plates were assigned to be stretched by 15% (maximal stretch), only the data obtained from the plates that were actually subjected to 14.8–15% maximal stretch were used for analysis.

Measurement of 45Ca2+ efflux from permeabilized cells. 45Ca2+ efflux from permeabilized cells was measured as previously described (16). Confluent monolayers of A7r5 cells grown on six-well collagen type I-coated silicone elastomer-bottomed culture plates were permeabilized by being incubated with 25 μg/ml saponin in 125 mM KCl, 25 mM NaCl, 2 mM MgCl2, and 10 mM HEPES, pH 6.9, at room temperature for 10 min. After 10 min, saponin was removed, and 2 μCi/ml 45Ca2+, 3 mM ATP (to facilitate uptake of 45Ca2+ into the intracellular Ca2+ stores), and 1 μM ruthenium red (to prevent loading of 45Ca2+ into the mitochondria) were added for 10 min (7, 9, 10, 13, 23, 33). No CaCl2 was added to the 45Ca2+ or saponin solutions to facilitate the selective uptake of the radioactive 45Ca2+ into the intracellular stores. The cells were then washed and incubated in isotonic efflux solution containing 125 mM KCl, 25 mM NaCl, 2 mM MgCl2, 0.2 mM CaCl2, 1 mM EGTA, 10 mM HEPES, pH 6.9, 150 mM free Ca2+, and 300 mosmol/kgH2O to mimic the intracellular milieu. This solution was collected and replaced with an equal volume of isotonic efflux solution at 30-s intervals for 2 min. The cells were then subjected to 7.5% average (15% maximal) cyclical stretch at 60 cycles/min, and isotonic efflux solution was continually collected and replaced with an equal volume of isotonic efflux solution at 30-s intervals. The assay was terminated by adding 5 μM A-23187, a Ca2+ ionophore, to release the remaining 45Ca2+ in the intracellular stores. Results are expressed as rate constant (fraction of remaining 45Ca2+ released per 30 s).

Staining for actin and microtubules. A7r5 cells were grown on 25-mm glass coverslips and incubated in the absence (control) and presence of either 10 μM cytochalasin D or 10 μM nocodazole in Earle’s solution containing 130 mM NaCl, 5.36 mM KCl, 26 mM HEPES, 0.8 mM MgSO4, 1.8 mM CaCl2, 1 mM NaH2PO4, and 10 mM glucose, pH 7.4, for 1 h at 37°C. After incubation, the cells were washed three times with Earle’s solution and fixed with 4% formaldehyde and then permeabilized with 0.5% Triton. The cells were washed three more times with Earle’s solution. To detect actin filaments and microtubules, cells were incubated with 10–6 M rhodamine phalloidin for 15 min or 1:25 dilution FITC-conjugated anti-α-tubulin for 1 h, respectively. After three more washes with Earle’s solution, the cells were visualized with a Zeiss LSM 310 microscope.

Statistics. Differences between groups of data were determined by using Tukey-Kramer one-way ANOVA or the paired t-test as appropriate. Data are reported as means ± SE. P < 0.01 was considered statistically significant.

RESULTS

Effect of cyclical stretch of intracellular Ca2+ stores. As shown in Fig. 1A, 45Ca2+ release was measured at 30-s intervals in isotonic efflux solution under non-stretch conditions and in the presence of cyclical stretch. 45Ca2+ rate constant was 0.1046 ± 0.002977 under control conditions and 0.230549 ± 0.004699 in the presence of cyclical stretch. Cyclical stretch resulted in an average increase in the 45Ca2+ rate constant by 0.125939 ± 0.003477 (P < 0.0001). The increase in 45Ca2+ efflux in response to cyclical stretch was measured in the presence of 1 μM thapsigargin, a Ca2+-ATPase inhibitor, which depletes the intracellular Ca2+ stores by inhibiting the active uptake of Ca2+ into the storage compartment (30). As shown in Fig. 1A, depletion of the intracellular stores by thapsigargin blocked the increase in 45Ca2+ efflux in response to cyclical stretch. This suggests that cyclical stretching of the intracellular Ca2+ stores results in a release of Ca2+ from a thapsigargin-sensitive store. Figure 1B depicts the efflux of 45Ca2+ as counts per minute in response to cyclical stretch and 6 μM IP3 from stores that have similar amounts of loading of 45Ca2+ into the stores. Both cyclical stretch and IP3 resulted in an increase in 45Ca2+ efflux. As shown in Fig. 2, an increase in the degree of cyclical stretch resulted in a progressive increase in the 45Ca2+ efflux.

Effect of blockade of IP3 channels. As shown in Fig. 3A, the 45Ca2+ rate constant increased by 0.21 ± 0.032 in response to IP3. When 45Ca2+ loaded cells were incubated with 50 μg/ml heparin, an inhibitor of IP3 channels, the 45Ca2+ rate constant decreased by 0.0272 ± 0.0099 in response to IP3. Thus the 45Ca2+ efflux in response to IP3 was completely blocked in the presence of heparin. As shown in Fig. 3B, heparin did not affect 45Ca2+ efflux.
We have shown previously that there is no increase in 45Ca2+ efflux in response to progressive increase in degree of cyclical stretch. This indicates that the release of Ca2+ from the intracellular stores, which in turn results in the increase in 45Ca2+ efflux in response to cyclical stretch. Application of 10 μM MgCl2, 10 μM BaCl2, 10 μM NiCl2, 10 μM CoCl2, and 10 μM MnCl2 did not affect the increase in 45Ca2+ efflux in response to cyclical stretch (data not shown).

Effect of actin filament and microtubule disruption. As shown in Fig. 6, A and B, control cells showed a dense array of actin filaments and a fine mesh of microtubules. As shown in Fig. 6, C and D, 10 μM cytochalasin D produced disruption of actin filaments and 10 μM nocodazole produced disruption of microtubules.

As shown in Fig. 7A, the change in 45Ca2+ rate constant in response to cyclical stretch was 0.13 ± 0.0148 in control cells and −0.0397 ± 0.003451 in the presence of 10 μM cytochalasin D (P < 0.0001). Thus, the disruption of actin filaments by 1-h preincubation with 10 μM cytochalasin D completely blocked the increase in 45Ca2+ efflux in response to cyclical stretch. However, as shown in Fig. 7B, the change in 45Ca2+ rate constant in response to cyclical stretch was 0.1376 ± 0.0104 in control cells and 0.121 ± 0.0134 in the presence of 10 μM nocodazole (P = 0.3654). The disruption of microtubules by 1-h preincubation with 10 μM nocodazole had no significant effect on the increase in 45Ca2+ efflux in response to cyclical stretch. This indicates that the actin microfilaments but not the microtubules play a role in the transmission of mechanical stimuli at the surface of the cells to the intracellular stores, which in turn results in the release of Ca2+ from the intracellular stores.

DISCUSSION

These data demonstrate that cyclical stretch results in a release of Ca2+ from the intracellular Ca2+ stores.
from a novel channel that is distinct from the IP$_3$, ryanodine, and NAADP channels. Our studies show that cyclical stretch results in an increase in $^{45}$Ca$^{2+}$ efflux from a thapsigargin-sensitive store that is proportional to the degree of cyclical stretch. There is an increase in $^{45}$Ca$^{2+}$ efflux in response to cyclical stretch despite the presence of inhibitors of the IP$_3$, ryanodine, and NAADP channels. The increase in $^{45}$Ca$^{2+}$ efflux in response to cyclical stretch is inhibited by 10 $\mu$M lanthanum and 10 $\mu$M gadolinium. Disruption of the actin filaments, but not disruption of microtubules, inhibits the increase in $^{45}$Ca$^{2+}$ efflux in response to cyclical stretch. Thus mechanical stimulation produced by cyclical stretch is transmitted to the intracellular stores via the actin microfilaments. This in turn results in a release of Ca$^{2+}$ from the intracellular stores via an as yet unidentified novel channel that is distinct from the IP$_3$, ryanodine, and NAADP channels.

These results are in agreement with previous reports that hypotonic swelling, another widely used model of mechanical stimulation, also results in the release of Ca$^{2+}$ from the intracellular stores in VSMC via a novel channel that is distinct from the IP$_3$, ryanodine, and NAADP channels (15, 16). However, mechanical stimulation produced by hypotonic swelling and direct stretch may not be entirely equivalent. In the hypotonic swelling model of mechanical stimulation, exposure to hypotonic solution is associated with exposure to a change in ion concentration and a change in osmolality. Therefore, in this model of mechanical stimulation, nonspecific effects of changes in ion concentration and osmolality on the release of Ca$^{2+}$ from the intracellular stores cannot be ruled out. The use of a cyclical stretch of cells grown on flexible membrane as a model of mechanical stimulus eliminates these confounding factors. Moreover, hypotonic swelling causes stretching of plasma membrane due to forces from within the cells, whereas direct stretch applies external force on the cells. Also, hypotonic swelling and direct stretch activate different ion channels in the plasma membrane. Hypotonic swelling predominantly activates Cl$^{-}$ channels (8, 34). On the other hand, stretching predominantly causes opening of nonselective, Gd$^{3+}$-sensitive cation channels (4, 19, 25). Therefore, the fact that two different models of mechanical stimulation, i.e., hypotonic swelling and cyclical stretch, result in a release of Ca$^{2+}$ from the intracellular stores independent of the IP$_3$, ryanodine, and NAADP channels further strengthens the evidence for a mechanosensitive Ca$^{2+}$ store.

Mechanical stimulation produced by hypotonic swelling also results in a release of Ca$^{2+}$ from the intracellular Ca$^{2+}$ stores independent of the IP$_3$, ryanodine-, and NAADP-sensitive stores in bovine aortic endothelial cells (10). Niggel et al. (18) showed that mechanical stimulation of C6 glioma cells by magnetic
forces resulted in a release of Ca²⁺ from the intracellular Ca²⁺ stores independent of the IP₃ and ryanodine channels. These data along with our findings strongly support the existence of a mechanosensitive Ca²⁺ store.

Because our data demonstrate that there is an increase in Ca²⁺ release from the intracellular stores in response to cyclical stretch and that this release is not inhibited by inhibitors of IP₃, ryanodine, and NAADP channels, it is possible that there is an as yet unidentified mediator of Ca²⁺ release that is released in response to cyclical stretch. It is also possible that cyclical stretch brings about a conformational change in the IP₃, ryanodine, and NAADP channels, rendering the pharmacological inhibitors of these channels ineffective. Therefore, Ca²⁺ release may still occur via the known channels of Ca²⁺ release in response to cyclical stretch but not be inhibited by their pharmacological inhibitors. It is also possible that the cytoskeletal network, which is linked to both the plasma membrane and the sarcoplasmic reticulum, the physiologically important Ca²⁺ store, transmits mechanical force from the surface of the cells to the intracellular Ca²⁺ stores without a chemical mediator and results in a direct release of Ca²⁺ from the intracellular stores. This is supported by our finding that the disruption of actin filaments by cytochalasin D inhibits the release of Ca²⁺ in response to cyclical stretch. The role of the actin cytoskeleton in the release of Ca²⁺ needs to be further studied. These findings are in contrast to previous findings that the release of Ca²⁺ from the intracellular stores in response to mechanical stimulation produced by hypotonic swelling is not inhibited by inhibitors of microfilaments or microtubules (Ref. 15 and unpublished observations). This apparent conflict may be explained by the fact that exposure of cells to a
hypotonic solution results in an influx of water into the cells, leading to dilution of the cytoplasm and thus exposure of the intracellular stores directly to a hypotonic environment. Exposure of intracellular stores to a hypotonic environment in turn leads to swelling of the intracellular stores (10). Thus, in the hypotonic swelling model of mechanical stimulation, the mechanical stimulation at the surface of the cells can be transmitted to the intracellular stores independent of the cytoskeleton.

Our data show that a 2-min incubation with 10 μM lanthanum inhibits the release of 45Ca2+ in response to cyclical stretch. Lanthanum and gadolinium did not inhibit the release of Ca2+ in response to mechanical stimulation produced by hypotonic swelling in VSMC (unpublished observations). However, longer preincubation (14 min) with higher concentrations of lanthanum (1 mM) and gadolinium (1 mM) did inhibit the release of Ca2+ in response to mechanical stimulation produced by hypotonic swelling in bovine aortic endothelial cells by about 75 and 57%, respectively (10). Because the intracellular stores are known to swell in response to hypotonic solution, it is possible that this osmotic perturbation results in an alteration of the configuration of the mechanosensitive ion channel in such a manner that it can no longer bind to lanthanum or gadolinium, and, thus, the release of Ca2+ in response to hypotonic swelling may be less sensitive to inhibition by lanthanum or gadolinium.

It has been shown that Ca2+ signaling mechanisms in A7r5 cells, a rat embryonic smooth muscle cell line, show marked similarity to those in VSMC from intact blood vessels. A7r5 cells have been extensively used to study Ca2+ signaling mechanisms and therefore serve as excellent models to study VSMC Ca2+ homeostasis (1, 14, 15, 16, 32). Therefore, our studies have been performed in A7r5 cells.

To produce cyclical stretching, we applied cyclical stretch to VSMC grown on flexible-bottomed culture plates via the Flexercell strain unit. This technique allows the application of reproducible and variable duration cyclical stretch on adherent cells. Also, because VSMC in vivo are subjected to pulsatile blood flow, this technique allows us to study the response to stretch of cells in vitro in a manner analogous to that experienced in vivo

The study to effect cyclical stretch of the intracellular Ca2+ stores, saponin-permeabilized cells were used. Saponin selectively permeabilizes the plasma membrane and not the membranes of the intracellular organelles, so when saponin-permeabilized cells loaded with 45Ca are subjected to cyclical stretch, we measure the release of Ca2+ from the intracellular Ca2+ stores (10). Because IP3 is not cell permeant, our data showing that IP3 results in a release of 45Ca2+ indicate that the A7r5 cells were indeed permeabilized. We have also shown previously that IP3 added to cells that are not treated with saponin does not result in an increase in 45Ca2+ efflux (16).

The cells are permeabilized, and hence the bathing fluid mimics the intracellular milieu. Therefore, the buffers used in these experiments have high KCl concentration. Because the plasma membranes of the cells are permeabilized, the voltage-sensitive Ca2+ channels in the plasma membrane are not activated in the presence of the high KCl concentration.

In summary, our studies show that mechanical stimulation of VSMC produced by cyclical stretch causes a release of Ca2+ via a novel channel independent of the IP3, ryanodine, and NAADP channels. Because VSMC are subjected to various mechanical stimuli such as shear stress and pulsatile stretch in vivo, this increase in [Ca2+], in response to mechanical stimulation may play a physiologically important role.

This research was supported by the funds from the Merit Review Entry Program of the Department of Veterans Affairs. Part of this work has been published in abstract form.

REFERENCES