Hypotonic swelling-induced \( \text{Ca}^{2+} \) release by an IP\(_3\)-insensitive \( \text{Ca}^{2+} \) store

Mohanty, Madhumita Jena, Maian Ye, Xingli Li, and Noreen F. Rossi. Hypotonic swelling-induced \( \text{Ca}^{2+} \) release by an IP\(_3\)-insensitive \( \text{Ca}^{2+} \) store. Am J Physiol Cell Physiol 281: C555–C562, 2001.—Hypotonic swelling increases the intracellular \( \text{Ca}^{2+} \) concentration \([\text{Ca}^{2+}]_i\) in vascular smooth muscle cells (VSMC). The source of this \( \text{Ca}^{2+} \) is not clear. To study the source of increase in \([\text{Ca}^{2+}]_i\), in response to hypotonic swelling, we measured \([\text{Ca}^{2+}]_i\) in fura 2-loaded cultured VSMC (A7r5 cells). Hypotonic swelling produced a 40.7-nM increase in \([\text{Ca}^{2+}]_i\), that was not inhibited by EGTA but was inhibited by 1 \( \mu \text{M} \) thapsigargin. Prior depletion of inositol 1,4,5-trisphosphate (IP\(_3\))-sensitive \( \text{Ca}^{2+} \) stores with vasopressin did not inhibit the increase in \([\text{Ca}^{2+}]_i\), in response to hypotonic swelling. Exposure of \( ^{45}\text{Ca}^{2+} \)-loaded intracellular stores to hypotonic swelling in permeabilized VSMC produced an increase in \(^{45}\text{Ca}^{2+} \) efflux, which was inhibited by 1 \( \mu \text{M} \) thapsigargin but not by 50 \( \mu \text{g}\text{/ml} \) heparin, 50 \( \mu \text{M} \) ruthenium red, or 25 \( \mu \text{M} \) thio-NADP. Thus hypotonic swelling of VSMC causes a release of \( \text{Ca}^{2+} \) from the intracellular stores from a novel site distinct from the IP\(_3\)-, ryanodine-, and nicotinic acid adenine dinucleotide phosphate-sensitive stores.

Intracellular calcium; vascular smooth muscle cells; inositol 1,4,5-trisphosphate; ryanodine

Various mechanical stimuli are known to cause an increase in \([\text{Ca}^{2+}]_i\) (7, 30, 35). It is commonly believed that the increase in \([\text{Ca}^{2+}]_i\) in response to mechanical stimulation is due to the influx of \( \text{Ca}^{2+} \) through stretch-activated channels in the plasma membrane (14, 33, 34). This belief is based on studies demonstrating that removal of extracellular \( \text{Ca}^{2+} \) inhibits the increase in \([\text{Ca}^{2+}]_i\) induced by mechanical stimulation. However, removal of extracellular \( \text{Ca}^{2+} \) promotes \( \text{Ca}^{2+} \) efflux and depletes the intracellular \( \text{Ca}^{2+} \) stores. Therefore, release of \( \text{Ca}^{2+} \) from the intracellular stores in response to mechanical stimulation is not definitively ruled out by these studies. Thus the possibility remains that the increase in \([\text{Ca}^{2+}]_i\), in response to mechanical stimulation in VSMC may result from the release of \( \text{Ca}^{2+} \) from the intracellular stores.

In bovine aortic endothelial cells, the increase in \([\text{Ca}^{2+}]_i\) in response to mechanical stimulation produced by hypotonic swelling is not due to the opening of \( \text{Ca}^{2+} \) channels in the plasma membrane but is due to the release of \( \text{Ca}^{2+} \) from the intracellular stores independent of the IP\(_3\) and ryanodine channels (16). Therefore, it is possible that the increase in \([\text{Ca}^{2+}]_i\) induced by hypotonic swelling in VSMC may also result from the release of \( \text{Ca}^{2+} \) from the intracellular stores independent of the known channels for \( \text{Ca}^{2+} \) release. The purpose of this study is to elucidate the mechanism(s) by which hypotonic swelling increases \([\text{Ca}^{2+}]_i\) in VSMC.

Hypotonic swelling is a convenient, reproducible, reversible, and widely used model of mechanical stimulation; therefore, we studied cultured A7r5 cells, a VSMC line, and used hypotonic swelling as a model of mechanical stimulation (10, 15, 16, 23, 24, 27).

Materials and Methods

Materials. A7r5 cells were obtained from American Type Culture Collection (Rockville, MD). Fura 2 was obtained from Molecular Probes (Eugene, OR). A-23187 was obtained from Calbiochem (San Diego, CA). All other chemicals were purchased from Sigma (St. Louis, MO).

Culture of A7r5 cells. A7r5 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum.

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serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 0.9% nonessential amino acids. Cells were subcultured every 5 to 7 days and used for experiments 5 to 7 days after plating.

Measurement of \([Ca^{2+}]_i\). Confluent A7r5 cells were washed with isotonic solution containing 130 mM NaCl, 5.36 mM KCl, 26 mM HEPES, 0.8 mM MgSO₄, 1.8 mM CaCl₂, 1 mM Na₂HPO₄, 10 mM glucose, pH 7.4, and 300 mosmol/kgH₂O and briefly treated with 0.25% trypsin and 0.53 mM EDTA to bring them into suspension. Cells were then washed to remove the trypsin and loaded with fura 2 by incubation in isotonic solution containing 2 μM fura 2-AM, 0.02% Pluronic F-127, and 0.1% bovine serum albumin for 2 h at room temperature. The cells were washed and reincubated in isotonic solution at room temperature for 30 min to allow hydrolysis of internalized fura 2-AM. The cells were then washed and kept in isotonic solution. \([Ca^{2+}]_i\) measurements were made in isotonic solution at room temperature with constant stirring to maintain homogeneity of the suspension. Fluorescence was measured with a spectrofluorimeter (SPEXFLUOROLOG II) at 510 nm with excitation wavelengths of 340 and 380 nm. The spectrofluorimeter has built-in software (DM3000) that converts fluorescence to \([Ca^{2+}]_i\) values using the following formula

\[
[Ca^{2+}]_i = K_d (R - R_{\min}) / (R_{\max} - R)(S_2/S_1)
\]

where \(K_d\) is the dissociation constant for fura 2, \(R\) is the ratio of the fluorescence at excitation wavelengths of 340 and 380 nm, \(R_{\max}\) is the ratio obtained when the cells are permeabilized with 0.1% Triton (high-Ca²⁺ condition), and \(R_{\min}\) is the ratio obtained when 60 mM EGTA was added to the cells to bind to Ca²⁺ (low-Ca²⁺ conditions). \(S_2\) and \(S_1\) are the values of fluorescence at an excitation wavelength of 380 nm with high-Ca²⁺ conditions and low-Ca²⁺ conditions, respectively (15). The fura 2-loaded cells were placed in isotonic solution until a stable baseline was obtained. A diluent lacking NaCl that contained 5.6 mM KCl, 0.8 mM MgSO₄, 1.8 mM CaCl₂, 10 mM d-glucose, and 67 mosmol/kg H₂O, pH 7.4, was then added to the isotonic solution in a 2:1.5 ratio to make the medium hypotonic (200 mosmol/kg H₂O). In all experiments involving \([Ca^{2+}]_i\), measurements of the osmolality of hypotonic solution was 200 mosmol/kg H₂O unless specified otherwise.

Measurement of \(45\text{Ca}^{2+}\) efflux from the intracellular \(Ca^{2+}\) stores in permeabilized cells. Confluent monolayers of cells grown on 12-well plates (Falcon, Lincoln Park, NJ) were permeabilized by incubating them with 25 μM saponin, 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 \(45\text{Ca}^{2+}\), 3 mM ATP (to facilitate uptake of \(45\text{Ca}^{2+}\) into the intracellular \(Ca^{2+}\) stores), and 1 μM ruthenium red (to prevent loading of \(45\text{Ca}^{2+}\) into the mitochondria) were added for 10 min. No CaCl₂ was added to the \(45\text{Ca}^{2+}\) or saponin solutions to facilitate the selective uptake of the radioactive \(45\text{Ca}^{2+}\) into the intracellular \(Ca^{2+}\) stores. The cells were then washed and incubated in isotonic solution that contained 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/kg H₂O to mimic the intracellular milieu. This was collected and replaced with an equal volume of isotonic solution at 30-s intervals. In studies involving hypotonic swelling, cells were incubated with hypotonic solution that contained 35 mM KCl, 25 mM NaCl, 2 mM MgCl₂, 0.2 mM CaCl₂, 1 mM EGTA, 10 mM HEPES at pH 6.9, 150 mM free Ca²⁺, and 145 mosmol/kg H₂O. The assay was terminated by adding 5 μM A-23187, a Ca²⁺ ionophore, to release the remaining \(45\text{Ca}^{2+}\) in the intracellular \(Ca^{2+}\) stores. Results are expressed as rate constant (fraction of remaining \(45\text{Ca}^{2+}\) released/30 s) (16).

Statistics. Differences between groups of data were determined using a 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 hypotonic swelling on \([Ca^{2+}]_i\). \([Ca^{2+}]_i\) increased from 62.8 nM in isotonic solution to 106.3 nM when the cells were placed in hypotonic solution (Fig. 1A; \(P < 0.0001, n = 18\)). The increase in \([Ca^{2+}]_i\) occurred immediately and reached a peak within 15 s. There was no change in \([Ca^{2+}]_i\) when the cells were left stirring in isotonic solution in a time-controlled experiment. As the cells were placed in solutions of progressively decreasing tonicity (253, 222, and 200 mosmol/kg H₂O), there was a progressive increase in the magnitude of increase in \([Ca^{2+}]_i\) (Fig. 1B). The increase in \([Ca^{2+}]_i\) in hypotonic solution reverted to baseline when the cells were put back in isotonic solution (Fig. 1C). Figure 1D depicts a time-controlled experiment that demonstrates that the increase in \([Ca^{2+}]_i\) is sustained when the cells are incubated in isotonic solution and then left in hypotonic solution.

These experiments indicate that the increase in \([Ca^{2+}]_i\) in response to hypotonic swelling is reversible and proportional to the degree of hypertonicity and that the response to hypotonic swelling is not due to irreversible cell damage.

Response to blocking of \(Ca^{2+}\) influx. To study the role of influx of \(Ca^{2+}\) from the extracellular medium, the increase in \([Ca^{2+}]_i\) in response to hypotonic solution was studied in the presence of 5.4 mM EGTA. Depletion of extracellular \(Ca^{2+}\) promotes efflux of \(Ca^{2+}\) and gradually depletes the intracellular \(Ca^{2+}\) stores. Hence, in this study, measurement of \([Ca^{2+}]_i\) was done 5 s after EGTA was added so that \([Ca^{2+}]_i\) was measured before the intracellular \(Ca^{2+}\) stores were depleted.

To verify that EGTA inhibits influx of \(Ca^{2+}\), the increase in \([Ca^{2+}]_i\) in response to vasopressin was observed. In the absence of EGTA, there was a biphasic increase in \([Ca^{2+}]_i\) in response to vasopressin, consisting of an initial rapid peak (that is known to be due to the release of \(Ca^{2+}\) from the extracellular \(Ca^{2+}\) stores (peak phase)) followed by a smaller sustained release of \(Ca^{2+}\) (that is known to be due to the influx of extracellular \(Ca^{2+}\) (plateau phase)) (38). The plateau phase of response to vasopressin was significantly inhibited after a 5-s preincubation with 5.4 mM EGTA, consistent with EGTA blockade of \(Ca^{2+}\) influx (126.7 ± 12.07 nM in the absence of EGTA and 30.76 ± 4.674 nM in the presence of EGTA) (Fig. 2A).

Figure 2B depicts the increase in \([Ca^{2+}]_i\) when the cells are incubated first in isotonic solution and then in hypotonic solution in the absence and presence of a 5-s preincubation with 5.4 mM EGTA. There was no inhibition of the increase in \([Ca^{2+}]_i\) in response to hypotonic swelling in the presence of EGTA.
These data indicate that the influx of extracellular Ca\(^{2+}\) cannot account for the increase in [Ca\(^{2+}\)]\(_i\) in response to hypotonic swelling.

Effect of depletion of intracellular Ca\(^{2+}\) stores. The increase in [Ca\(^{2+}\)]\(_i\) in response to hypotonic solution was studied in the presence of 1 \(\mu\)M thapsigargin, a Ca\(^{2+}\)-ATPase inhibitor that depletes the intracellular Ca\(^{2+}\) stores by inhibiting the active uptake of Ca\(^{2+}\) into the storage compartments (36). Depletion of intracellular Ca\(^{2+}\) stores causes secondary influx of Ca\(^{2+}\) from the extracellular medium. To block this secondary influx of Ca\(^{2+}\), 5.4 mM EGTA was added to thapsigargin-treated cells before the addition of hypotonic solution or vasopressin.

Figure 3A depicts the change in [Ca\(^{2+}\)]\(_i\) when the cells are first incubated in isotonic solution and then in 10 nM vasopressin in the absence and presence of a 30-min preincubation with 1 \(\mu\)M thapsigargin. The increase in [Ca\(^{2+}\)]\(_i\) in response to vasopressin was completely inhibited by thapsigargin. This indicates that thapsigargin indeed depletes intracellular Ca\(^{2+}\) stores in these cells.

Figure 3B depicts the change in [Ca\(^{2+}\)]\(_i\) when the cells are incubated first in isotonic solution and then in hypotonic solution in the absence and presence of 1 \(\mu\)M thapsigargin. The increase in [Ca\(^{2+}\)]\(_i\) in response to hypotonic swelling was significantly inhibited in thapsigargin-treated cells \((P < 0.0001)\). This indicates that the increase in [Ca\(^{2+}\)]\(_i\) in response to hypotonic swelling is largely due to the release of Ca\(^{2+}\) from a thapsigargin-sensitive pool of the intracellular Ca\(^{2+}\) stores. The incomplete inhibition was not due to influx of extracellular Ca\(^{2+}\) since these experiments were performed in the pres-
This increase in response to hypotonic swelling is indicative that vasopressin-sensitive (IP3) stores were depleted at that time (Fig. 4A). When the cells were stimulated first with vasopressin and then 30 s later with hypotonic solution, when vasopressin-sensitive (IP3) stores were still depleted, [Ca2+]i increased by 50.2 ± 7.11 nM in response to hypotonic solution (Fig. 4B). This increase in response to hypotonic swelling is similar in magnitude to the response to hypotonic solution in the absence of prior stimulation with vasopressin. This suggests the presence of two different stores: one sensitive to IP3 and the other to hypotonic swelling.

Effect of direct swelling of the intracellular Ca2+ stores. To study whether direct swelling of the intracellular Ca2+ stores causes a release of Ca2+, 45Ca2+ efflux from the intracellular Ca2+ stores was measured in saponin-permeabilized A7r5 cells. As shown in Fig. 5A, 45Ca2+ release was measured at 30-s intervals in isotonic solution and then in hypotonic solution. The 45Ca2+ rate constant increased by 0.115 ± 0.009 in hypotonic solution. This suggests that direct swelling of the intracellular Ca2+ stores with hypotonic solution causes a release of Ca2+.

As shown in Fig. 5B, 45Ca2+ rate constant increased by 0.305 ± 0.025 in 6 μM IP3 solution. IP3 causes a release of Ca2+ via IP3-sensitive channels of the intracellular Ca2+ stores. Because IP3 is not cell membrane permeant, the release of 45Ca2+ in response to IP3 indicates that the A7r5 cells were indeed permeabilized. When IP3 was added to cells not treated with saponin, the change of 45Ca2+ rate constant was 0.011 ± 0.0071. This indicated that IP3 added extracellularly had no effect.

When the intracellular Ca2+ stores were depleted with thapsigargin, there was no increase in 45Ca2+ efflux in response to IP3, and the increase in 45Ca2+ efflux in response to hypotonic solution was blocked (Fig. 5, A and B).

Effect of blockade of IP3, ryanodine, and NAADP channels on 45Ca2+ efflux in response to hypotonic stimuli. When 45Ca2+-loaded cells were exposed to 50 μg/ml of heparin, an inhibitor of IP3 channels, the change in 45Ca2+ rate constant in response to IP3 was 0.305 ± 0.025 in the absence and -0.0185 ± 0.0221 in the presence of heparin. Thus the 45Ca2+ efflux in response to IP3 was completely blocked in the presence of heparin. However, there was no inhibition of the response to hypotonic solution in the presence of heparin (Fig. 6).

When 45Ca2+-loaded cells were exposed to either 10 μM ryanodine or 1 μM cADP-ribose, the 45Ca2+ rate constant decreased by 0.020 ± 0.0025 and 0.0097 ± 0.0082, respectively. Thus there was no increase in 45Ca2+ efflux in response to ryanodine and cADP-ribose. This is consistent with previously reported results that the A7r5 cells do not express ryanodine receptors. Furthermore, 50 μM ruthenium red, an inhibitor of the ryanodine channel, did not block the response to hypotonic solution (Fig. 6).
In the presence of 25 μM thio-NADP, an inhibitor of NAADP-sensitive channels, there was no inhibition of 45Ca2+ efflux in response to hypotonic solution (Fig. 6). This indicates that the release of 45Ca2+ in response to hypotonic solution was from the intracellular Ca2+ stores and was independent of the IP3, ryanodine, and NAADP channels.

Change in ion concentration. The composition of the isotonic solution differs from that of the hypotonic solution only in that the concentration of KCl is 125 mM in isotonic solution and 35 mM in hypotonic solution. To investigate whether the release of 45Ca2+ in response to hypotonic solution is due to the swelling of the intracellular Ca2+ stores or to the exposure of the cells to a lower concentration of KCl, permeabilized cells were placed in standard isotonic solution and then in an isotonic solution that contained 35 mM KCl and 160 mM sucrose, with all other constituents remaining unchanged.

When the permeabilized cells were placed in isotonic solution and then in an isotonic solution that contained 35 mM KCl and 160 mM sucrose, there was no further increase in the rate of 45Ca2+ efflux (Fig. 7). This indicates that the increase in 45Ca2+ efflux in response to hypotonic solution is not due to a change in the concentration of KCl.

DISCUSSION

These data demonstrate that hypotonic swelling results in a release of Ca2+ from the intracellular Ca2+ stores from a novel site independent of the IP3-, ryanodine-, and NAADP-sensitive stores. Our studies show that hypotonic swelling results in an increase in [Ca2+]i that is reversible, reproducible, and proportional to the degree of hypotonicity. This increase in [Ca2+]i was not blocked by inhibiting the influx of Ca2+ but was inhibited by depletion of the intracellular Ca2+ stores. Depletion of the IP3-sensitive stores did not affect the release of [Ca2+]i in response to hypotonic solution, indicating that the increase in [Ca2+]i was independent of the IP3-sensitive stores. There was an increase in 45Ca2+ efflux in response to hypotonic swelling despite the presence of inhibitors of the IP3, ryanodine, and NAADP channels. This further supports the existence of a yet unidentified novel channel that leads to a release of Ca2+ from the intracellular Ca2+ stores in response to mechanical stimulation produced by hypotonic swelling.

These results differ from previously reported results that show that the release of Ca2+ in response to mechanical stimulation is either due to influx of Ca2+ or due to both influx and release of Ca2+ from the intracellular Ca2+ stores (7, 35). In these studies, cells were preincubated for 10–60 min with EGTA. However, this duration of incubation with EGTA promotes...
Ca$^{2+}$ efflux and depletes the intracellular Ca$^{2+}$ stores as well. Therefore, these studies do not definitively exclude that the release of Ca$^{2+}$ in response to mechanical stimulation is from the intracellular Ca$^{2+}$ stores. In our study, measurement of Ca$^{2+}$ in response to hypotonic swelling or vasopressin was done 5 s after EGTA was added so that the [Ca$^{2+}$]$i$ was measured when Ca$^{2+}$ influx was inhibited but before the intracellular Ca$^{2+}$ stores were depleted.

Our studies indicate that hypotonic swelling causes a 44-nM increase in [Ca$^{2+}$]$i$. It has been shown that rises in [Ca$^{2+}$]$i$, of this magnitude increase the myogenic tone (19, 22). Thus hypotonic swelling can produce physiologically significant increases in [Ca$^{2+}$]$i$.

The magnitude of increase in [Ca$^{2+}$]$i$ in response to hypotonic swelling may appear less than that which is produced by agonists such as vasopressin. However, in our studies we have used supraphysiological levels of vasopressin (10 nM vasopressin). This was done to deplete the vasopressin-sensitive stores (Fig. 4, A and B). The response to more physiological levels of vasopressin (1.5–6 pmol) is closer to that in response to hypotonic swelling (3). Also, response to other vasoagonists in the physiological concentration range is 40 nM. Hence, the increase in [Ca$^{2+}$]$i$, of 44 nM in response to hypotonic swelling may be physiologically significant.

Because our data demonstrate that the increase in [Ca$^{2+}$]$i$ in response to hypotonic swelling is due to a release from intracellular Ca$^{2+}$ stores, which is distinct from the IP$_3$-, ryanodine-, and NAADP-sensitive stores, it is possible that there is a yet unidentified mediator of Ca$^{2+}$ release that is released in response to hypotonic swelling.

Conversely, it is possible that no messenger is needed for the release of Ca$^{2+}$ from the intracellular Ca$^{2+}$ stores in response to hypotonic swelling. For example, when cells swell in response to hypotonic swelling, it leads to stretching of the plasma membrane. It is possible that the cytoskeletal network, which is linked to both the plasma membrane and the sarcoplasmic reticulum, the physiologically important Ca$^{2+}$ store, transmits mechanical force from the surface of the cells to the intracellular Ca$^{2+}$ stores without a chemical mediator and results in a direct release of Ca$^{2+}$ (11, 20, 37). The role of the cytoskeleton in release of Ca$^{2+}$ needs to be further elucidated.

Furthermore, during hypotonic swelling, influx of water dilutes the cytoplasm and thus the intracellular Ca$^{2+}$ stores are directly exposed to decreased toxicity of the cytoplasm (24). It has been shown that the endoplasmic reticulum, the physiologically important intracellular Ca$^{2+}$ reservoir, exhibits hypotonic swelling (4). Therefore, it is possible that direct exposure of the intracellular Ca$^{2+}$ stores to hypotonicity causes swelling of the intracellular Ca$^{2+}$ stores, resulting in release of Ca$^{2+}$ from the intracellular Ca$^{2+}$ stores. Hence, in the hypotonic model of mechanical stimulation, the cytoskeletal involvement may not be required for the release of Ca$^{2+}$ from the intracellular Ca$^{2+}$ stores. However, during mechanical stimulation produced by direct stretch, the cytoskeleton may play a role in the transmission of mechanical force from the surface of the cells to the intracellular Ca$^{2+}$ stores.

To study the direct effect of swelling of the intracellular Ca$^{2+}$ stores, saponin-permeabilized cells were studied. Saponin selectively permeabilizes the plasma membrane and not the membranes of the intracellular organelles so that when saponin-permeabilized cells are incubated in hypotonic medium, osmotic forces are eliminated across the plasma membrane but not in the membranes limiting the intracellular Ca$^{2+}$ stores (16). Hence, when saponin-permeabilized cells are incubated in hypotonic medium, it causes direct swelling of the intracellular Ca$^{2+}$ stores.

The cells are permeabilized and hence the bathing fluid mimics the intracellular milieu. Therefore, the buffers used in these experiments have a high KCl concentration. Because the plasma membranes of the cells are permeabilized, the voltage-sensitive Ca$^{2+}$ channels in the plasma membrane are not activated in the presence of the high KCl concentration.

The studies in permeabilized cells demonstrate that the release of $^{45}$Ca$^{2+}$ in response to hypotonic swelling is not from the IP$_3$-, ryanodine-, and NAADP-sensitive channels and is independent of the change in ion concentration in the medium. However, with our experimental design, we cannot rule out a direct independent effect of lower osmolality rather than swelling of the intracellular Ca$^{2+}$ stores as an underlying cause for $^{45}$Ca$^{2+}$ release.

Various methods have been devised to produce mechanical stimulation in vitro including hypotonic swelling, stretching of Silastic membranes, extrusion of buffer from a pipette, magnetic forces, flow-induced shear stress, and application of suction with a pipette. These various mechanical stimuli are not physiologically equivalent. For instance, hypotonic swelling causes stretching of the 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 (12, 39). However, hypotonic swelling also activates Ca$^{2+}$-activated K$^+$ channels in rabbit coronary VSMC and stretch-activated cation channels in brain capillaries (21, 29). On the other hand, direct stretch predominantly causes opening of nonselective, Gd$^{3+}$-sensitive cation channels (6, 26, 32). However, application of direct stretch also activates Ca$^{2+}$-activated K$^+$ channels in mesenteric and pulmonary artery smooth muscle cells and Cl$^-$ channels in isolated human arterial myocytes (8, 18, 31). Although mechanical stimulation produced by hypotonic swelling and direct stretch may not be entirely equivalent, hypotonic swelling is a widely used, convenient, and reproducible model of mechanical stimulation (10, 15, 16, 23, 24, 27). Therefore, we chose to use hypotonic swelling as a model of mechanical stimulation.

Our studies in permeabilized cells are in agreement with those reported by Missiaen et al. (24), which demonstrate that the increase in $^{45}$Ca$^{2+}$ efflux in re-
sponse to hypotonic solution is independent of IP₃ and ryanodine channels in permeabilized A7r5 cells. In a previous study, it was shown that in bovine aortic endothelial cells, hypotonic swelling causes a release of Ca²⁺ from the intracellular Ca²⁺ stores independent of the IP₃-, ryanodine-, and NAADP-sensitive stores (16). Niggel et al. (25) showed that mechanical stimulation of C6 glioma cells by magnetic forces caused a release of Ca²⁺ from the intracellular Ca²⁺ stores independent of the IP₃ and ryanodine channels. This further strengthens the evidence for a mechanosensitive Ca²⁺ store.

In summary, our studies show that mechanical stimulation produced by hypotonic swelling causes a release of Ca²⁺ from the intracellular Ca²⁺ stores from a novel site independent of the IP₃-, ryanodine-, and NAADP-sensitive stores. 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 (5).

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