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Mechanosensitive Calcium Entry and Mobilization in Renal A6 Cells

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Abstract. Using spectrofluorescence imaging of fura-2 loaded renal A6 cells, we have investigated the generation of the cytosolic Ca^{2+} signal in response to osmotic shock and localized membrane stretch. Upon hypotonic exposure, the cells began to swell prior to a transient increase in $[Ca^{2+}]_i$ and the cells remained swollen after $[Ca^{2+}]_i$ had returned towards basal levels. Exposure to 2/3rd strength Ringer produced a cell volume increase within 3 min, followed by a slow regulatory volume decrease (RVD). The hypotonic challenge also produced a transient increase in $[Ca^{2+}]$ after a delay of 22 sec. Both the RVD and $[Ca^{2+}]_i$ response to hypotonicity were inhibited in a Ca^{2+}-free bathing solution and by gadolinium (10 μM), an inhibitor of stretch-activated channels. Stretching the membrane by application of subatmospheric pressure (-2 kPa) inside a cell-attached patch-pipette induced a similar global increase in $[Ca^{2+}]_i$ as occurred after hypotonic shock. A stretch-sensitive $[Ca^{2+}]_i$ increase was also observed in a Ca^{2+}-free bathing solution, provided the patch-pipette contained Ca^{2+}. The mechanosensitive $[Ca^{2+}]_i$ response was by gadolinium (10 μM) or Ca^{2+}-free pipette solutions, even when Ca^{2+} (2 mM) was present in the bath. Long-term (>10 min) pretreatment of the cells with thapsigargin inhibited the $[Ca^{2+}]_i$ response to hypotonicity. These results provide evidence that cell swelling or mechanical stimulation can activate a powerful amplification system linked to intracellular Ca^{2+} release mechanisms.

Key words: Stretch-activated Ca^{2+} entry — Cell volume regulation — A6 cells — Ca^{2+} Fluorescence imaging — thapsigargin

Introduction

Animal cells initially swell in hypotonic media, but their volume is subsequently regulated by loss of KCl and water triggered by a myriad of signaling pathways (Lang et al., 1998). A dominant role is attributed to variations in the intracellular Ca^{2+} concentration in the activation of this regulatory volume decrease (Razani-Boroujerdi, Partridge & Sopori, 1994). An increase in cytosolic Ca^{2+} accompanies cell-swelling in different types of epithelial cells (Wong & Chase, 1986; Christensen, 1987; Wong, Debell & Chase, 1990; McCarty & O'Neil, 1992; Ehrenfeld, Raschi & Brochiero, 1994; Mooren & Kinne, 1994; Tinel Hanna, Wehner & Sauer, 1994). The generation of this cytosolic $[Ca^{2+}]$ response to hypotonic exposure involves extracellular Ca^{2+} entry and/or Ca^{2+} release from intracellular stores. A mechanosensitive $[Ca^{2+}]_i$ rise has been described in different cell types, including fibroblasts (Henkart & Nelson, 1979), endothelial cells (Naruse & Sokabe, 1993), human intestine (Okada, Hazama & Yan, 1990), mammary cells (Henkart & Nelson, 1979), airway epithelial cells (Sanderson, Charles & Dirkse, 1990) and renal A6 cells (Kawahara & Matsuzaki, 1992).

In the present study, using spectrofluorescence imaging of Fura-2 loaded A6 cells, a distal renal cell line from *Xenopus laevis*, the source of the intracellular Ca^{2+} signal and its mechanism of control in response to osmotic shock were investigated and compared to the Ca^{2+} signal produced by a localized membrane stretch. We observed that the increase in cytosolic $[Ca^{2+}]$ associated with a cell swelling is due to a Ca^{2+} entry via a stretch-activated channel and subsequent release from thapsigargin-sensitive stores. Importantly we show that activation of single stretch-activated calcium channels is sufficient to trigger an amplified $Ca^{2+}{}_i$ signal. Thus $Ca^{2+}{}_i$ signaling operates with high gain in response to cell swelling and membrane stress.

Materials and Methods

A6 Cell Culture

A6 cells, a cell line derived from the *Xenopus laevis* nephron were purchased from American Type Culture Collection (Rockville, MD).

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The cells were grown at 28°C on plastic tissue culture flasks (Corning, NY) in a modified culture medium for amphibian cells (NCTC 135, Gibco, NY) containing 10% fetal calf serum (Gibco), 0.6% penicillin and 1.0% streptomycin (Gibco), and gassed with 5% CO_2. After the cells had formed a polarized confluent monolayer, they were dispersed by exposure to 0.25% trypsin (Trypsin-EDTA, Gibco) for 5 min at 37°C. Following centrifugation, the cell pellet was resuspended in culture medium and subcultured (> million of cells per flask) or resuspended in a normal Ringer solution for the experiments.

Intracellular Ca^{2+} Measurements

Isolated A6 cells were loaded with the acetoxy-methyl ester of fura-2 (5 μM) dissolved in DMSO, for 30 min at room temperature. The final DMSO concentration was < 0.1% in Ringer solution. The dye-loaded cells were washed in standard Ringer solution and deposited on a glass coverslip, mounted in a miniperfusion chamber and placed on the stage of a Nikon epifluorescent microscope (Diaphot TMD). The light from a Xenon lamp was filtered through alternating 340 nm and 380 nm filters. The emitted fluorescence produced after the excitation of fura-2 was filtered at 510 nm and detected with a video camera (Darkstar, Photonics Sciences, UK). The video images were digitized, recorded and analyzed using an image analysis program (STARWISE FLUO system, IMSTAR Paris, France). The fluorescence obtained at each excitation wavelength (I_{340} and I_{380}) depends upon the level of Ca^{2+} binding to fura-2. Intracellular Ca^{2+} concentration was measured from the fluorescence ratio I_{340}/I_{380}. Calibration of the dye was performed using a range of EGTA-buffered Ca^{2+} solutions of the fura-2 free acid using the equation of Grynkeiwicz et al. (1985):

$$[Ca^{2+}]_i = K'(R - R_{min})/(R_{max} - R),$$

where K' is the product of the dissociation constant of the Ca^{2+} fura-2 complex and a constant related to the optical characteristics of the system. R is the experimental ratio of I_{340}/I_{380} from which the background fluorescence has been subtracted, and R_{min} and R_{max} are the values of R in the presence of zero and saturating calcium respectively. K', R_{min} and R_{max} were obtained using fura-2 free acid in a range of EGTA-buffered Ca^{2+} solutions.

The intracellular Ca^{2+} changes were determined under two different experimental procedures designed to mechanically stress the cell membrane. Localized membrane stretch was produced by application of suction inside a patch-pipette after formation of a giga-ohm seal on an isolated A6 cell. Cell swelling was produced by exposure of isolated cells to a 2/3rd strength hypotonic Ringer solution superfused through a micropipette positioned within 10 μm of the cell. In other experiments, to avoid possible shear stress effects, a slow exchange of the entire bath solution was made. These two methods of producing an osmotic shock produced changes in intracellular Ca^{2+} of similar time course and magnitude.

Single-Cell Volume Determination

Isolated A6 cells were seeded on Petri dishes in 1 ml normal Ringer solution, and placed on the inverted microscope stage (Nikon Diaphot TMD). Video images of cells recorded with a camera (WAT 902, WATEC, Japan) connected to a video frame grabber (model LG3, Scion Corporation). Images were recorded in real time, stored and analyzed on a Macintosh computer using the public domain NIH Image program (v1.57ppc), developed at the U.S. National Institutes of Health and available from the Internet by anonymous ftp from zippy.nimh.nih.gov or on floppy disk from the National Technical Information Service, Springfield, Virginia, part number PB95-500195GEI. Assuming a spherical shape of isolated cells, the relative volume change (V/V_0) was calculated from the cross-sectional surface area before (S_0) and during swelling (S) from the relation: $V/V_o = (S/S_0)^{3/2}$ (Ross & Cahalan, 1995). The A6 cells were exposed to a hypotonic solution by perfusion (10 ml/min) of 2/3rd strength Ringer (extracellular Ca^{2+} concentration was maintained constant at 2 mM).

Cell volume changes were also detected by analyzing the intensity of fura-2 fluorescence signals. The two excitation wavelengths of fura-2 permit a ratiometric determination of cytosolic $[Ca^{2+}]$ independent of the dye concentration. A rise in intracellular $[Ca^{2+}]$ produces an increase of the fluorescent intensity measured at 340 nm and a decrease at 380 nm. Thus a reduction of fluorescence at both 340 nm and 380 nm, without a change of the ratio value, indicates a dilution or a leakage of the dye, without a change in $[Ca^{2+}]$. Since this phenomenon was observed upon hypotonic exposure of the cells, we conclude that the decrease of dye concentration corresponds to a dilution of the dye, due to the cell swelling. Using this technique a simultaneous measurement of cell swelling and intracellular Ca^{2+} is possible.

Solutions

The standard amphibian Ringer solution had the following composition (in mM): 120 NaCl, 3.7 KOH, 6 HEPES, 2 $CaCl_2$, 1 $MgCl_2$ and pH 7.4. Osmolarity was adjusted to 250 mOsM by adding glucose. The hypotonic perfusion solution was made by diluting the standard Ringer with distilled water and $CaCl_2$ was added to maintain the Ca^{2+} concentration at 2 mM (2/3rd strength Ringer). The Ca-free Ringer solution contained (in mM): 120 NaCl, 3.7 KOH, 10 HEPES, 5 EGTA (250 mOsM), and pH adjusted at 7.4. Fura 2/AM was supplied by Molecular Probes Gadolinium (III) chloride hexahydrate was obtained from Aldrich Chemical. Thapsigargin was purchased from Sigma Chemical.

Data are given as mean ± SE, and n is the number of experiments. Statistical significance was determined from Student t-tests as indicated in the text.

Results

Effect of Hypotonicity on Cell Volume and Cytosolic Calcium

Exposure of A6 cells to 2/3rd strength Ringer solution produced a cell swelling and an increase in intracellular calcium concentration ($[Ca^{2+}]_i$). The time course of changes in cell volume and $[Ca^{2+}]_i$ were compared. The experiments were started 10 min after plating the isolated A6 cells on plastic Petri dishes. No significant changes in cell surface area were observed under control conditions when A6 cells were bathed in normal isotonic Ringer solution for extended periods of time. The initial diameter of isolated cells was 25 ± 0.5 μm and remained constant at 26 ± 0.5 μm ($n = 7$) measured after 30 min. However, within 3.4 ± 0.2 min of exposure to 2/3rd strength Ringer solution, cell volume increased by 43 ± 2% ($n = 19$), followed by a slow regulatory volume decrease (Fig. 1). Over the initial 15 min of hypotonic exposure, the cell volume had undergone a volume regulatory decrease of 19 ± 3% ($n = 19$) (Fig. 1).

Exposure to hypotonic shock also produced a tran-

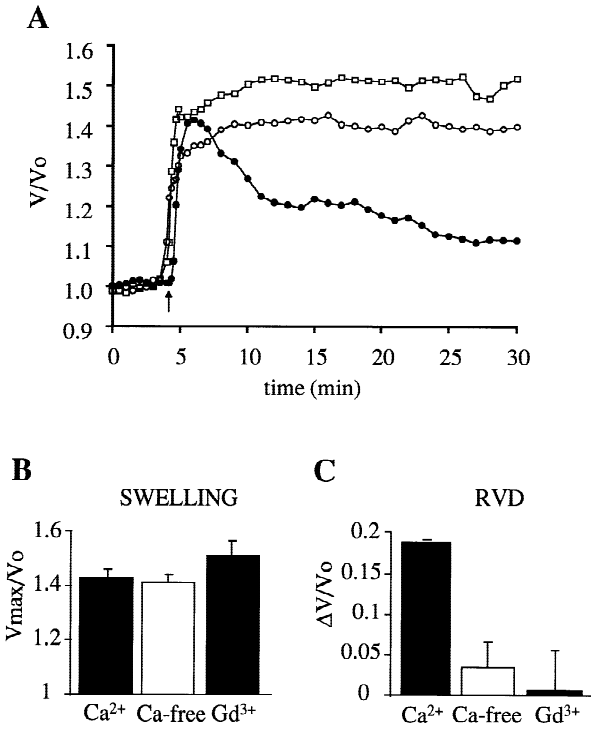

Fig. 1. Cell swelling and volume regulation during a hypotonic shock (2/3rd strength Ringer). The relative volume (V/V_o) was calculated from the initial volume, V_O, measured at the beginning of the experiment ($t = 0$) and the volume, V, measured at each subsequent time point. (*A*) Three typical experiments are shown for cells exposed to calcium-containing bathing solution (●), to Ca^{2+}-free solution (○), and to 10 mM gadolinium (□). The arrow indicates the start of hypotonic exposure of the cells ($t = 4$ min). (*B*) Comparison of the maximum relative volume increase (V_{max}) in the presence of calcium-containing hypotonic solution ($n = 19$), in a Ca-free bathing solution ($n = 10$) and after gadolinium treatment ($n = 14$). (*C*) Comparison of the RVD response in cells bathed in calcium-containing hypotonic solution ($n = 19$), Ca^{2+}-free bathing solution ($n = 10$) and gadolinium ($n = 19$). ΔV corresponds to the difference between V_{max} and V measured 15 min after the beginning of hypotonic exposure.

sient and large increase in $[Ca^{2+}]_i$ (Fig. 2). Comparison of cell volume and Ca^{2+} variations after a hypotonic shock showed that small volume changes, as low as 5%, can elicit the $[Ca^{2+}]_i$ rise (Fig. 2). However, the cell volume and $[Ca^{2+}]_i$ changes were dissociated in time. As shown in Fig. 2, the cells began to increase in volume prior to any increase in $[Ca^{2+}]_i$. Furthermore, during continued hypotonic exposure, the cells were still increasing in volume, whereas $[Ca^{2+}]_i$ had returned towards basal levels. The dissociation between cell swelling and Ca^{2+} transients is also apparent from the experiment shown in Fig. 3, where measurements of relative changes in cell volume and calcium were obtained simultaneously in the same cell. Two phases in the cellular responses were observed. An initial reduction of fluorescence intensity at both 340 nm and 380 nm, which corresponds to dilution of the dye due to the cell swelling, was observed (phase a). No changes in $[Ca^{2+}]_i$ were recorded during this time. After a delay of 22.5 ± 5 seconds (phase b), the fluorescence intensity increased at 340 nm and decreased at 380 nm, which translates to a $[Ca^{2+}]_i$ increase from 151 ± 4 nM to a peak value of 510 ± 21 nM ($n = 42$). This effect on $[Ca^{2+}]_i$ was observed in 94% of cells exposed to a hypotonic shock. The calcium response was transient and, after the peak, $[Ca^{2+}]_i$ levels decreased towards resting levels of 175 ± 5 nM, with a mean half-time of 40 ± 10 sec ($n = 11$). It is clear from these experiments that the increase in intracellular Ca^{2+} lags behind the initial cell swelling phase and the transient increase in $[Ca^{2+}]_i$ is over, while cell volume is still increasing.

Effects of External Ca^{2+} and Gadolinium on $[Ca^{2+}]_i$ During a Hypotonic Shock

The contribution of extracellular calcium to the rise in $[Ca^{2+}]_i$ after hypotonic exposure of A6 cells was investigated. The substitution of normal Ringer (2 mM Ca^{2+}) in the bath with a Ca-free-EGTA solution did not produce a detectable change in baseline $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ in control was 170 ± 14 nM, and was unchanged at 182 ± 14 nM measured 10 min after incubation in Ca-free-EGTA Ringer solution ($n = 27$, $P > 0.5$). However, after exposure to a hypotonic shock produced in the absence of external Ca^{2+}, (2/3rd strength Ca-free-EGTA Ringer solution), only 52% of cells responded with a blunted increase in $[Ca^{2+}]_i$. The remaining 48% of cells gave no calcium response. In responding cells exposed to calcium-free solutions, the mean absolute increase in $[Ca^{2+}]_i$ was reduced to 264 ± 29 nM ($n = 27$) compared to an increase of 428 ± 12 nM ($n = 31$, $P < 0.01$) observed in the same pool of cells exposed to extracellular calcium. These results show that Ca^{2+} entry through the plasma membrane is one of the components of the rise in intracellular Ca^{2+} during cell swelling (Fig. 4).

One possible route for Ca^{2+} entry during cell swelling is via a stretch-activated channel. We investigated the effect of pretreatment of A6 cells with gadolinium (Gd^{3+}), an inhibitor of stretch-activated channels, on the Ca^{2+} response to a hypotonic shock (Fig. 4). After Gd^{3+} (10 μM) treatment, only 58% of the cells responded to a hypotonic shock with calcium changes that were reduced and asynchronous, whereas 94% of cells responded in non-gadolinium-treated paired experiments on the same cell batches (Fig. 4). The mean increase of $[Ca^{2+}]_i$ produced by hypotonic exposure of responding cells was reduced to 228 ± 19 nM ($n = 7$) in the presence of Gd^{3+} compared to an increase of 330 ± 38 nM ($n = 39$) in control experiments ($P < 0.05$). These results support the involvement of volume-activated and Gd^{3+}-sensitive channels in Ca^{2+} entry during hypotonic exposure of the cells.

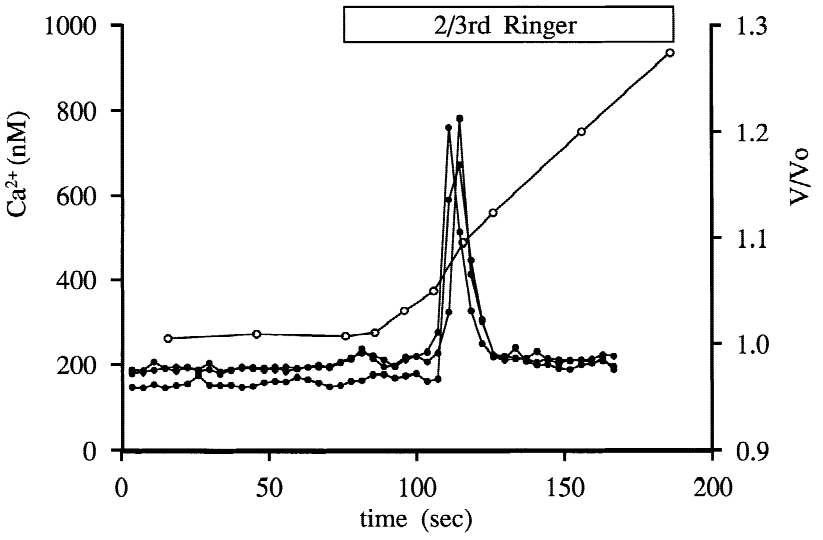

Fig. 2. Comparison of typical $[Ca^{2+}]_i$ (●) and cell volume (○) variations during the first minutes of exposure to a hypotonic solution (2/3rd Ringer). Note the recovery of intracellular $[Ca^{2+}]$ to resting levels before the end of the hypotonic exposure and before the maximum volume increase.

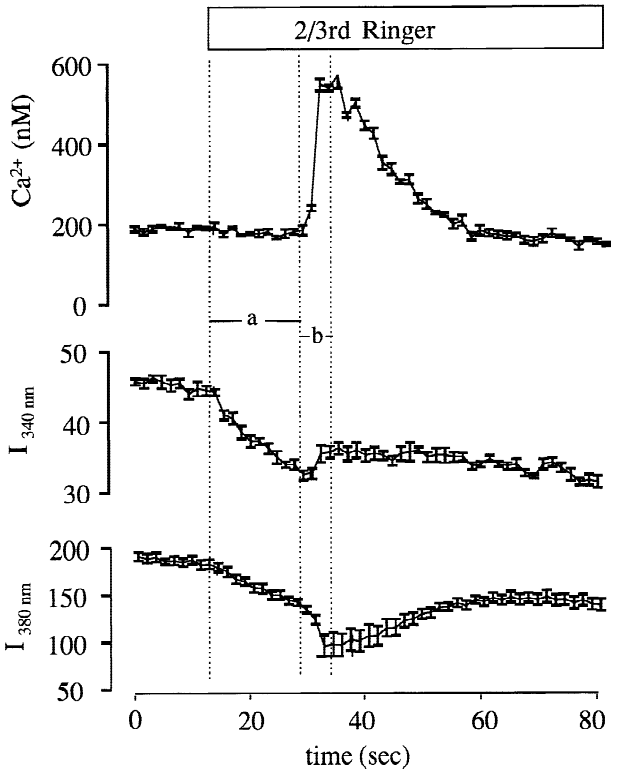

Fig. 3. Representative experiment obtained in isolated A6 cells showing $[Ca^{2+}]_i$ and fura-2 fluorescence intensity measured at 340 nm and 380 nm as a function of time, before and after exposure to hypotonic solution (2/3rd strength Ringer). The $[Ca^{2+}]_i$ rise was observed after a delay of 18 sec. Cell swelling had already occurred over this time period as indicated by the decrease in fluorescence intensity at the two excitation wave lengths.

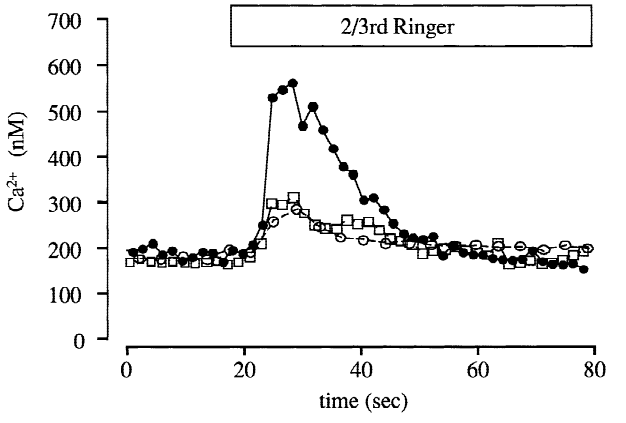

Fig. 4. Effects of external $[Ca^{2+}]_o$ and gadolinium (10 mM) on the $[Ca^{2+}]_i$ response to a hypotonic shock (2/3rd Ringer solution). The $[Ca^{2+}]_i$ response to a hypotonic shock is shown for cells bathed in calcium-containing solutions ($[Ca^{2+}]_o$ = 2 mM) (●), in calcium-free solution (○), and in solutions containing gadolinium and calcium (□).

Effects of External Ca^{2+} and Gadolinium on Cell Swelling and RVD

The effect of removal of external calcium or exposure to Gd^{3+} on the cell volume responses to hypotonic exposure of A6 cells was investigated. Removal of external Ca^{2+} from the bath solution did not significantly affect the time course of the swelling response or the maximal volume increase of cells exposed to a 2/3rd strength solution. In Ca-free-EGTA solution, the maximal volume increase, reached after 3.6 ± 0.4 min of hypotonic exposure of the cells, was 41 ± 2% (n = 10) (Fig. 1). The change in cell volume and the time taken to reach the peak volume increase are therefore similar to that found in control Ca^{2+} containing solutions.

Exposure of cells to Gd^{3+} (10 μM) did not affect the cell swelling response to hypotonic shock. A maximal volume increase of 51 ± 5% (n = 14) was reached after 4.0 ± 0.6 min of exposure to a hypotonic solution containing Gd^{3+} (Fig. 1).

We verified that a cell volume increase still occurred in cells in which the swelling-induced rise in $[Ca^{2+}]_i$ was

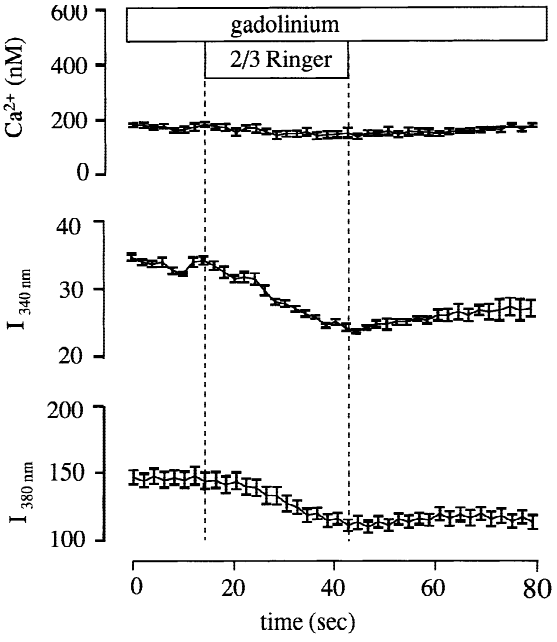

Fig. 5. Gadolinium (10 μM) effect on fura-2 fluorescence intensity at 340 and 380 nm in response to a hypotonic shock (2/3rd strength Ringer) with Ca^{2+} in the bathing solution in a cell where the $[Ca^{2+}]_i$ level did not change. The decrease of fluorescence intensity at 340 and 380 nm indicated the dilution of the dye due to the cell swelling.

completely abolished by Gd^{3+}. Fura-2 fluorescence intensity measurements at 340 nm and 380 nm show cell swelling occurring in the presence of Gd^{3+} although the $[Ca^{2+}]_i$ level remained constant throughout the period of osmotic shock (Fig. 5).

Removal of external Ca^{2+} or addition of Gd^{3+} (10 μM) in the bath solution inhibited regulatory volume decrease after cell swelling (Fig. 1). When cells were exposed to a Ca^{2+}-free solution or to Gd^{3+}, the volume measured 15 min after hypotonic exposure was not significantly reduced (calcium free: $\Delta V/V_0 = 3.5 \pm 3\%$, $n = 10$, $P > 0.5$ and Gd^{3+}: $\Delta V/V_0 = 0.6 \pm 0.5\%$, $n = 14$, $P > 0.5$).

These results confirm the primary role of calcium entry through Gd^{3+}-sensitive channels in generating the RVD response. An increase in $[Ca^{2+}]_i$ is necessary to initiate RVD but a maintained elevated $[Ca^{2+}]_i$ is not required for restoration of cell volume once RVD is initiated.

Effects of Localized Membrane Stretch on $[Ca^{2+}]_i$

The role of stretch-activated Ca^{2+} channels in the calcium response, was examined by testing the effect of localized stretch of the membrane on $[Ca^{2+}]_i$. We show that suction inside a cell-attached patch-pipette causes a transient increase in intracellular calcium (Fig. 6*A*). The data are summarized in Table 1. Intracellular $[Ca^{2+}]$ increased 3-fold within 1 sec of application of membrane stretch and subsequently decreased within 30 sec to a plateau value 2-fold higher (296 ± 16 nM, $n = 10$) than basal while the stretch was maintained. The calcium response occurred instantaneously with the onset of membrane stretch and was transient despite the maintenance of stretch (Fig. 6*A*). In the first seconds of membrane stretch, the increase in $[Ca^{2+}]_i$ was localized under the patch pipette, before spreading rapidly to more distant regions within the cell (Fig. 6*B*).

Effects of External Ca^{2+} and Gadolinium on the Stretch-activated Ca^{2+} Response

The role of external Ca^{2+} in the mechanosensitive calcium response was investigated using Ca^{2+}-free Ringer solution in the patch pipette. The increase in $[Ca^{2+}]_i$ normally elicited by membrane stretch was not observed when the patch pipette contained a Ca^{2+}-free Ringer solution (cells bathed in standard Ringer solution) (Fig. 7, Table 1). Thus, the stretch-induced increase in intracellular $[Ca^{2+}]$ was absolutely dependent upon the presence of Ca^{2+} in the patch-pipette solution.

These results suggest the involvement of stretch-activated channels in the calcium response to membrane deformation. In addition, a highly localized Ca^{2+} entry through mechanosensitive channels appears sufficient to trigger a global cytosolic increase in Ca^{2+} following mechanical stretching of the membrane patch. As shown in Fig. 7 and summarized in Table 1, a twofold increase in $[Ca^{2+}]_i$ over basal could be produced by stretching a small area of cell membrane even when the bath solution did not contain Ca^{2+}, provided Ca^{2+} was present in the patch pipette.

The role of the stretch-activated channel in this response was further investigated using Gd^{3+}. The effect of localized membrane stretch to produce an increased $[Ca^{2+}]_i$ was completely abolished by adding Gd^{3+} (10 μM) to the patch-pipette solution (Fig. 7, Table 1).

This results indicate that calcium entry through stretch-activated, calcium permeable and Gd^{3+} -sensitive channels triggers a powerful amplification system to produce a global cytosolic change in $[Ca^{2+}]_i$.

Thapsigargin Effect on $[Ca^{2+}]_i$

Our finding, that exposure to a calcium-free solution or to gadolinium did not completely abolish the $[Ca^{2+}]_i$ response to hypotonic shock, indicates that extracellular Ca^{2+} is not the unique source for the increased $[Ca^{2+}]_i$ following cell swelling. Intracellular Ca^{2+} stores must also be implicated in generating the volume and mechanosensitive $Ca^{2+}{}_i$ signal. To test this hypothesis, we investigated the effect of cell swelling on $[Ca^{2+}]_i$ in A6 cells after treatment with thapsigargin, an inhibitor of the Ca-ATPase pump in endoplasmic reticulum. Addition of

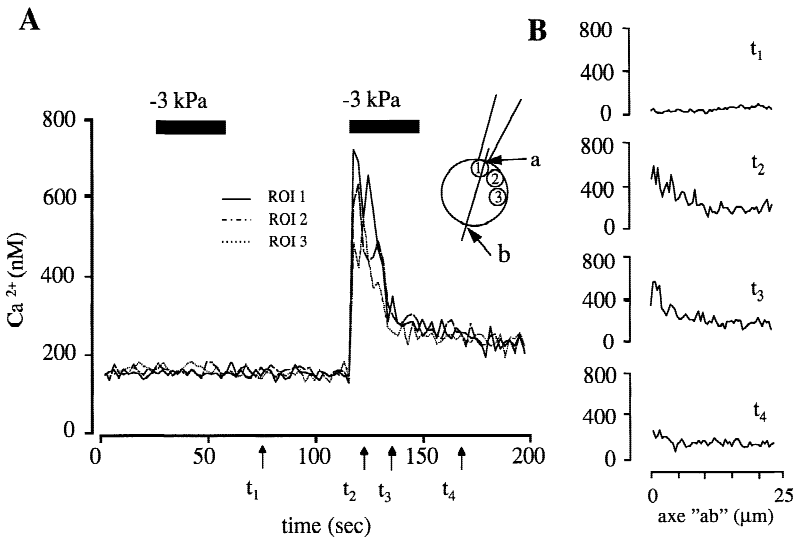

Fig. 6. Effect of application of subatmospheric pressure (black bars, -3kPa) inside a cell-attached patch pipette on $[Ca^{2+}]_i$. (*A*) $[Ca^{2+}]_i$ measured in 3 regions of interest (ROI) in the same cell in response to pipette suction. The cell-attached seal was made at the first pressure pulse (first pressure bar) and did not produce a change in $[Ca^{2+}]_i$. However, after achieving the giga-ohm seal, a subsequent pressure pulse (second pressure bar) caused a rapid transient rise in $[Ca^{2+}]_i$ in all 3 regions of interest. (*B*) $Ca^{2+}{}_i$ imaged along the axis 'a-b' at times t_1 to t_4 in the same cell as in (*A*).

Table 1. Effects of external Ca^{2+} and Gd^{3+} on the intracellular Ca^{2+} response to membrane stretch

Conditions Pipette/bath	(a) Basal	(b) Stretch	(c) $\Delta[Ca^{2+}]_i$	*n*
2 Ca^{2+}/2 Ca^{2+}	147 ± 12	581 ± 32	434 ± 44	10
Ca^{2+} free/2 Ca^{2+}	171 ± 21	174 ± 25	3 ± 46	20
2 Ca^{2+}/Ca^{2+} free	263 ± 32	543 ± 28	280 ± 60	20
2 Ca^{2+} + Gd^{3+}/2 Ca^{2}	176 ± 16	172 ± 15	−4 ± 31	31

The presence of Ca^{2+} (mM) or Gd^{3+} (10 μM) in pipette and bath solutions are shown. For each experimental condition, the basal $[Ca^{2+}]_i$ (a), the maximum $[Ca^{2+}]_i$ reached during applicationof localized stretch (b) and the mean $[Ca^{2+}]_i$ change (c) are given in nM.

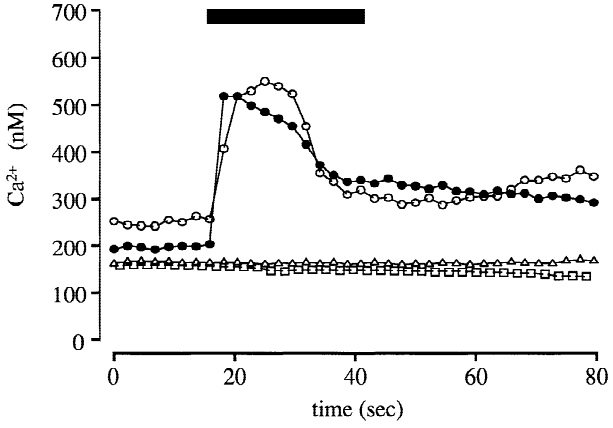

Fig. 7. Effect of calcium and gadolinium on $[Ca^{2+}]_i$ changes induced by localized stretch of the membrane using a patch pipette. A subatmospheric pressure (-3 kPa) was applied inside a cell-attached patch pipette filled with the same normal Ringer solution as the bath (●), with normal Ringer in the pipette, and Ca-free Ringer in the bathing solution (○), with a Ca-free solution in the pipette and normal Ringer in bath (▲), and with normal Ringer containing gadolinium in the pipette and normal Ringer in bath (✻).

thapsigargin (100 nM) to the bath solution produced a significant two-fold increase in $[Ca^{2+}]_i$ over basal (Fig. 8, Table 2). Thapsigargin treatment reduced the increase in $[Ca^{2+}]_i$ produced by a hypotonic shock (Fig. 8*A*). The degree of inhibition of the calcium response depended on the duration of the thapsigargin treatment prior to a hypotonic challenge. Figure 8*B* and Table 2 show the effects of hypotonic shock on $[Ca^{2+}]_i$ at various times following thapsigargin. A hypotonic exposure after 5 min treatment with thapsigargin, produced a normal transient increase in $[Ca^{2+}]_i$ which was not significantly different from non-thapsigargin treated cells.

After a 10-min exposure to thapsigargin, the effect of a hypotonic shock to produce an increase in $[Ca^{2+}]_i$ was significantly reduced by 3.3-fold compared to control. Following 20 min of thapsigargin treatment, a hypotonic shock failed to produce any significant variation in intracellular calcium.

These experiments highlight the importance of calcium mobilization from thapsigargin-sensitive stores in generating the calcium signal in response to cell swelling. Furthermore, since extracellular calcium was shown to be necessary to initiate this response, these results, taken together, indicate the involvement of a calcium entry-induced calcium release mechanism (CICR).

Exposure to a hypotonic solution, after 5-to-10 min thapsigargin treatment, produced an initial transient $[Ca^{2+}]_i$ increase (which was blunted after 10 min thapsigargin), followed by a rapid decrease in $[Ca^{2+}]_i$ towards basal levels (Fig. 8 and Table 2). With longer exposures to thapsigargin (20 min), a hypotonic shock produced a decrease in $[Ca^{2+}]_i$ without any observable initial increase in $[Ca^{2+}]_i$ (Table 2).

The hypotonic-induced fall in $[Ca^{2+}]_i$ may be due to Ca^{2+} efflux across the plasma membrane or reuptake into thapsigargin-insensitive stores. This result suggests that cell swelling not only mobilizes intracellular Ca^{2+} but also stimulates mechanisms to terminate the Ca^{2+} signal.

Table 2. Effect of the duration of thapsigargin treatment on the $[Ca^{2+}]_i$ response to a hypotonic challenge (2/3rd dilution)

(a) Basal	(b) Thapsigargin	(c) Hypotonic	(d) $\Delta[Ca^{2+}]_i$	(e) Return	*n*
199 ± 6	359 ± 7 (5 min)	697 ± 14	339 ± 21 (*)	159 ± 8	23
185 ± 1	338 ± 7 (10 min)	447 ± 33	108 ± 40 (**)	242 ± 12	20
164 ± 11	431 ± 5 (20 min)	435 ± 6	4 ± 11 (**)	161 ± 10	35

Intracellular $[Ca^{2+}]$ (nM) was measured following three different durations (5, 10, 20 min) of thapsigargin treatment prior to hypotonic exposure. The basal $[Ca^{2+}]_i$ and the plateau value of $[Ca^{2+}]_i$ reached after thapsigargin treatment are shown in columns (a) and (b), respectively. The maximum $[Ca^{2+}]_i$ level and the mean $[Ca^{2+}]_i$ variation, obtained in response to hypotonic exposure (after thapsigargin) are shown in columns (c) and (d), respectively. Prolonged hypotonic exposure produced a fall in intracellular calcium and the $[Ca^{2+}]_i$ levels measured 5 min after the beginning of hypotonic exposure are given in column (e).
Statisitical significance (*) $P > 0.5$ and (**) $P < 0.05$ in comparison to $\Delta[Ca^{2+}]_i = 359 \pm 25$ nM ($n = 42$) obtained without thapsigargin.

Discussion

In this study we have measured intracellular Ca^{2+} variations after hypotonic shock or localized stretch of the plasma membrane in renal A6 cells. The hypotonic shock produced an increase in cell volume consistent with previous reports of osmotic effects in A6 cells (Ehrenfeld et al., 1994; De Smet et al., 1995). The simultaneous measurements of cell volume and $[Ca^{2+}]_i$ also showed a significant delay between the onset of cell swelling and changes in $[Ca^{2+}]_i$. Comparison of volume and Ca^{2+} changes after a hypotonic shock showed that the $[Ca^{2+}]_i$ increase was initiated after a 5% increase in cell volume. The calcium response was rapid and transient (duration 30 sec), compared to the volume increase which attained a maximum value only 2 min after exposure to hypotonic shock. Intracellular Ca^{2+} had returned to a basal level before volume regulation (RVD) started. Despite the apparent temporal uncoupling of Ca^{2+} and volume changes, our data show that intracellular calcium plays a central role in the control of volume regulation of A6 cells. The regulatory volume decrease of A6 cells was less effective in calcium-free-EGTA solution or with gadolinium in the external bath, conditions which inhibited the normal calcium response to a hypotonic shock.

The mechanisms involved in the rise of $Ca^{2+}{}_i$ after hypotonic exposure are complex. In our experiments on A6 cells, the changes to intracellular Ca^{2+} produced by a hypotonic shock or membrane stretch are consistent with the rise reported in osmotically swollen toad urinary bladder (Wong & Chase, 1986), proximal tubule (Beck et al., 1991; McCarty & O'Neil, 1991) or A6 cells (Kawahara & Matsuzaki, 1992; Ehrenfeld et al., 1994). The reduced $Ca^{2+}{}_i$ rise following hypotonic shock in the absence of extracellular Ca^{2+} and the inhibition of the osmotic-induced Ca^{2+} response by thapsigargin, demonstrate that the rise in $[Ca^{2+}]_i$ during cell swelling is dependent both on plasma-membrane entry of Ca^{2+} and release of calcium from intracellular stores.

Previous studies in the proximal tubule (McCarty & O'Neil, 1991) have demonstrated an important contribution of Ca^{2+} release from intracellular stores in the calcium response after a hypotonic shock, and this conclusion is supported by our experiments. Repetitive membrane stretches never produced the same large $[Ca^{2+}]_i$ response (*data not shown*) indicating that Ca^{2+} is mobilized from a limited store. Furthermore, long-term treatment with thapsigargin, which causes emptying of intracellular Ca^{2+} stores, produced an inhibition of the $[Ca^{2+}]_i$ increase in response to hypo-osmotic shock

External Ca^{2+} was necessary to produce a maximum increase in $[Ca^{2+}]_i$ after membrane stretch or cell-swelling and also to activate regulatory volume decrease. These observations are consistent with the dependence of the intracellular Ca^{2+} signal on extracellular Ca^{2+} described in osmotically swollen intestinal epithelial cells (Hazama & Okada, 1988), amphibian urinary bladder (Davis & Finn, 1987; Wong, Debell & Chase, 1990), MDCK cells (Wong et al., 1990.), proximal tubule cells (Suzuki et al., 1990) and nonpigmented ciliary epithelial cells (Civan et al., 1992). In addition, the inhibitory effect of gadolinium on the stretch and swelling-induced $[Ca^{2+}]_i$ variations and on RVD suggests the involvement of a mechanosensitive calcium channel in this response. Stretch-activated calcium permeable channels have been described in different epithelial cell types (Christensen, 1987; Filipovic & Sackin, 1991; Okada et al., 1990), including the basolateral membranes of A6 cells the basolateral membrane of A6 cells (Urbach et al., 1993). The present data are consistent with the hypothesis that a mechanosensitive calcium channel mediates Ca^{2+} entry during swelling of A6 cells.

We have shown, using patch electrodes, that a localized membrane stretch produces a rapid and large increase in $[Ca^{2+}]_i$, equivalent to that observed under cell swelling conditions. This suggests that a localized membrane stretch can trigger a powerful amplification cascade for calcium signaling. Thus, Ca^{2+} entry via stretch-

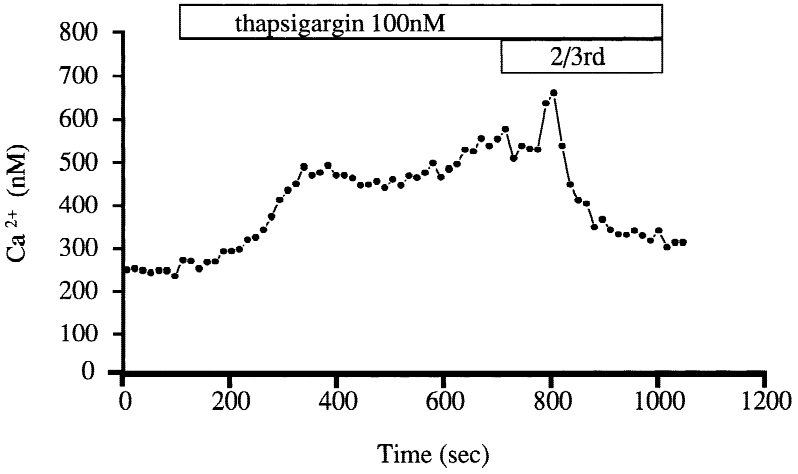

Fig. 8. Thapsigargin (100 nM) effect on the swelling induced $[Ca^{2+}]_i$ increase. Typical calcium response in a single A6 cell exposed to a hypotonic solution (2/3rd) 10 min after thapsigargin treatment. Note the rapid decrease in $[Ca^{2+}]_i$ induced by hypotonic shock.

sensitive calcium channels may provide the initial calcium signal to produce stimulation of calcium release from thapsigargin-sensitive stores (calcium-entry-induced calcium release "CICR"). The calcium sensitivity of IP_3-sensitive receptors of calcium stores (Lino, 1990) could also be implicated in this phenomenon. CICR may also be reinforced by $Ca^{2+}{}_i$ effects on calcium entry across the plasma membrane (Calcium-Release Activated Calcium entry "CRAC"), and by the positive feedback effect of depletion of calcium stores on Ca^{2+} entry (capacitive calcium entry "CCE") as proposed in excitable and nonexcitable cells (Randriamampita & Tsien, 1993; Razani-Boroujerdi, Partridge & Sopori, 1994). Cell swelling and membrane stretch may also activate other mechanosensitive transduction pathways which mobilize Ca^{2+} from cytosolic stores. In Ehrlich cells and cardiomocytes, cell-swelling or membrane stretch induces IP_3 production (Dassouli et al., 1993; Svane & Hoffmann, 1992). Thus, a mechanosensitive IP_3 synthesis may act synergistically with calcium entry to stimulate calcium mobilization from intracellular stores during cell swelling, thus amplifying the calcium signal initiated by calcium entry through stretch-sensitive channels.

In summary, we have shown that mechanical stimulation of a small patch of membrane can activate a global rise in intracellular Ca^{2+}, indicating a powerful amplification system linked to intracellular Ca^{2+} release mechanisms. We conclude that a mechanosensitive calcium entry is the pathway for Ca^{2+} signaling during cell swelling in A6 cells. However, the calcium response is transient and terminated before regulatory volume decrease occurs. The calcium signal may, therefore, be permissive for RVD while other mechanosensitive events (e.g., cytoskeleton reorganization) intervene in producing a full RVD response.

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