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Calcium-Dependent Control of Volume Regulation in Renal Proximal Tubule Cells: I. Swelling-Activated Ca^{2+} Entry and Release

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Summary. The mechanism of Ca^{2+}-dependent control of hypotonic cell volume regulation was investigated in the isolated, nonperfused renal proximal straight tubule. When proximal tubules were exposed to hypotonic solution with 1 mM Ca^{2+}, cells swelled rapidly and then underwent regulatory volume decrease (RVD). This treatment resulted in an increase in intracellular free calcium concentration ($[Ca^{2+}]_i$) by a mechanism that had two phases: the first was a transient increase from baseline (136 nM) to a peak (413 nM) that occurred in the first 15–20 sec, but was followed by a rapid decay toward the pre-swelling levels. The second phase was characterized by a sustained elevation of $[Ca^{2+}]_i$ above the baseline (269 nM), which was maintained over several minutes. The dependence of these two phases on extracellular Ca^{2+} was determined. Reduction of bath $[Ca^{2+}]$ to 10 or 1 μM partially diminished the transient phase, but abolished the sustained phase completely, such that $[Ca^{2+}]_i$ fell below the baseline levels during RVD. It was concluded that the transient increase resulted predominantly from swelling-activated release of intracellular Ca^{2+} stores and that the sustained phase was due to swelling-activated Ca^{2+} entry across the plasma membrane. Ca^{2+} entry probably also contributed to the transient increase in $[Ca^{2+}]_i$. The time dependence of swelling-activated Ca^{2+} entry was also investigated, since it was previously shown that RVD was characterized by a "calcium window" period (<60 sec), during which extracellular Ca^{2+} was required. Outside of this time period, RVD would inactivate and could not be reactivated by subsequent addition of Ca^{2+}. It was found that the Ca^{2+} permeability did not inactivate over several minutes, indicating that the temporal dependence of RVD on extracellular Ca^{2+} is not due to the transient activation of a Ca^{2+} entry pathway.

Key Words cell swelling · regulatory volume decrease · volume regulation · intracellular calcium · calcium release · calcium entry · fura-2 · renal proximal tubule

Introduction

Most mammalian cells are capable of at least partial regulation of cell volume under hypotonic conditions. The process by which swollen cells extrude ions to allow the removal of the excess water, thereby allowing a return toward normal volume, is referred to as regulatory volume decrease (RVD). In most cases, RVD is achieved by the activation of separate conductances for K^+ and Cl^- [9, 19]. As KCl leaves the cell, water follows by osmosis, allowing the cell to shrink toward the pre-swelling volume.

The control of these conductances during RVD has been the subject of many studies in recent years. Calcium serves a dominant role as modulator in most tissues studied, including epithelia, Ehrlich ascites tumor cells, and erythrocytes (for recent reviews *see* [5, 9, 17, 19]). However, it is not clear what the relative contributions are of Ca^{2+} entering across the plasma membrane and Ca^{2+} released from intracellular stores. Previous studies from this laboratory [25] have shown that RVD in the rabbit renal proximal straight tubule is strongly dependent upon the extracellular calcium concentration ($[Ca^{2+}]_o$), that RVD is inhibited by calcium channel blockers, and that cell swelling is associated with an increase in intracellular free calcium concentration ($[Ca^{2+}]_i$). Furthermore, McCarty and O'Neil [25] demonstrated that the action of Ca^{2+} on RVD is highly regulated with respect to time, and that intracellular $[Ca^{2+}]$ undergoes spontaneous fluctuations even before swelling.

The present study had three main objectives: (i) to characterize more fully the roles of swelling-induced Ca^{2+} entry and swelling-induced Ca^{2+} release on RVD; (ii) to determine if the dependence of RVD upon $[Ca^{2+}]_o$ reflects variable control of $[Ca^{2+}]_i$; and (iii) to determine the time dependence of the activation of the swelling-induced Ca^{2+} entry pathways. In the accompanying paper [24], we characterize the pathways by which Ca^{2+} enters during swelling. Portions of these data have been presented in abstract form [26].

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Materials and Methods

Tissue Preparation

The isolated, nonperfused straight portion (S_2 segment) of the rabbit kidney proximal tubule was used for these studies [25]. Since the tubule lumen is collapsed in nonperfused tubules, this preparation allows the isolation of the transport pathways in the basolateral membrane, which is where the dominant volume-activated conductances are located in this tissue [37], without interference from transport pathways in the luminal (apical) membrane. This preparation also affords considerable improvement in the ability to measure cell volumes, since, as described before [25], the lumen remains collapsed and the tissue assumes the equivalent geometry of a cylinder.

The protocols for tubule isolation were detailed previously [25]. Briefly, female New Zealand white rabbits were placed under dual anesthesia, a laparotomy was performed, and the viscera were cooled with chilled saline. The left kidney was excised and sagittal slices (~1–2 mm thick) were made and placed in dissection medium (*see* Solutions) at 17°C. Individual proximal tubules (S_2 portion) were removed from strips of cortical tissue, and were trimmed to ~0.5 mm length. Tubule segments were transferred in dissection medium to a chamber on the stage of a Nikon Diaphot microscope, and the ends were crimped into glass pipettes. Tubules were then lowered onto a spot in the center of the chamber, which had been pretreated with either poly-L-lysine (3 mg/ml) or Cell-Tak (0.5 mg/ml), in order to minimize tubule movements during fluorescence excitation cycles.

Solutions

Nonbicarbonate-containing solutions were used in this study (*see* Table 1), since previous work indicated that RVD in the rabbit PST is bicarbonate-independent [37]. All solutions used were bubbled with 100% O_2 or room air[1], and adjusted to pH 7.4. Osmolalities averaged 293.0 ± 4.3 (SD) and 157.5 ± 5.8 mOsmolal for the isotonic and hypotonic solutions, respectively, as determined by vapor pressure osmometry. The dissection solutions were made hypertonic by the addition of 50 mM mannitol [25]. Hypertonic solutions were used during the initial dissection of tubules from kidney slices to reduce the cell swelling which may be a consequence of hypoxic conditions. Solutions of approximately 1 or 10 μM $[Ca^{2+}]$ were prepared by isohydric mixture of Ca-EGTA and K_2-EGTA, in proportions calculated based upon association constants, with corrections for the effects of temperature and ionic strength (*see* legend to Table 1 and [25]).

Unless stated otherwise, all reagents were obtained from Sigma Chemical (St. Louis, MO). Fura-2-acetoxymethylester (fura-2/AM) and ionomycin were obtained from Calbiochem (La Jolla, CA). FCCP was from Aldrich (Milwaukee, WI). Fura-2 pentapotassium salt and Pluronic F-127 (product of BASF-Wyandotte) were from Molecular Probes (Eugene, OR). Tissue culture supplements were obtained from Gibco Laboratories (Grand Island, NY). Cell-Tak was from Collaborative Research (Bedford, MA).

Intracellular Calcium Measurement

Measurements of $[Ca^{2+}]_i$ were made using the fluorescent calcium chelator fura-2 and a dual-wavelength microspectrofluorimetry work station, as described previously [25]. The system was built by the authors around a Nikon Diaphot inverted microscope with quartz optics. Fura-2 fluorescence was determined by alternating excitation at 340 and 380 nm with a filter wheel and recording the emission at 510 nm. Tubule images were made by adding a red filter to the brightfield light path of the microscope. Fluorescence emission and tubule image were separated by a dichroic mirror, where the green fura-2 emission irradiated a photomultiplier tube and the red brightfield image was reflected to a video camera, in a manner similar to that used by Foskett [12]. Thus, this system allows measurements of cell volume and $[Ca^{2+}]_i$ to be made for the same cells simultaneously (in the case of the nonperfused PST, this corresponds to 20–25 cells), at a best-speed of one fluorescence ratio per 2.03 sec. The work station was controlled by a Hewlett Packard computer, using software written by one of the authors (N.A.M.).

Dye Loading

Tubules were loaded with the dye by incubation with the membrane-permeant form, fura-2/AM, as described previously [25] with minor modifications. After measurement of autofluorescence (background), tubules were bathed with incubation solution [Dissection/incubation solution] (Table 1) containing a dye emulsion at room temperature. The dye was prepared in a test tube by 10 sec sonication of 4 μl of fura-2/AM (in DMSO) with 2 μl of Pluronic F-127 (10% wt/wt in DMSO). Five hundred microliters of incubation solution were then added, and the mixture was sonicated for 30 sec. The mixture was diluted to 2.0 ml with incubation buffer (final fura-2/AM concentration 15 μM), and this volume was flushed through the microscope chamber containing the tubule by use of a plastic syringe. Incubation proceeded for 9–10 min with occasional mixture of the chamber volume by repeatedly withdrawing 0.1 ml of the chamber volume into the syringe and then rapidly pushing this volume back into the chamber. This "refluxing" appeared to overcome the unstirred layer effect imposed upon the tubule by its placement at the bottom of the chamber. The process was repeated for another two dye incubations for a total of ~30 min. Fluorescence measurements during the loading procedure indicated that the dye was converted easily to its Ca^{2+}-sensitive form, since the fluorescence ratio typically reached a plateau after 10 min of incubation. Further loading increased the total signal strength without changing the ratio significantly. Following dye loading, tubules were brought to 30°C over 10 min and then equilibrated 15 min in the isotonic control solution at 37°C. This modified loading procedure led to a greater signal-to-noise ratio than the previous protocol [25] and avoided extensive dye leakage and sequestration during the loading period.

[1] In some cases, solutions were bubbled with room air, in order to avoid complete depletion of CO_2, since proximal tubule cell metabolism is enhanced in the presence of CO_2 (L.J. Mandel, *personal communication*). This did not affect the measured levels of intracellular calcium or ability to undergo cell volume regulation. For instance, baseline $[Ca^{2+}]_i$ was 138 ± 17 nM (n = 13) in the presence of 100% O_2 and 134 ± 14 nM (n = 11) in presence of room air.

Table 1. Composition of solutions

	Control Isotonic	Hypotonic	Dissection/incubation	Fura-2/Ca_i^{2+} manipulation
NaCl	145	70	145	0
KCl	5	5	5	5
$CaCl_2$	1	1[a]	0*	0
$MgCl_2$	1	1	2	1
Na_2PIPES	0	0	0	5
NaH_2HPO_4	1.5	1.5	2.5	0.5
Choline-Cl	0	0	0	140
Mannitol	0	0	50	0–12[b]
ATP and adenosine	0	0	1	0
Bovine serum albumin (%)	0.1	0.1	0.1	0
Organic substrates[c]	+	+	+	–

Values in mmoles/liter unless otherwise noted.

[a] The 10 μM $[Ca^{2+}]_o$ solution contained 19 μM total Ca and 10 μM total EGTA, pH 7.4, and the 1 μM $[Ca^{2+}]_o$ solution and dissection/incubation solution contained 460 μM total Ca and 500 μM total EGTA, pH 7.4.

[b] The concentration of mannitol in the manipulation solutions (with Ca, EGTA, Mn or no additions, *see* text) was adjusted to maintain isosmolality.

[c] Solutions indicated (+) also contained 5 mM glucose, 5 mM Na-acetate, 2.5 mM L-alanine, and 1 mM Na-butyrate. Tissue culture supplements were added at 10% of the concentration used in tissue culture: MEM amino acids with glutamine, MEM nonessential amino acids, and sodium pyruvate solution (Gibco Laboratories, Grand Island, NY).

In vivo Calibration

After most of the experiments, the responsiveness of the intracellular dye to Ca^{2+} was determined by the same procedure as described previously [25]. Briefly, the critical end-points of the dye fluorescence ratio were determined by exposing the tissue to a low Na^+ manipulation solution (Table 1) with ionomycin (1–10 μM) and either EGTA (10 mM), to measure the minimum fluorescence ratio (R_{min}), or saturating concentrations of Ca^{2+} (5 mM), to measure the maximum fluorescence ratio (R_{max}). FCCP (1–10 μM) was also added to disrupt ATP-dependent Ca^{2+} extrusion during measurement of R_{max}. The experiment was ended by exposure to manipulation solution with 1 mM $MnCl_2$, to quench all deesterified cytoplasmic fura-2. The unquenched fluorescence is a measure of Ca^{2+}-independent dye fluorescence, which, in this tissue, is usually not significantly greater than the autofluorescence and, hence, could be used as a background measurement.

Fura-2 fluorescence ratios were converted to intracellular free calcium concentrations by the method of Grynkiewicz et al. [16], using the following formula:

$$[Ca^{2+}] = \beta \cdot K_d[(R - R_{min})/(R_{max} - R)] \quad (1)$$

where R is the ratio at any time and β is the ratio of the fluorescence emission intensity in Ca^{2+}-depleting and Ca^{2+}-saturating conditions with excitation at 380 nm. K_d is the Ca^{2+}-dissociation constant of the dye and was determined previously (using the same system and under the same conditions) to be 218 nM [25]. The in vitro calibration procedure was not performed on tubules that exhibited rapid leakage of the dye. While fura-2 is a "ratio" type of probe, leakage of the dye over the 5 min required to go from Ca^{2+}-depleting to Ca^{2+}-saturating conditions would result in an inappropriate value for β. Therefore, the calibration procedure was performed only on tubules that did not exhibit significant dye leakage over this time period. Average values (± SD) for the constants used in Eq. (1) are as follows: $R_{max} = 1.901 \pm 0.060$, $R_{min} = 0.235 \pm 0.011$, $\beta = 16.0 \pm 1.5$ ($n = 19$). This value of β is similar to the β value found in the in vitro calibration ($\beta = 15.6 \pm 1.2$) described previously [25], indicating that the intracellular dye behaves as if it were localized in an aqueous solution.

Cell Volume Measurements

Images of nonperfused tubules were recorded on videotape for later measurements of cell volume. Since the lumen of the nonperfused PST remains collapsed, cell volumes could be easily calculated by measuring the cross-sectional diameter of the tubule. This was done at three randomly chosen points along the ~45 μm of tubule length in the field of view of the microscope, as displayed on a video monitor. Volumes at any time (V) were calculated relative to the original baseline volume in isotonic solution immediately before swelling (V_o) as follows:

$$(V/V_o) = (\pi r_t^2 L)/(\pi r_o^2 L) \quad (2)$$

where r_o and r_t are the tubule radii in isotonic medium and at time t, respectively, and L is an arbitrary length of tubule. Since the cell volume changes are manifest predominantly by a change in the apical-to-basolateral dimension without a change in length [21], Eq. (2) was simplified by ignoring the length terms. The extent of volume regulation after swelling is presented as

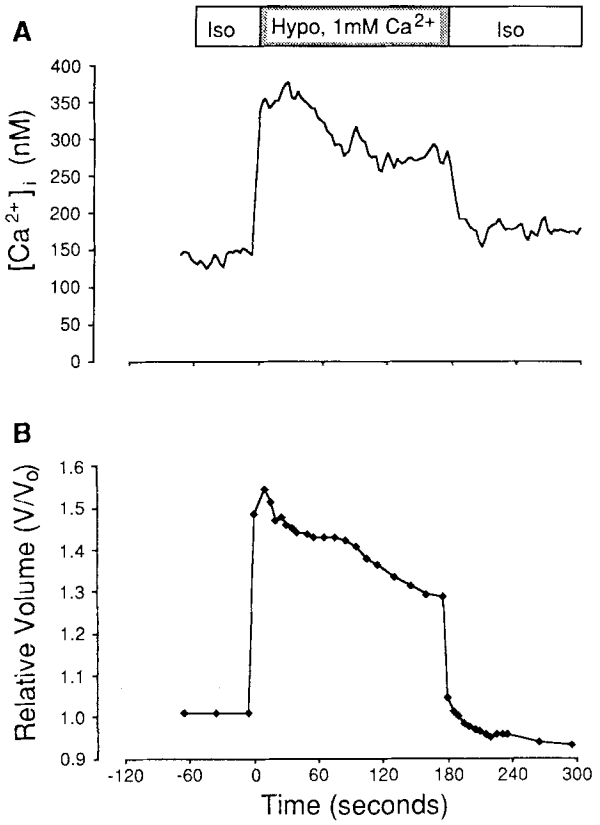

Fig. 1. Response of an isolated proximal tubule to hypotonicity in the presence of normal $[Ca^{2+}]_o$ in a typical experiment. At time zero, the superfusate (bath perfusion solution) was switched from isotonic (*Iso*) to hypotonic with 1 mM $[Ca^{2+}]_o$ (*Hypo*), followed by return to isotonic solution (*Iso*) at 180 sec. (*A*) Intracellular free calcium concentration ($[Ca^{2+}]_i$) was determined from background-corrected fluorescence of fura-2 using a dual-wavelength microspectrofluorimeter. Individual points are not shown, but were generated every 2–3 sec throughout the duration of the experiment. When exposed to hypotonic solution, $[Ca^{2+}]_i$ rose rapidly to a peak and then fell to an elevated plateau. Upon return to isotonic solution, $[Ca^{2+}]_i$ returned slowly to the original baseline. (*B*) Simultaneous measurement of cell volume during the same time period as in *A*. Cell volume is expressed as the relative volume (V/V_o). When exposed to hypotonic solution, cells swelled to a peak volume and then underwent RVD. Upon return to isotonic solution, cell volume dipped below the isotonic volume, due to loss of osmolytes during RVD

$$\% \text{ RVD} = \{(V_{peak} - V_{reg})/(V_{peak} - V_o)\} \cdot 100 \quad (3)$$

where V_{peak} and V_{reg} are the peak and regulated (plateau) relative volumes, respectively, and $V_o = 1$ by definition. Therefore, % RVD signifies the magnitude of the return from the peak swollen volume V_{peak} back toward the isotonic volume V_o, such that 0% RVD indicates no volume regulation and 100% RVD indicates a complete return to isotonic volume, in the continued presence of hypotonic solution. The coefficient of variation for the volume measurement was determined to be 0.4%, by repeated measurements of the volume of a swollen tubule.

Table 2. Summary of baseline intracellular calcium concentration[a]

n	Average $[Ca^{2+}]_i$ (nM)	Range $[Ca^{2+}]_i$ (nM)
67	146 ± 4	81–243

[a] Data given represent the baseline intracellular free calcium concentration under isotonic conditions, calculated as the average of the last 30 sec of measurements before experimental treatment.

Statistics

All values are reported as mean ± SEM, unless otherwise indicated, where *n* is the number of tubules. Significance was determined with the *t* test (paired or unpaired, as indicated), with $P \leq 0.05$ considered indicative of significance.

Results

Response of Cell Volume and $[Ca^{2+}]_i$ to Swelling

An example of the control response to swelling is given in Fig. 1. Upon exposure to hypotonic solution containing 1 mM Ca^{2+}, proximal straight tubules (PSTs) were observed to swell to a peak volume, always within the first 45 sec. Peak relative volume in control swelling experiments averaged 1.392 ± 0.015 ($n = 24$), signifying a 39.2% increase in cell volume over the isotonic volume. Over the course of the next three minutes, cells underwent RVD to return the cell volume to a new quasi-plateau volume (V_{reg}) of 1.272 ± 0.015. This represents an average % RVD of 31.0 ± 2.7. It is expected that cells would continue to undergo RVD for several minutes in the continued presence of hypotonic solutions, as shown by other investigators [8, 23, 36]. The finding of decreased RVD in these experiments relative to previous data is likely due to the shortened duration of experiments in the present study. Experiments in this study were limited to 3-min duration in order to reduce the effects of dye leakage.

Concomitant measurements of fura-2 fluorescence were performed in order to determine the relationship between the effects of swelling on cell volume and on $[Ca^{2+}]_i$. These data indicated that $[Ca^{2+}]_i$ rose from a baseline of 136 ± 4 nM to 413 ± 15 nM during the initial swelling phase, and then relaxed to a plateau level that averaged 269 ± 9 nM ($n = 24$) (*see* Tables 2 and 3). This elevated plateau was maintained for the duration of the exposure to the hypotonic solution. Thus, the $[Ca^{2+}]_i$ response

Table 3. Summary table of the dependence of RVD and $[Ca^{2+}]_i$ on $[Ca^{2+}]_o$[a]

Treatment	Baseline $[Ca^{2+}]_i$	Peak[b] $\Delta[Ca^{2+}]_i$	Plateau[b] $\Delta[Ca^{2+}]_i$	% RVD	n
Hypo, 1 mM Ca^{2+}	136 ±4	276 ±15	132 ±10	31.0 ±2.7	24
Hypo, 10 μM Ca^{2+}	136 ±12	130[c] ±18	−85[c] ±7	8.0[c] ±2.7	3
Hypo, 1 μM Ca^{2+}	140 ±8	71[c] ±13	−64[c] ±9	5.2[c] ±2.0	9

[a] Values are mean ± SEM.

[b] Peak $\Delta[Ca^{2+}]_i$ and plateau $\Delta[Ca^{2+}]_i$ (nM) are given as the change with respect to baseline values measured immediately before swelling, in the presence of 1 mM Ca^{2+}. Peak values are the highest value recorded while plateau values are from the average measurements during the last 20 sec of a 3-min treatment, and thus include variations around the mean due to fluctuations. For all treatments, the isotonic medium before swelling always contained 1 mM Ca^{2+}.

[c] $P \leq 0.05$ compared to controls with 1 mM Ca^{2+}.

to swelling appears to consist of two phases: an initial transient phase represented by an increase of 276 ± 15 nM over baseline, followed by a sustained phase where $[Ca^{2+}]_i$ was maintained at 132 ± 10 nM above baseline. Upon returning to the isotonic solution, $[Ca^{2+}]_i$ returned to the baseline values within 2–3 min.

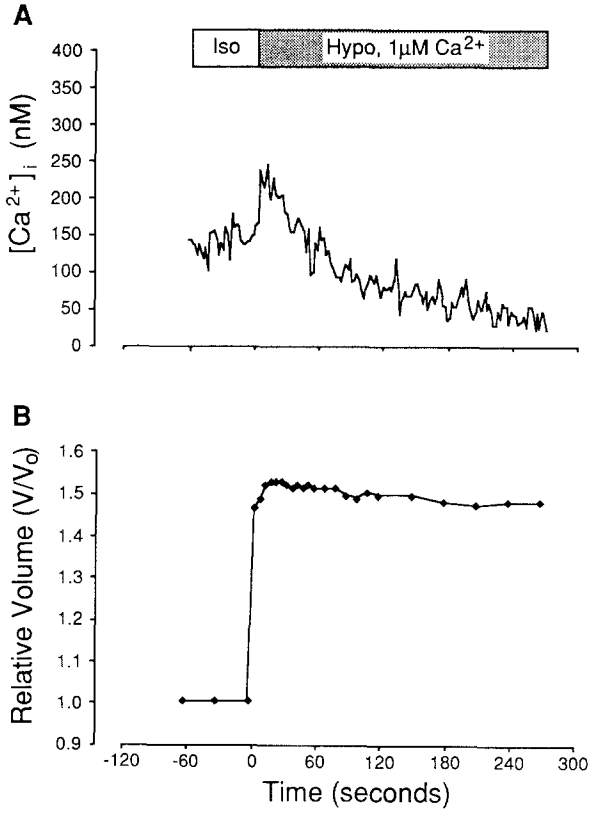

Fig. 2. Simultaneous measurements of $[Ca^{2+}]_i$ (*A*) and cell volume (*B*) during exposure to hypotonic solution with 1 μM Ca^{2+}. Following a transient increase, $[Ca^{2+}]_i$ fell below the original baseline. RVD was inhibited under these conditions

Dependency upon $[Ca^{2+}]_o$

When tubules were swollen in hypotonic solutions containing only 1 μM Ca^{2+} (Fig. 2) or 10 μM Ca^{2+} (*data not shown*), RVD was diminished significantly.[2] In the presence of low $[Ca^{2+}]_o$, particularly 1 μM, Ca^{2+} influx should be limited. Nonetheless, the transient increase in $[Ca^{2+}]_i$, while reduced in magnitude, was still clearly distinguishable above the spontaneous fluctuations, even in exposures to 1 μM Ca^{2+} hypotonic solution. In contrast, the late phase of the normal intracellular calcium response was completely abolished in the presence of low $[Ca^{2+}]_o$, such that the plateau level in *all* cases was below the baseline $[Ca^{2+}]_i$. The data for these experiments are summarized in Table 3.

It is important to note that the actual extent of Ca^{2+} influx during exposure to hypotonic solutions in the presence of 1 mM Ca^{2+} (as reflected by the sustained elevation of $[Ca^{2+}]_i$), is related to the difference between the plateau $[Ca^{2+}]_i$ in these experiments and the plateau $[Ca^{2+}]_i$ in the presence of low external $[Ca^{2+}]$. Since $[Ca^{2+}]_i$ consistently fell below baseline in low Ca^{2+} hypotonic solutions, the magnitude of Ca^{2+} influx in control treatments is considerably greater than that shown by calculation relative to the baseline pre-swelling $[Ca^{2+}]_i$. In the case of the low Ca^{2+} experiments, it is probable that the fall below baseline $[Ca^{2+}]_i$ is due to the same mechanisms by which $[Ca^{2+}]_i$ is maintained low in the isotonic state. Hence, the Ca^{2+} extrusion action of the plasma membrane Ca^{2+}-ATPase combined with Ca^{2+} resequestration into intracellular stores may be able to lower $[Ca^{2+}]_i$ under conditions of reduced Ca^{2+} influx. Also, the influx of water into

[2] Our experience indicates that for PST cells, the most appropriate condition to evaluate the Ca^{2+} dependence of RVD is in the presence of *low* $[Ca^{2+}]_o$ (down to 1 μM), rather than *zero* $[Ca^{2+}]_o$. This is based on a variety of observations, including the finding that zero Ca^{2+} solutions cause marked morphological changes in PST cells. These changes were not observed if cells were exposed to solutions containing either 1 or 10 μM Ca^{2+}. Furthermore, due to the low conductance rates of Ca^{2+} entry pathways in mammalian cells, Ca^{2+} influx at 1 μM $[Ca^{2+}]_o$ would be miniscule.

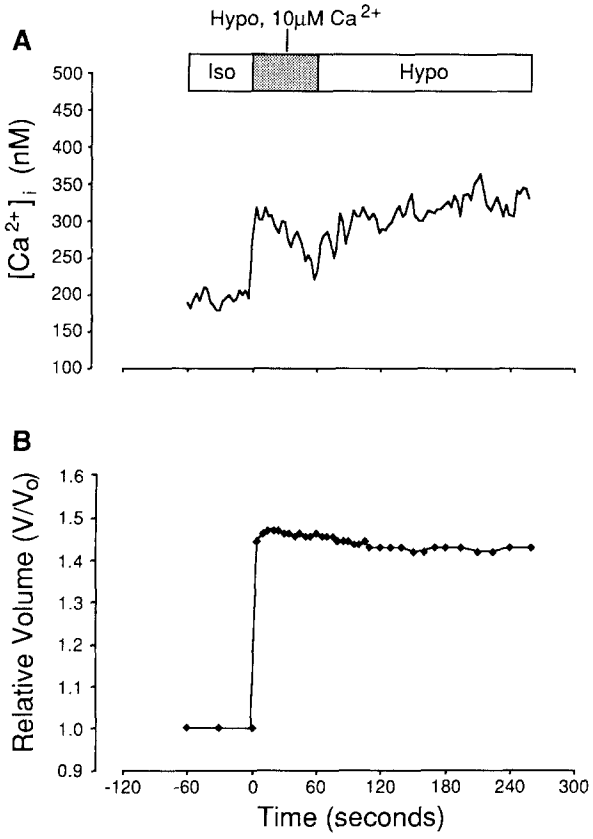

Fig. 3. Temporal dependence of the Ca^{2+} entry pathway during a 60-sec calcium window experiment. Tubules were swollen in 10 μM $[Ca^{2+}]_o$ hypotonic solution for 60 sec before $[Ca^{2+}]_o$ was returned to normal (1 mM). (*A*) $[Ca^{2+}]_i$. (*B*) Cell volume. After the transient peak, $[Ca^{2+}]_i$ fell toward baseline. Returning to 1 mM $[Ca^{2+}]_o$ after 60 sec led to an increase in $[Ca^{2+}]_i$ to a plateau value that was not different from that observed in control swelling experiments with 1 mM $[Ca^{2-}]_o$. In spite of the maintained Ca^{2+} entry pathway, RVD did not occur

the cytoplasm during hypotonic swelling would be expected to dilute the cellular content of Ca^{2+}.

Time-Dependence of the $[Ca^{2+}]_i$ Response

Previous experiments [25] indicated that the RVD mechanism in PST requires extracellular calcium to be present during the early part of the swelling response. When tubules were initially swollen in low Ca^{2+} hypotonic solutions, returning to normal $[Ca^{2+}]_o$ after 1 min did not lead to RVD. Since those experiments did not include measurements of $[Ca^{2+}]_i$, it has remained unclear if this temporal dependence was due to inactivation of the Ca^{2+} entry pathway or due to another form of desensitization. For this reason, those experiments were repeated in the present study, with concomitant measurements of $[Ca^{2+}]_i$.

As shown in Fig. 3, when PSTs were exposed to 10 μM Ca^{2+} hypotonic solution, cell volume initially increased and then was held in the swollen state due to lack of sufficient extracellular calcium (Fig. 3*B*). Intracellular calcium rose during the initial phase and then began to fall toward baseline (Fig. 3*A*). When $[Ca^{2+}]_o$ was raised to 1 mM after 1 min, in the continued presence of hypotonicity, $[Ca^{2+}]_i$ rose again to a plateau level of 259 ± 30 nM, well above baseline. Since $[Ca^{2+}]_i$ continued to be sensitive to $[Ca^{2+}]_o$ after 1 min, this indicates that the Ca^{2+} entry pathway is still active at this time. In spite of this late increase in $[Ca^{2+}]_i$, however, RVD did not occur. In contrast, when the period of incubation in low Ca^{2+} hypotonic solution was reduced to only 30 sec, return to normal $[Ca^{2+}]_o$ caused a transient dip in cell volume and secondary swelling (as previously described [25]), followed by normal RVD (Fig. 4). It is presently not known why, under these conditions, the cells underwent this transient dip in volume, which was observed in each experiment. Nonetheless, the cell volume response was accompanied by an increase in $[Ca^{2+}]_i$ that was indistinguishable from the response in the 1-min case, since the plateau level was similar (292 ± 32 nM). Thus, it appears that the swelling-induced Ca^{2+} entry pathway does not inactivate in PST during continued exposure to hypotonic conditions. These data are summarized in Table 4.

Effects of Ionomycin + Ca^{2+} on Isotonic Cell Volume and $[Ca^{2+}]_i$

It has been shown in Ehrlich ascites tumor cells [20], frog urinary bladder [7], and lymphocytes [15] that treatment with a Ca^{2+} ionophore in the presence of external calcium results in considerable cell shrinkage, even under isotonic conditions. The shrinkage was attributed to the activation of Ca^{2+}-dependent K^+ and Cl^- conductances, which resulted in movement of K^+ and Cl^- down their electrochemical gradients, followed by water. This phenomenon was investigated in the PST to determine if the ion conductances that are operational in the steady state are under similar control to those in Ehrlich cells, frog urinary bladder, and lymphocytes. Upon treatment with 2–5 μM ionomycin in isotonic solution containing 1 mM Ca^{2+}, $[Ca^{2+}]_i$ increased rapidly within 20 sec to a peak at 392 ± 38 nM above baseline, and then fell to a lower level (Fig. 5*A*). In spite of such a considerable rise in $[Ca^{2+}]_i$, however, cell volume was largely unaffected (Fig. 5*B*). In the case shown, ionomycin treatment was followed by quenching of the fura-2 signal with Mn^{2+}.

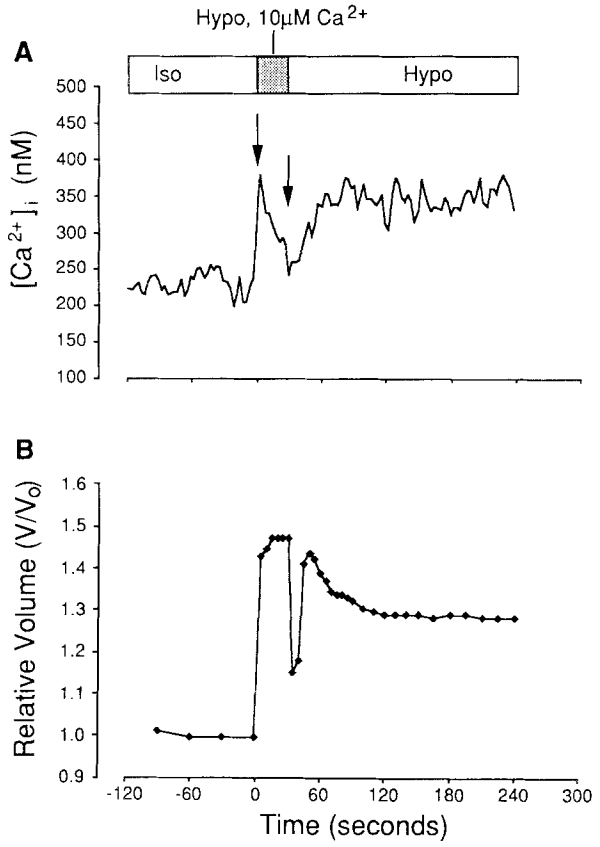

Fig. 4. Temporal dependence of the Ca^{2+} entry pathway during a 30-sec calcium window experiment. Tubules were swollen in 10 μM $[Ca^{2+}]_o$ hypotonic solution for 30 sec before $[Ca^{2+}]_o$ was returned to normal (1 mM). (*A*) $[Ca^{2+}]_i$. (*B*) Cell volume. After the transient peak, $[Ca^{2+}]_i$ fell toward baseline. Returning to 1 mM $[Ca^{2+}]_o$ after 30 sec led to an increase in $[Ca^{2+}]_i$ to a plateau value that was not different from that observed in control swelling experiments with 1 mM $[Ca^{2+}]_o$. The figure indicates the transient dip in cell volume immediately after restoration of $[Ca^{2+}]_o$ to normal values, followed by secondary swelling and normal RVD. The anomalous transient dip was seen in each of three experiments

Na^+/Ca^{2+} Exchange

Many cells have a countertransporter in their plasma membranes which serves to link the movement of Na^+ into the cell with the movement of Ca^{2+} out from the cell [31]. Since solutions were made hypotonic by the partial removal of NaCl (Table 1), it was necessary to determine if a Na^+/Ca^{2+} exchange activity contributed to the observed changes in $[Ca^{2+}]_i$. Reducing extracellular $[Na^+]$ by 75 mM would be expected to have an effect on the kinetic operation of this exchanger, since the driving force for its activity is generated by the driving force for passive Na^+ movements, either into or out of the cell.

Table 4. Temporal dependence of RVD and $[Ca^{2+}]_i$

Duration of 10 μM Ca^{2+} hypo (sec)	10 μM Ca^{2+} hypo	1 mM Ca^{2+} hypo		
	Peak $\Delta[Ca^{2+}]_i$	Peak $\Delta[Ca^{2+}]_i$	Plateau $\Delta[Ca^{2+}]_i$	% RVD
60	202	200	94[ns]	1.3[a]
(n = 3)	±58	±39	±20	±1.3
30	214	227	151[ns]	27.9[ns]
(n = 3)	±46	±59	±11	±5.6

Tubules were swollen in the 10 μM Ca^{2+} hypotonic solution for the duration indicated (left column) before switching to the 1 mM Ca^{2+} hypotonic solution. During the initial swell in low Ca^{2+}, $[Ca^{2+}]_i$ reached the peak level indicated in the second column (Peak $\Delta[Ca^{2+}]_i$). $[Ca^{2+}]_i$ then fell to 131 ± 64 nM in 30 sec or 88 ± 53 nM in 60 sec. Subsequent exposure to 1 mM Ca^{2+} elicited the peak increases shown in the third column (Peak $\Delta[Ca^{2+}]_i$), which fell to a plateau at the value shown in the fourth column (Plateau $\Delta[Ca^{2+}]_i$). Values for $[Ca^{2+}]_i$ are in nM, with Δ values relative to the original baseline in isotonic conditions. Baseline $[Ca^{2+}]_i$ = 165 ± 25 nM and 140 ± 41 nM for 60 and 30 sec treatments. No RVD was exhibited during the exposures to 10 μM Ca^{2+} in either case (average %RVD = 0 for 30 sec and 1.3 for 60 sec).

In the 30-sec experiments, switching to the 1 mM Ca^{2-} solution was followed by rapid shrinkage, reswelling, and subsequent RVD (*see* Fig. 4*B*). During the transient shrinkage, cells lost 38.9 ± 12.4% of the swollen volume.

[a] $p \leq 0.05$ or not significant [(ns)] compared to control swells in 1 mM Ca^{2+}.

Accordingly, the post-experiment intracellular calibration was preceded in several tubules by first switching to the manipulation solution containing 12 mM Na^+ and 1 mM Ca^{2+}, representing more than a 10-fold drop in $[Na^+]$ while maintaining $[Ca^{2+}]_o$ constant. As shown in Fig. 6, this treatment did not lead to a dramatic rise in $[Ca^{2+}]_i$ as seen in hypotonic solutions. Rather, $[Ca^{2+}]_i$ appeared to rise slowly over the course of a few minutes. When $[Ca^{2+}]_o$ was further increased to 5 mM, the rate of change of $[Ca^{2+}]_i$ increased. This response to reduction in $[Na^+]$, therefore, showed a pattern distinctly different from that seen during swelling. Furthermore, the Ca^{2+} channel blockers verapamil and nifedipine, which are not known to inhibit Na^+/Ca^{2+} exchange at the concentrations used [34], essentially abolish the swelling-induced Ca^{2+} entry. Therefore, it is likely that Na^+/Ca^{2+} exchange does not contribute significantly to the observed increase in $[Ca^{2+}]_i$ under the conditions used in other experiments in this study, although a partial contribution of this exchanger to Ca^{2+} entry during swelling cannot be fully ruled out with the present data.

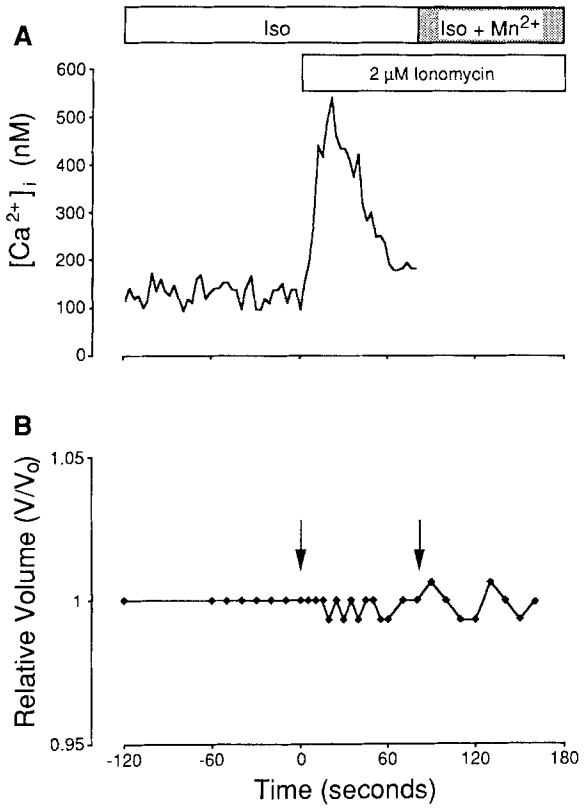

Fig. 5. Lack of an effect of ionomycin + Ca^{2+} on cell volume in the isotonic state. (*A*) $[Ca^{2+}]_i$, and (*B*) cell volume. Note the change of scale. At time zero, the superfusate was switched to *Iso* with 2 μM ionomycin (left arrow). Although $[Ca^{2+}]_i$ rose dramatically, cell volume was unaffected. This response was seen in each of three experiments. When 1 mM $MnCl_2$ was included to quench fura-2, thereby abolishing fura-2 fluorescence, cell volume displayed distinct fluctuations (second arrow)

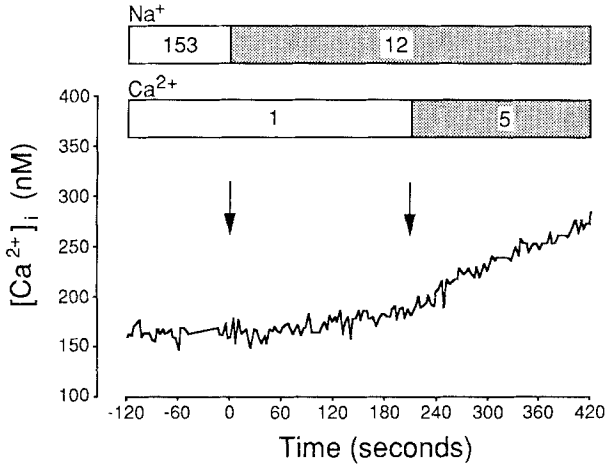

Fig. 6. Lack of an abrupt affect of reduction of bathing medium $[Na^+]$ on $[Ca^{2+}]_i$. A contribution by Na^+/Ca^{2+} exchange to the swelling-induced rise in $[Ca^{2+}]_i$ was investigated by measuring the effect of isotonic reduction of Na from 153 to 12 mM (NaCl for choline-Cl replacement), in the presence of 1 mM Ca^{2+} (left arrow). An abrupt rise in $[Ca^{2+}]_i$ was not seen. Rather, $[Ca^{2+}]_i$ rose slowly at a rate that was increased by augmenting $[Ca^{2+}]_o$ to 5 mM (second arrow)

Discussion

Swelling-Activated Ca^{2+} Entry and Release

The data presented in this study indicate that hypotonic cell volume regulation in renal proximal straight tubules involves a change in intracellular calcium. Similar results have been shown in toad bladder cell primary cultures [38], proximal convoluted tubule (PCT) primary cultures [33], and osteosarcoma cells [39]. The pattern of the $[Ca^{2+}]_i$ increase seen in isolated tubules differs considerably from the pattern in these other cells types. In osteosarcoma cells, hypotonic shock caused $[Ca^{2+}]_i$ to increase from 142 to 498 nM over the course of about 30 sec. $[Ca^{2+}]_i$ then fell slowly toward control, but did not plateau, at least not over the course of 2–3 min shown by the authors [39]. In toad bladder cells, swelling caused $[Ca^{2+}]_i$ to increase from 97 to 354 nM. After reaching the peak, $[Ca^{2+}]_i$ fell to 163 nM within 2 min, but then it continued to fall in the representative experiment shown [38]. In PCT primary cultures [33], swelling did not cause a transient increase in $[Ca^{2+}]_i$. Instead, $[Ca^{2+}]_i$ slowly increased from 96 to 468 nM over the course of 2–3 min, and then remained at roughly the same level. In all three cases, the observed rise in $[Ca^{2+}]_i$ was dependent upon extracellular Ca^{2+}.

In contrast, swelling-induced changes in $[Ca^{2+}]_i$ in the PST cells in this study were characterized by two distinct phases (Fig. 1*A*). The initial transient increase in $[Ca^{2+}]_i$ was of a similar magnitude to that shown for toad bladder cells and osteosarcoma cells, and was initiated concomitantly with the osmometric phase of the cell volume change (Fig. 1*B*). However, the rate of decay from the transient peak appears to be greater in PST than in these other cells. Following the peak and early decay in $[Ca^{2+}]_i$, PST cells indicate a sustained increase in $[Ca^{2+}]_i$ above baseline levels, at least for 3–7 min. The maintenance by PST of an elevated $[Ca^{2+}]_i$ for several minutes in the continued presence of hypotonicity is similar to that seen in PCT primary culture cells [33] (although the PCT primary culture data should be viewed with caution since $[Ca^{2+}]_i$ did not return to baseline after > 17 min in isotonic wash). Furthermore, the plateau level of $[Ca^{2+}]_i$ after swelling in PST was somewhat more sensitive to changes in extracellular Ca^{2+} concentration than was the initial

transient. Although we did not directly test an involvement of Na^+/Ca^{2+} exchange, the observed changes in $[Ca^{2+}]_i$ in PST were not likely due to activation of this transporter, since the time course for Ca^{2+} entry by Na^+/Ca^{2+} exchange appears to be too slow (Fig. 6). Also, data presented in the accompanying paper [24] indicate that the rise in $[Ca^{2+}]_i$ is inhibited by Ca^{2+} channel blockers, which would not likely inhibit Na^+/Ca^{2+} exchange at the concentrations used [34]. However, further studies will be needed to fully evaluate this issue.

It thus appears that there may be two mechanisms leading to swelling-induced increases in $[Ca^{2+}]_i$ in PST: one that is solely dependent upon Ca^{2+} entry from the extracellular space, and one that is transient and likely predominantly reflects release of Ca^{2+} from intracellular stores, although a contribution from Ca^{2+} entry cannot be excluded. It may be that PCT primary culture cells do not have a mechanism leading to rapid swelling-induced Ca^{2+} release, but rather only a slowly activating Ca^{2+} entry mechanism. Osteosarcoma cells, on the other hand, may only have a Ca^{2+} entry mechanism that activates rapidly and then inactivates. Toad bladder cells may be similar to osteosarcoma cells without any apparent Ca^{2+} release, although Ca^{2+} release during swelling in low Ca^{2+} hypotonic solutions in toad bladder cells may have been masked by the extensive pre-incubation in nearly Ca^{2+}-free isotonic solution (15 min at 200 nM $[Ca^{2+}]_o$). PST cells appear to have both swelling-induced Ca^{2+} release and Ca^{2+} entry mechanisms. It is likely that the initial transient increase in $[Ca^{2+}]_i$ is due to combined contributions of release and entry, while the sustained level of increased cell calcium is due to sustained rates of Ca^{2+} entry. Further evidence of swelling-induced Ca^{2+} entry is given in the accompanying paper [24].

The initial transient increase in $[Ca^{2+}]_i$ in PST cells occurs concomitantly with the rapid phase of cell swelling. The decay from the transient peak to the plateau $[Ca^{2+}]_i$ level is roughly associated with the most rapid phase of RVD. However, a significant degree of RVD occurs after the plateau $[Ca^{2+}]_i$ level is reached, as shown in Fig. 1.

Mechanism of Activation of Ca^{2+} Entry and Ca^{2+} Release

Swelling-induced release of Ca^{2+} from intracellular stores in Ehrlich ascites tumor cells has been proposed by Hoffmann and coworkers [20]. It is unclear what might be the source of Ca^{2+} in this case. These experiments were based upon the action of the ionophore A23187, which has been shown to rapidly uncouple mitochondrial respiration [27]. Mitochondrial uncoupling is associated with spontaneous release of Ca^{2+} from Ehrlich cell mitochondria [11]. Thus, the mitochondria may have served as the A23187-releasable store of Ca^{2+} in the experiments by Hoffmann. However, mitochondrial Ca^{2+} stores are usually considered a nonphysiological sink of Ca^{2+}, where Ca^{2+} accumulation occurs in pathophysiological states [4].

Intracellular storage sites for Ca^{2+} are now typically considered to be the endoplasmic reticulum or an associated organelle such as the calciosome [35]. How swelling can lead to release of Ca^{2+} from these sites is unknown. Three possibilities may be considered, however. First, various stimuli lead to Ca^{2+} release by the production of inositol polyphosphates, via hydrolysis of membrane phosphatidylinositol lipids [2, 3]. The breakdown species of greatest interest, inositol 1,4,5-trisphosphate (IP_3), has been shown to cause release of Ca^{2+} from a storage site that may be the endoplasmic reticulum. It is possible that swelling induces membrane phosphatidylinositol hydrolysis, leading to production of IP_3 and subsequent release of PST Ca^{2+} stores. Suzuki et al. [32] have shown that the levels of inositol polyphosphates are tripled within the first minute of hypotonic shock in cultured PCT cells. Preliminary evidence for swelling-activated phosphatidylinositol turnover has also been found in PST exposed to hypotonic solutions (R.G. O'Neil, *unpublished observations*).

Proximal tubule cells have been shown to be responsive to stimulation by a variety of agents, resulting in alteration of $[Ca^{2+}]_i$. The response of cultured proximal tubule cells to bradykinin [1] is very similar to the response to swelling, in that bradykinin induces a transient increase in $[Ca^{2+}]_i$ independently of extracellular calcium. $[Ca^{2+}]_i$ then relaxes to an elevated plateau that is abolished in Ca^{2+}-free media, such that the plateau $[Ca^{2+}]_i$ is below the original baseline when stimulated in the absence of external Ca^{2+}. Angiotensin II has a similar effect on $[Ca^{2+}]_i$, supposedly via a similar mechanism [1, 14]. Phenylephrine also causes rapid release of stored Ca^{2+} in cultured proximal tubule cells [13]. Bradykinin, angiotensin II, and phenylephrine all stimulate Ca^{2+} release through the inositol polyphosphates pathway, indicating that this biochemical mechanism is operative in proximal tubule cells.

An alternative mechanism for Ca^{2+} release may be the operation of voltage-dependent, IP_3-insensitive calcium channels in endoplasmic reticulum, as described recently in rat exocrine pancreas [29]. The channels open with hyperpolarization to positive voltages (endoplasmic side relative to cytoplasm). As cytoplasmic contents become diluted during swelling, it is conceivable that the voltage across the

endoplasmic reticulum membrane may change. In this way, the voltage-sensitive Ca^{2+} release channel could play a role in the swelling-induced Ca^{2+} release in PST.

Finally, ion channels that are sensitive to stretch may pass Ca^{2+} (*see below*). It may be that the channels present in the membranes of intracellular organelles, such as the endoplasmic reticulum [30], are stretch activatable. Thus, dilution of the cytoplasm during swelling could lead to swelling of the organelles and distention of these intracellular membranes, leading to Ca^{2+} release.

A current favorite mechanism proposed for swelling-activated Ca^{2+} entry is the operation of stretch-activated channels in the plasma membrane. Membrane stretch may serve as the trigger for opening of channels that are permeable to Ca^{2+} [10, 40]. Stretch-activated cation channels, which may pass calcium, are present in proximal tubule [28] and may serve as the volume-sensitive Ca^{2+} entry pathway. Alternatively, the swelling-activated Ca^{2+} entry pathway may be a voltage-activated channel, similar to the voltage-gated, DHP-sensitive Ca^{2+} channels of excitable tissues. It is known that the voltage across the basolateral plasma membrane in PST exposed to hypotonic solution undergoes a transient hyperpolarization followed by a prolonged depolarization [37]. This depolarization could serve to open voltage-activated Ca^{2+} channels, leading to substantial Ca^{2+} influx [22]. While voltage-activated Ca^{2+} channels have not been recorded in renal tubules, their presence is suggested by indirect means [6]. Future studies will focus on the mechanism of activation of this entry pathway [24].

Sustained Activation of the Ca^{2+} Entry Pathway

Previous experiments [25] indicated that RVD in the proximal straight tubule requires extracellular Ca^{2+} to be present early in the response to swelling. This phenomenon was originally termed the "calcium window" effect, since it appeared that there was a window in time after the initiation of swelling, during which extracellular calcium was capable of activating RVD. Outside of this window, augmenting the level of extracellular calcium did not restart RVD. The experiments shown in Figs. 3 and 4 represent an investigation into the question of whether this temporal dependence is due to inactivation of the Ca^{2+} entry pathway. Swelling in 10 μM Ca^{2+} hypotonic solution caused an initial transient rise in $[Ca^{2+}]_i$ followed by decay toward baseline levels (or below). Subsequently returning $[Ca^{2+}]_o$ to 1 mM after 30 or 60 sec led to a secondary increase in $[Ca^{2+}]_i$, up to a plateau level similar to that reached in typical control experiments in 1 mM Ca^{2+} hypotonic solution. This indicates that after 30 or 60 sec from the initiation of swelling, the measured $[Ca^{2+}]_i$ is a function of the driving force for Ca^{2+} entry. Furthermore, these data show that the Ca^{2+} entry pathway activates immediately during swelling and does not inactivate in the continued presence of hypotonic medium.

In Ehrlich ascites tumor cells, evidence has been presented that RVD occurs by the activation of separate Ca^{2+}-dependent K^+ and Cl^- permeabilities [18]. In this tissue, cell shrinkage can be induced under isotonic conditions and in the presence of quinine by the addition of ionophores A23187 and gramicidin. A23187 raises $[Ca^{2+}]_i$ (presumedly) and gramicidin produces a cation transport pathway that overcomes the inhibition of the endogenous cation pathway by quinine. It was found that as the delay between addition of A23187 and gramicidin was prolonged in Ca^{2+}-free medium, gramicidin caused less and less shrinkage [18]. Hoffmann and coworkers attributed this effect to the transient Ca^{2+} activation of the Cl^- permeability, P_{Cl}, since the ionophore-induced increase in $[Ca^{2+}]_i$ would not be sustained in Ca^{2+}-free medium. As the P_{Cl} inactivated with time, the ability to undergo cell shrinkage also inactivated [18]. It is clear that a similar mechanism does not occur in PST. While the swelling-induced release of Ca^{2+} from intracellular stores is transient, the Ca^{2+} entry pathway is not. Therefore, inactivation of another part of the volume-regulatory mechanism must be responsible for the calcium window phenomenon. In contrast, Davis and Finn [7] found that when frog urinary bladder cells were swollen in Ca^{2+}-free media, returning $[Ca^{2+}]_o$ to normal after 5 min in hypotonic solution, but prior to the peak swelling, led to RVD, although studies were not performed to look for a calcium window of longer duration.

In spite of the fact that intracellular calcium in PST cells responded to a change in $[Ca^{2+}]_o$ equally well after 30 and 60 sec, RVD was only activated when Ca^{2+} was returned after 30 sec (*compare* Fig. 3*B* with Fig. 4*B*). With a return to normal $[Ca^{2+}]_o$ after 30 sec, RVD proceeded to a degree that was indistinguishable from that in control swelling experiments with 1 mM Ca^{2+} (Table 4). When $[Ca^{2+}]_o$ was returned after 60 sec, RVD was diminished to the same extent as observed in swelling experiments with 10 μM Ca^{2+}. Thus, RVD was inactivated and insensitive to the level of $[Ca^{2+}]_i$ after 60 sec.

Two conclusions can be drawn from these experiments. First, it is apparent that some portion of the

mechanism that usually links a rise in $[Ca^{2+}]_i$ to cell shrinkage undergoes inactivation within 60 sec of swelling. Second, these data (and previous data [25]) also indicate that the activation of the RVD process in proximal straight tubule cells involves more than just calcium. This conclusion is supported by the lack of significant cell shrinkage induced by ionomycin + Ca^{2+} under isotonic conditions when $[Ca^{2+}]_i$ alone is elevated (Fig. 5). In this respect, Ca^{2+} may serve a modulatory function in controlling RVD, such that the actions of Ca^{2+} comprise only one part of a multi-branched pathway that leads to activation of the RVD process (*see* [24]).

The results in this study show that cell swelling leads to an increase in intracellular free calcium concentration by a dual mechanism involving both Ca^{2+} entry and Ca^{2+} release from intracellular stores. Transient Ca^{2+} release occurs immediately upon swelling but does not generate sufficient cytosolic calcium ions to support sustained volume regulation. However, the initial release of Ca^{2+} from intracellular stores may turn out to be important in the chain reaction leading to activation of the K^+ and Cl^- conductances which underlie regulatory volume decrease. Ca^{2+} entry is also activated early on during swelling, but does not inactivate over several minutes. The sustained activation of the Ca^{2+} entry pathway affords continued generation of cytosolic Ca ions, which appears to be responsible for sustained activation of the K^+ and/or Cl^- conductances that lead to RVD. The Ca^{2+} entry pathway, therefore, is the basis for the extracellular Ca^{2+} dependence of RVD in this tissue. In the accompanying paper [24], the Ca^{2+} entry pathway is characterized by its inhibition by Ca^{2+} channel blockers, and is related to the control of steady-state $[Ca^{2+}]_i$ as well as swelling-induced changes in $[Ca^{2+}]_i$.

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