Hypotonically Induced Calcium Release from Intracellular Calcium Stores

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Osmotic cell swelling induced by hypotonic stress is associated with a rise in intracellular Ca\(^{2+}\) concentration, which is at least partly due to a release of Ca\(^{2+}\) from internal stores. Since osmotic influx of water dilutes the cytoplasmic milieu, we have investigated how nonmitochondrial Ca\(^{2+}\) stores in permeabilized A7r5 cells respond to a reduction in cytoplasmic tonicity. We now present experimental evidence for a direct Ca\(^{2+}\) release from the stores when exposed to a hypotonic medium. The release is graded, but does not occur through the inositol trisphosphate or the ryanodine receptor. Ca\(^{2+}\) seems to be released through the passive leak pathway, and this phenomenon can be partially inhibited by divalent cations in the following order of potency: Ni\(^{2+}\) > Co\(^{2+}\) > Mn\(^{2+}\) > Mg\(^{2+}\) > Ba\(^{2+}\). This release also occurs in intact A7r5 cells. This novel mechanism of hypotonically induced Ca\(^{2+}\) release is therefore an inherent property of the stores, which can occur in the absence of second messengers. Intracellular stores can therefore act as osmosensors.

Most cells exposed to anisosmotic solutions activate volume regulatory processes to prevent damage by cell swelling or shrinkage (1–3). Osmotic cell swelling in response to hypotonic stress is associated with a rise in intracellular [Ca\(^{2+}\)], which is at least partly due to a release of Ca\(^{2+}\) from internal stores (2–13). The link between changes in external osmotic concentration and internal Ca\(^{2+}\) release is not known: inositol trisphosphate (3) and arachidonate (5) may be involved, but it is also possible that no messenger is needed (2). Since the influx of water dilutes the cytoplasm, we have investigated how the Ca\(^{2+}\) stores in permeabilized A7r5 cells respond to a reduction in cytoplasmic tonicity. We now present experimental evidence for a direct Ca\(^{2+}\) release from the nonmitochondrial stores when exposed to a hypotonic medium. This hypotonically induced Ca\(^{2+}\) release through the passive leak pathway is an inherent property of the stores, which can occur in the absence of second messengers.

MATERIALS AND METHODS

A7r5 cells, an established cell line derived from embryonic rat aorta, were used between the 7th and the 18th passage after receipt from the American Type Culture Collection (Bethesda, MD) and subcultured weekly by trypsinization. The cells were cultured at 37 °C in a 9% CO\(_2\) incubator in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 3.8 mM l-glutamine, 0.9% (w/v) nonessential amino acids, 85 IU ml\(^{-1}\) penicillin, and 85 μg ml\(^{-1}\) streptomycin. The cells were seeded for the 46Ca\(^{2+}\) fluxes in 12-well dishes (Costar, 4 cm\(^2\)) at a density of approximately 104 cells cm\(^{-2}\) and for the intracellular [Ca\(^{2+}\)] measurements in Coverglass Chambers (Nunc Inc., Naperville, IL) at a density of 5 × 105 cells cm\(^{-2}\).

46Ca\(^{2+}\) fluxes on monolayers of saponin-permeabilized A7r5 cells (3 × 106 cells/4-cm2 well) at 25 °C were done as described (14). The stores were loaded for 40 min in 120 mM KCl, 30 mM imidazole (pH 6.8), 5 mM MgCl\(_2\), 5 mM ATP, 0.44 mM EGTA, 10 mM Na\(_2\)Glc, and 150 mM free Ca\(^{2+}\) (50 μCi ml\(^{-1}\)). The cells were then washed twice in an isosmotic efflux medium containing 60 mM KCl, 120 mM mannitol, 30 mM imidazole (pH 6.8), 1 mM EGTA, and 2 μM thapsigargin (measured osmolality of 300 mosm/kg H\(_2\)O). 1 ml of medium was then added at time 0 and replaced every 6 s or every 2 min. Osmolality changes were induced by changing the mannitol concentration to prevent changes in ionic concentration and ionic strength.

For the 46Ca\(^{2+}\) fluxes on monolayers of intact A7r5 cells (3 × 106 cells/4-cm2 well), the cells were loaded for 60 min in a modified Krebs solution containing 135 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl\(_2\), 11.6 mM Hepes (pH 7.3), 11.5 mM glucose, and 1.5 mM CaCl\(_2\) (50 μCi ml\(^{-1}\)) at 25 °C. The efflux was performed in a medium containing 65 mM NaCl, 120 mM mannitol, 5.9 mM KCl, 1.2 mM MgCl\(_2\), 11.6 mM Hepes (pH 7.3), 11.5 mM glucose, and 2 mM EGTA. The hypotonic shock was induced by removing the mannitol.

Single-cell intracellular [Ca\(^{2+}\)] measurements were performed using a laser-scanning MRC-1000 system (Bio-Rad, Hertfordshire, UK) attached to an inverted Nikon Diaphot 300 epifluorescence microscope with a CF Fluor 40 × (numerical aperture = 1.3) oil immersion objective. The cells were incubated for 30 min with 5 μM Indo-1/AM dissolved in 50% ethanol and then further incubated for 1 h in the absence of Indo-1. During the experiment at 25 °C, the cells were continuously superfused from a pipette placed on top of the cell. The solutions were the same as for the 46Ca\(^{2+}\) fluxes in intact cells.

RESULTS AND DISCUSSION

Permeabilized A7r5 cells loaded to equilibrium with 45Ca\(^{2+}\) slowly lost their 45Ca\(^{2+}\) during incubation in an isosmotic (300 mosm/kg H\(_2\)O) Ca\(^{2+}\)-free medium. A long-lasting reduction in medium osmolality to 180 mosm/kg H\(_2\)O transiently increased the rate of Ca\(^{2+}\) release (Fig. 1A). This effect occurred despite the reduced concentration gradient for Ca\(^{2+}\) across the store membrane as a result of the decreased luminal [Ca\(^{2+}\)] by osmotic H\(_2\)O influx. This 40% reduction in tonicity is of the same order as used experimentally in intact cells (range 30–50%, Refs. 4–12). The release could be elicited again after reloading the stores with 46Ca\(^{2+}\) (data not shown). Increasing the osmolality to 420 mosm/kg H\(_2\)O had no effect but the returning to isosmotic solution again resulted in a transient, although less pronounced, Ca\(^{2+}\) release (Fig. 1B).

Ca\(^{2+}\) release through the inositol trisphosphate (InsP\(_3\)) receptor (15) and ryanodine receptor (16, 17) is graded, i.e. continuous submaximal stimulation is unable to completely empty the entire Ca\(^{2+}\) pool. The hypotonically induced Ca\(^{2+}\) release is also graded: a long-lasting moderate decrease in tonicity released less Ca\(^{2+}\) than a more pronounced decrease (Fig. 2A).
Graded responses allow increment detection (18). Increment detection for the hypotonically induced Ca\textsuperscript{2+} release is shown in Fig. 2B: decreasing the osmolality stepwise resulted in concomitant phases of Ca\textsuperscript{2+} release. The hypotonically induced Ca\textsuperscript{2+} release (up to 60% of the total store Ca\textsuperscript{2+} content in Fig. 2) originated from the InsP\textsubscript{3}-sensitive store, since the InsP\textsubscript{3}-insensitive compartment in permeabilized A7r5 cells contains only about 5% of the stored Ca\textsuperscript{2+} (14). Activation of the InsP\textsubscript{3} receptor by endogenous InsP\textsubscript{3} formation was, however, not involved. First, a hypotonic challenge after 20 min (less filled stores) released more Ca\textsuperscript{2+} and therefore resulted in a lower Ca\textsuperscript{2+} content at 30 min than a challenge at 0 min (full stores, Fig. 3A). This larger Ca\textsuperscript{2+} release from less filled stores is in contrast with the less complete InsP\textsubscript{3}-induced Ca\textsuperscript{2+} release from partially depleted stores (14, 19–21). A second argument against the involvement of the InsP\textsubscript{3} receptor is that 10\textmu M thimerosal, a pharmacological activator of InsP\textsubscript{3} receptors (22–26), did not stimulate the hypotonically induced Ca\textsuperscript{2+} release (Fig. 3B). A third argument indicating that the InsP\textsubscript{3} receptor was not involved is that the release was unaffected by the presence of 50\mu g ml\textsuperscript{-1} heparin (14, 19–21), added from time 0 onward. Typical for 4 experiments.

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**FIG. 1.** Effect of anisosmotic solutions on the rate of Ca\textsuperscript{2+} release from permeabilized A7r5 cells. A illustrates the effect of lowering the medium osmolality by leaving out 120 \textmu M mannitol (filled bar). B shows the effect of a short-lasting increase in mannitol concentration from 120 to 240 \textmu M (hatched bar). Typical for 4 experiments.

**FIG. 2.** Hypotonically induced Ca\textsuperscript{2+} release is a graded process. The permeabilized cells were first incubated in efflux medium containing 120 \textmu M mannitol, and the Ca\textsuperscript{2+} content of the stores was followed as a function of time. In A, the mannitol concentration was reduced to 80 \textmu M (●), 40 \textmu M (▲), or 0 \textmu M (□) after 14 min. In B, the mannitol concentration was reduced to 80 \textmu M after 14 min and to 0 \textmu M after 28 min. The dashed lines are the control efflux curves in isosmotic medium. Typical for 4 experiments.

**FIG. 3.** Hypotonically induced Ca\textsuperscript{2+} release in permeabilized A7r5 cells does not occur through the InsP\textsubscript{3} or ryanodine receptor. A shows how the Ca\textsuperscript{2+} content decreased during incubation in isosmotic efflux medium and how this Ca\textsuperscript{2+} content was affected by removing all mannitol for 2 min, either at time 0 (●) or after 20 min (○). B illustrates the effect of removing the mannitol in the absence (●) and in the presence of 10\mu M thimerosal (▲) or 10\mu M ruthenium red (○), added from time 0 onward. The inset shows the effect of removing the mannitol in the absence (●) and in the presence of 50\mu g ml\textsuperscript{-1} heparin (○), added from time 0 onward. Typical for 4 experiments.
release (27–29) (data not shown).

Ryanodine receptors were not involved in the release because 10 μM ruthenium red was without effect (Fig. 3B) and because less Ca2+ was released from filled stores (Fig. 3A), while ryanodine receptors are stimulated by luminal Ca2+ (30). Ca2+ was not released via Ca2+ pumps, because the presence or absence of 2 μM thapsigargin, a blocker of pump-mediated Ca2+ release (31), did not affect the hypotonically induced Ca2+ release. Phospholipase A2 and C blockers (10 μM 4-bromophenacyl bromide, 10 μM manoaide) had no effect (data not shown), indicating that endogenous production of arachidonic acid or InsP3 was not involved. Also, modulators of microfilaments or microtubules (10 μM phalloidin, 50 μM cytochalasin B, 50 μM taxol, 10 μM demecolcine) had no effect (data not shown).

Divalent cations (2 mM) inhibited the hypotonically induced Ca2+ release with the following order of potency: Ni2+ > Mn2+ > Mg2+ > Ba2+ (Fig. 4A). The inhibition by 2 mM Mg2+ (Fig. 4B) and the other cations (data not shown) was more effective at moderate decreases in tonicity. These ions also decreased the passive InsP3-independent Ca2+ leak with the same order of potency (Fig. 4A, inset). We therefore propose that Ca2+ was released through this passive leak pathway. The overall inhibition by these cations was relatively small and actually became even smaller when the [Ni2+] was increased from 2 to 10 mM (Fig. 4B, inset).

The hypotonically induced Ca2+ release also occurred in intact A7r5 cells incubated in Ca2+-free medium. A 40% reduction in extracellular osmolality induced a transient increase in intracellular [Ca2+] in 60% of the cells investigated (closed symbols in Fig. 5A), this Ca2+ did not come from outside since the external medium contained no Ca2+. To discriminate whether this [Ca2+] increase represented a Ca2+ release from internal stores or an inhibited Ca2+ extrusion, we investigated the effect of hypotonic stress on the rate of 45Ca2+ release from intact A7r5 cells. Fig. 5B shows an enhanced rate of 45Ca2+ extrusion during the hypotonic shock, indicating that Ca2+ release from intracellular stores and not inhibition of the extrusion caused the [Ca2+] increase in the intact cell. 40% of the cells showed no rise in intracellular [Ca2+] in response to the hypotonic challenge (open circles in Fig. 5A), although a subsequent vasopressin stimulation (10 μM) could release internal Ca2+.

We conclude that hypotonically induced Ca2+ release through the passive leak pathway is an inherent property of the intracellular stores. Although this phenomenon is not mediated by classical Ca2+ channels such as InsP3 receptors and ryanodine receptors, it is possible that, in the intact cell, released Ca2+ subsequently activates phospholipase C or A2, thereby generating InsP3 or arachidonic acid and its metabolites. These second messengers may provide a positive feedback loop for the internal Ca2+ release or alternatively activate the necessary mechanisms for volume recovery.
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