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Calcium-Dependent Control of Volume Regulation in Renal Proximal Tubule Cells: II. Roles of Dihydropyridine-Sensitive and -Insensitive Ca^{2+} Entry Pathways

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Summary. The Ca^{2+} entry pathways in the basolateral plasma membrane of the isolated, nonperfused proximal straight tubule (PST) of rabbit kidney were investigated using fura-2 fluorescence microscopy. Under isotonic conditions, reduction of bath $[Ca^{2+}]$ from 1 mM to 1 μM caused intracellular free calcium concentration ($[Ca^{2+}]_i$) to fall close to zero. Treatment with 10 μM verapamil, a calcium channel blocker, had a similar effect. Treatment with verapamil or low Ca^{2+} also induced fluctuations in cell volume. However, isotonic treatment with 10 μM nifedipine, a dihydropyridine (DHP)-type calcium channel blocker, did not affect $[Ca^{2+}]_i$ or cell volume, indicating that the endogenous Ca^{2+} entry pathway is verapamil-sensitive but DHP-insensitive. When cells were exposed to hypotonic solutions in the presence of 1 mM Ca^{2+}, they swelled and underwent normal RVD while $[Ca^{2+}]_i$ increased transiently to a peak before decreasing to a late phase plateau level above the baseline level (*see* McCarty, N.A., O'Neil, R.G. 1991. *J. Membrane Biol.* **123**:149–160). When cells were swollen in the presence of verapamil or low bath $[Ca^{2+}]$, RVD was abolished and $[Ca^{2+}]_i$ fell well below the baseline during the late phase response. In contrast, when cells were swollen in the presence of nifedipine, RVD and the late phase rise in $[Ca^{2+}]_i$ were abolished, but $[Ca^{2+}]_i$ did not fall below the baseline level in the late phase, indicating that nifedipine inhibited the swelling-induced Ca^{2+} entry but that Ca^{2+} entry by another pathway was undisturbed. It was concluded that PST cells are characterized by two Ca^{2+} permeability pathways in the basolateral membrane. Under both isotonic and hypotonic conditions, Ca^{2+} entry occurs at a slow rate via a verapamil-sensitive, DHP-insensitive "baseline" Ca^{2+} entry pathway. Cell swelling activates a separate DHP-sensitive, verapamil-sensitive Ca^{2+} entry pathway, which is responsible for the supply of Ca ions to the Ca^{2+}-dependent mechanism by which cell volume regulation is achieved.

Key Words intracellular calcium · volume regulation · regulatory volume decrease · nifedipine · dihydropyridine · verapamil · calcium fluctuation · renal proximal tubule · kidney tubule

Introduction

Upon exposure to hypotonic medium, many cells achieve regulatory volume decrease (RVD) by the activation of separate conductive pathways for K^+ and for Cl^- [2, 6]. It is clear that Ca^{2+} plays an important role in the control of this response, since RVD has been shown in many cells to be Ca^{2+} dependent [15]. On the basis of the dependency of RVD upon extracellular Ca^{2+}, it has been assumed in most cases that extracellular calcium serves as the source for an increase in intracellular calcium, requiring Ca^{2+} to enter across the plasma membrane by some unknown pathway.

RVD in the rabbit proximal straight tubule (PST) is highly dependent upon the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$), with half-maximal inhibition of the ability to undergo RVD being produced by only a 10-fold reduction in $[Ca^{2+}]_o$ to 100 μM [11]. This inhibition of RVD by low Ca^{2+} was mimicked by swelling in the presence of the calcium channel blockers verapamil, lanthanum, and nifedipine, implicating a role for Ca^{2+} entry. In the accompanying paper [13], it was shown that cell swelling in PST leads to an increase in $[Ca^{2+}]_i$ that is characterized by two distinct phases. The first is a transient maximal increase in $[Ca^{2+}]_i$ (from 136 to 413 nM) which is partly related to release of Ca^{2+} from intracellular stores, but is not sustained and decays rapidly toward baseline levels. The second phase is a sustained elevation of $[Ca^{2+}]_i$ above pre-swelling levels (269 nM), which does not decay over several minutes. This sustained phase is supported by Ca^{2+} entry across the plasma membrane by an unknown mechanism. It is the sustained phase that is responsible for the sensitivity of RVD in PST to extracellular calcium reduction [13].

The present study had the following goals: (i) to characterize the Ca^{2+} entry pathways of PST cells in the steady state; (ii) to characterize the swelling-induced Ca^{2+} entry pathway with pharmacological inhibitors; and (iii) to determine the relationship between Ca^{2+} entry pathways and the

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control of cell volume. Portions of these data have been presented in abstract form [12].

Materials and Methods

General

Isolated, nonperfused rabbit proximal straight tubules (S_2 segment) were used in this study. All aspects of renal tissue preparation and dissection of individual tubules were the same as those described in the accompanying paper [13].

Solutions

All solutions used in this study were the same as those outlined in the companion paper [13], except as described below. Verapamil and nifedipine were added to the normal hypotonic solution containing 1 mM Ca^{2+}, to a final concentration of 10 μM in each case. Verapamil stock solutions were prepared by the dissolution of verapamil-HCl (Sigma Chemical, St. Louis, MO) in distilled water at 10 mM concentration. Nifedipine stocks were prepared at 10 mM concentration in ethanol. Nifedipine was obtained from Calbiochem (La Jolla, CA). Care was taken to avoid exposure of solutions containing these drugs to ambient light, since they are light-labile compounds. Neither of these compounds exhibited fluorescence in the range of excitation and emission wavelengths used in fura-2 studies. In experiments where tubules were incubated in the presence of nifedipine or verapamil, these compounds were added to the normal isotonic solution with 1 mM Ca^{2+}. In experiments involving pre-exposure to low Ca^{2+} or Ca^{2+} channel blockers, pre-incubation was performed for 1.5 to 3 min.

Measurement of Intracellular Ca^{2+} and Cell Volume

Intracellular calcium was measured using the fura-2 fluorescence ratioing technique, as described in detail previously [11], and in brief in the accompanying manuscript [13]. Cells were loaded with fura-2, and $[Ca^{2+}]_i$ was measured by excitation at 340 and 380 nm while the fluorescence emission at 510 nm was recorded with a microspectrofluorimetry work station [11]. All aspects of dye loading, in vitro calibration, and calculation of free calcium concentration were as described [13]. Tubule images were recorded by a video camera and stored on tape for subsequent analysis of cell colume. Cell volumes were calculated from the diameter of the tubule measured at three points along the length of the tubule in the field of view (~45 μm). A major advantage of the microspectrofluorimetry work station used in this study is that it allows simultaneous measurements of $[Ca^{2+}]_i$ and cell volume from the same group of cells. Thus, variations in one parameter can be correlated with variations in the other.

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.

Table 1. Effects on $[Ca^{2+}]_i$ and cell volume of Ca^{2+} channel blockers and low $[Ca^{2+}]_o$ under isotonic conditions

Treatment	Baseline $[Ca^{2+}]_i$	Plateau $[Ca^{2+}]_i$	Plateau[a] $\Delta[Ca^{2+}]_i$	Volume changes	n
1 μM Ca^{2+}	165 ±23	66[b] ±10	−99 ±23	Yes	5
Verapamil, 10 μM	179 ±22	125[b] ±10	−54 ±21	Yes	4
Nifedipine, 10 μM	154 ±33	169[ns] ±30	15 ±9	No	4

Values for $[Ca^{2+}]_i$ are in nM.
Tubules were pre-incubated in low Ca^{2+} or in the presence of verapamil or nifedipine for 1.5 to 3 min.
[a] Plateau $\Delta[Ca^{2+}]_i$ (nM) is given as the change in $[Ca^{2+}]_i$ with respect to baseline values measured immediately before the treatments indicated. Plateau values are from the average measurements during the last 20 sec of a 3-min treatment.
[b] $p \leq 0.05$ or not significant ([ns]) compared to baseline (paired t test).

Results

Ca^{2+} Entry Pathways in the Isotonic State

Prevous work [11] indicated that RVD was inhibited by the calcium channel blockers verapamil, lanthanum, and nifedipine, implicating the involvement of dihydropyridine-sensitive Ca^{2+} channels in Ca^{2+} entry during swelling and volume regulation. For this study, the activity of these Ca^{2+} permeability pathways during the steady-state isotonic condition before swelling was determined. The results are summarized in Fig. 1 and Table 1.

When $[Ca^{2+}]_o$ was reduced to 1 μM in isotonic solution, $[Ca^{2+}]_i$ fell rapidly below the baseline level, usually reaching a plateau minimum in about 3 min (Fig. 1*A*). This effect on $[Ca^{2+}]_i$ was immediate. The dramatic fall in $[Ca^{2+}]_i$ induced by a 10^3-fold drop in $[Ca^{2+}]_o$ indicates that baseline $[Ca^{2+}]_i$ is determined, in part, by at least a small rate of Ca^{2+} entry even before swelling. Exposure to 10 μM verapamil in isotonic solution with 1 mM Ca^{2+} had a strikingly similar effect (Fig. 1*C*), indicating that verapamil may have blocked the endogenous Ca^{2+} entry pathway. However, treatment with 10 μM nifedipine in isotonic solution did not cause a reduction in $[Ca^{2+}]_i$ (Fig. 1*E*) and, hence, may not act on the same Ca^{2+} channels as verapamil in the isotonic state.

Since the microspectrofluorimetry work station used in this study allows simultaneous measurements to be made of $[Ca^{2+}]_i$ and cell volume, it was possible to determine the effects of alterations in the Ca^{2+} entry pathway on cell volume in the isotonic state. As shown in Fig. 1*B*, exposure to isotonic

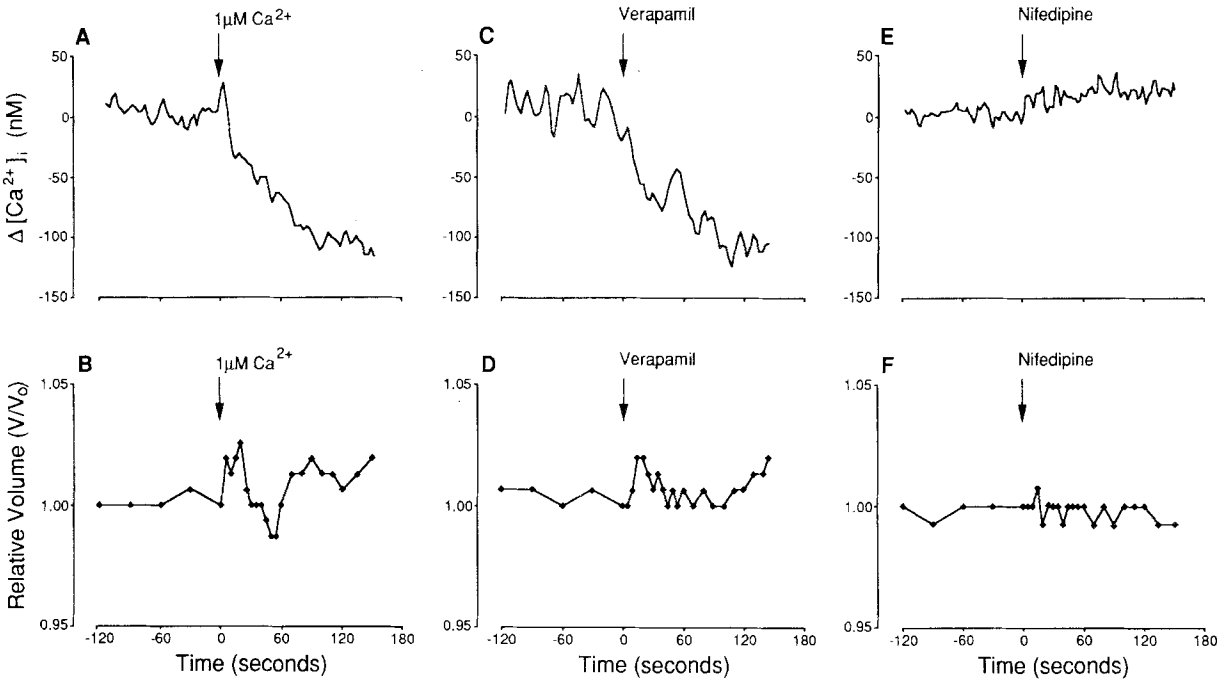

Fig. 1. Composite figure showing the effects of bath $[Ca^{2+}]$ reduction and Ca^{2+} channel blocker administration upon $[Ca^{2+}]_i$ (*A,C,E*) and cell volume (*B,D,F*), measured simultaneously in the steady state. Isotonic exposure to 1 μM Ca^{2+} (*A,B*) led to a rapid drop in $[Ca^{2+}]_i$ which was associated with induction of fluctuations in cell volume. Administration of 10 μM verapamil (*C,D*) had similar effects. Administration of 10 μM nifedipine (*E,F*), a dihydropyridine, was largely without effects on $[Ca^{2+}]_i$ and volume.

solution with 1 μM Ca^{2+} caused immediate variations in cell volume. In each of five cases, the initial fall in $[Ca^{2+}]_i$ induced by 1 μM bath Ca^{2+} was associated with cell swelling (up to 3%). Following a variable period of volume fluctuation, the end of the incubation period was characterized by a general trend for increased cell volume above the baseline isotonic volume. The same results were seen in four experiments using exposure to verapamil (Fig. 1*D*). The initial drop in $[Ca^{2+}]_i$ was associated with cell swelling (up to 2.5%), followed by volume fluctuation and a final trend toward a sustained increase in cell volume. In contrast, cell volume was largely unaffected by exposure to nifedipine in the isotonic state (Fig. 1*F*), indicating that the Ca^{2+} entry pathway that is active in the isotonic state is verapamil sensitive and nifedipine insensitive.

Ca^{2+} Entry Pathways during Cell Swelling

In the companion paper [13], it was shown that acute exposure to hypotonic solution with 10 μM or 1 μM Ca^{2+} caused release of Ca^{2+} from internal stores. Further evidence for this swelling-induced Ca^{2+} release is given in Fig. 2. Pre-incubation in 1 μM Ca^{2+} isotonic solution led to a rapid fall in $[Ca^{2+}]_i$, as above. When tubules were then swollen in hypotonic solution, a transient increase in $[Ca^{2+}]_i$ was seen that averaged 86 nM above the pre-swelling level (Table 2). Since extracellular $[Ca^{2+}]$ was already reduced below the point where swelling-induced Ca^{2+} entry supports elevation of $[Ca^{2+}]_i$, the observed transient increase is likely due to release of stored Ca^{2+}. The transient increase shown without pre-incubation in low bath Ca^{2+} (71 nM [13]) is not different from the increase of 86 nM observed with pre-incubation ($p = 0.548$, unpaired t test), indicating that the transient increase in the absence of pre-incubation is not due to entry of residual calcium from an unstirred layer around the tubule [11]. Thus, the source of calcium for this part of the response is intracellular. However, the transient increase was followed by a drop in $[Ca^{2+}]_i$ back to the pre-incubation baseline before swelling (61 nM, Table 2), which is well below the normal isotonic baseline values in the presence of 1 mM Ca^{2+}. RVD was inhibited during swelling in the presence of only 1 μM Ca^{2+}.

Likewise, after pre-incubation in 10 μM verapamil, swelling in hypotonic solution with 1 mM Ca^{2+} and 10 μM verapamil also led to a transient increase in $[Ca^{2+}]_i$ above the new baseline (Fig. 3*A*). Following the transient, $[Ca^{2+}]_i$ fell to the same pre-incubation baseline level as before swelling, or below, indicating that swelling-induced Ca^{2+} entry was

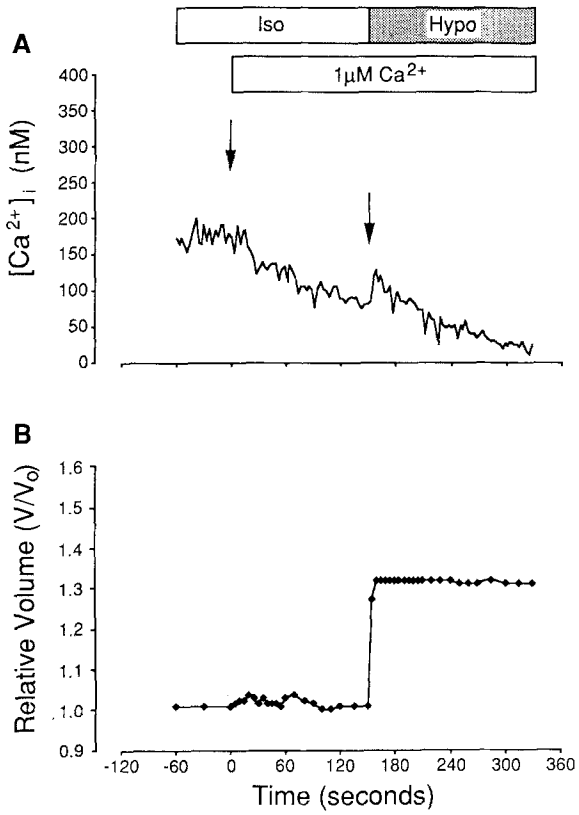

Fig. 2. Swelling-activated Ca^{2+} release and Ca^{2+} entry in tubules pre-exposed to 1 μM Ca^{2+}. (*A*) $[Ca^{2+}]_i$, and (*B*) relative cell volume. Pre-incubation in low Ca^{2+} isotonic solution led to $[Ca^{2+}]_i$ reduction and volume fluctuations, as in Fig. 1. Subsequently, swelling in low Ca^{2+} hypotonic solution (*Hypo*) led to a transient increase in $[Ca^{2+}]_i$, due to release from intracellular Ca^{2+} stores, followed by a fall to the pre-swelling baseline. RVD was inhibited under these conditions.

inhibited by verapamil, while Ca^{2+} release was not affected by verapamil (Table 2). RVD was also inhibited by swelling in the presence of verapamil (Fig. 3*B*). This inhibitory effect of verapamil on RVD was previously shown to be due to blockade of Ca^{2+} entry, since the Ca^{2+} ionophore ionomycin could overcome this block [11].

In previous studies, nifedipine inhibited RVD only partially at 10 μM concentration when the tubule was not pre-incubated with the drug [11]. This was attributed to lack of sufficient time for nifedipine binding to occur. In the present study, the actions of nifedipine were compared in experiments with (Fig. 4) or without (Fig. 5) pre-incubation. When tubules were swollen in the presence of 1 mM Ca^{2+} and 10 μM nifedipine without pre-incubation, $[Ca^{2+}]_i$ rose by 103 nM and then fell to a plateau that was slightly above the original baseline ($p = 0.048$, paired t test) (Table 2). RVD was partially inhibited (%RVD = 8.5). When tubules were pre-incubated with nifedipine before swelling, the transient $[Ca^{2+}]_i$ increase was 76 nM above baseline, and was followed by a fall to a level indistinguishable from baseline ($p = 0.420$, paired t test). RVD was completely inhibited by nifedipine with pre-incubation (0.4% RVD). Thus, pre-incubation caused only a minor enhancement of nifedipine blockade of the initial transient increase in $[Ca^{2+}]_i$ but improved the block of nifedipine on the late plateau phase and, therefore, apparently improved the block of RVD. It is also apparent from these data that 1.5 to 3 min is sufficient time for effective binding of nifedipine.

The actions of nifedipine on the plateau level of $[Ca^{2+}]_i$ in a 3-min exposure to hypotonic solution also appears to differ from that observed for verapamil or low Ca^{2+}. Using pooled data from nifedipine-treated tubules with and without pre-incubation, the plateau $[Ca^{2+}]_i$ was 179 ± 8.8 nM ($n = 12$), compared to 115 ± 26 ($n = 4$) in tubules swollen with verapamil pre-incubation (unpaired t test, $p < 0.01$), or nifedipine-treated tubules compared to pooled data from 1 μM Ca^{2+}-treated tubules with and without pre-incubation, where plateau $[Ca^{2+}]_i = 70 \pm 7.5$ nM ($n = 14$, unpaired t test, $p < 0.001$). Thus, these data demonstrate that while nifedipine does block a component of swelling-induced Ca^{2+} entry, a nifedipine-insensitive component of Ca^{2+} entry remains which is inhibitable by verapamil and low Ca^{2+} bathing solutions. In this regard, it would have been helpful to see the combined effects of nifedipine and verapamil on the late phase of the swelling-induced increase in $[Ca^{2+}]_i$, experiments not performed in the present study. In one experiment, however, a tubule was swollen in the presence of nifedipine and EGTA to chelate exogenous Ca^{2+}. In this case, $[Ca^{2+}]_i$ fell well below the pre-swelling baseline, indicating additive effects over the nifedipine-inhibited Ca^{2+} entry. Furthermore, these data show that swelling-induced Ca^{2+} entry occurs by a pathway that is sensitive to verapamil and to the 1,4-dihydropyridine type Ca^{2+} channel blocker nifedipine. Thus, it appears that the endogenous Ca^{2+} entry pathway that is active in the isotonic state may differ from the pathway that is activated upon swelling.

These experiments also indicate that swelling-induced Ca^{2+} entry, and not swelling-induced Ca^{2+} release, is responsible for establishing the magnitude of cell volume regulation in hypotonic media. This relationship is indicated in Fig. 6, which shows that the extent of RVD (%RVD) is correlated with the extent of swelling-induced Ca^{2+} influx, as indicated by the correlation coefficient between %RVD and the change in the plateau level of $[Ca^{2+}]_i$ over baseline ($r = 0.756$, $p \ll 0.01$). In contrast, the correlation coefficient of the relationship between the *peak*

Table 2. Effects on $[Ca^{2+}]_i$ and RVD of Ca^{2+} channel blockers and low $[Ca^{2+}]_o$

Treatment	Baseline $[Ca^{2+}]_i$	Peak[b] $\Delta[Ca^{2+}]_i$	Plateau		% RVD
			$[Ca^{2+}]_i$	$\Delta[Ca^{2+}]_i$	
1 μM Ca^{2+}, pre-incubated (n = 5)	66[a] ±10	86 ±23	61 ±15	−7 ±13	4.8 ±2.0
Verapamil, pre-incubated (n = 4)	125[a] ±10	136 ±20	115 ±26	−10 ±13	0.7 ±0.7
Nifedipine, pre-incubated (n = 4)	169[a] ±30	76 ±2	189 ±22	20 ±21	0.4 ±0.4
Nifedipine, no pre-incubation (n = 8)	147[c] ±9	103 ±17	174 ±8	27 ±12	8.5 ±2.9

Values for $[Ca^{2+}]_i$ are in nM.
Tubules were either pre-incubated for 1.5–3.0 min in the conditions given or not pre-incubated, as indicated. The baseline $[Ca^{2+}]_i$ was taken during the last 20 sec of the indicated treatment.
[a] Baseline values indicated are repeated for comparison, from Table 1.
[b] Peak $\Delta[Ca^{2+}]_i$ is given as the maximum change in $[Ca^{2+}]_i$ with respect to baseline values measured immediately before swelling. Peak values are the highest values recorded during the treatment.
[c] All values other than the baseline $[Ca^{2+}]_i$ in *Nifedipine, no pre-incubation* were significantly different from control experiments in 1 mM Ca^{2+} and the absence of inhibitors ($p \leq 0.05$, unpaired t test) (data from [13]).

change in $[Ca^{2+}]_i$ and %RVD is $r = -0.096$. These observations indicate the extreme dependence of the RVD mechanism upon a *sustained* increase in $[Ca^{2+}]_i$. In the absence of a sustained increase in $[Ca^{2+}]_i$, the RVD machinery is incapable of effecting sufficient solute efflux to achieve RVD. However, it may be that the transient release of intracellular Ca^{2+} serves some other function, such as limiting the initial extent of swelling in response to hypotonic shock.

Ca^{2+} Fluctuations

It was observed previously that intracellular calcium undergoes spontaneous fluctuations, even before swelling [11]. These fluctuations were investigated further in the present study, in an effort to determine their origin. As shown in Fig. 7, $[Ca^{2+}]_i$ fluctuations around the mean are consistently seen in the isotonic state. The amplitude of the fluctuations in the example given is approximately 40 nM, but this varied between tubules (range 15 to 70 nM). Upon exposure to hypotonic solution with 1 mM Ca^{2+}, the fluctuations continued with an increased amplitude of approximately 70 nM. The peak-to-peak period did not differ between isotonic and hypotonic conditions. Over ~150 sec, 9 waves occurred: period = 17.1 ± 2.7 sec (SD) and period = 17.6 ± 2.4 sec, in isotonic and hypotonic conditions, respectively. As shown in Figs. 2*A* through 5*A*, $[Ca^{2+}]_i$ fluctuations continued to occur in the presence of low bath Ca^{2+} and in the presence of Ca^{2+} channel blockers verapamil and nifedipine. In many cases, the $[Ca^{2+}]_i$ fluctuations appeared to dampen as $[Ca^{2+}]_i$ fell considerably below baseline, close to zero. Some treatments may have affected the period of the fluctuations, such that the waves became more "flickery" (e.g., nifedipine in Fig. 5*A*). However, since the fluctuations were maintained in the presence of these blockers, it would appear that they have their origin at an intracellular site and may be related to release and sequestration of Ca^{2+} into intracellular stores. Ca^{2+} channel blockers and bath $[Ca^{2+}]$ reduction may impose their limited effects by affecting the resupply of $[Ca^{2+}]_i$ from the extracellular milieu. It is important to note that under no circumstance were the spontaneous fluctuations in $[Ca^{2+}]_i$ directly associated with fluctuations in cell volume. This is in contrast to the hormone-induced changes in $[Ca^{2+}]_i$ in parotid cells, where $[Ca^{2+}]_i$ oscillations are of much lower frequency and are very closely associated with changes in cell volume [3].

Discussion

Upon exposure to hypotonic solution, proximal straight tubule (PST) cells swell to a peak volume and then undergo a regulatory volume decrease (RVD) over the course of the next several minutes. As in most mammalian cells, RVD is achieved by the activation of separate conductive pathways for

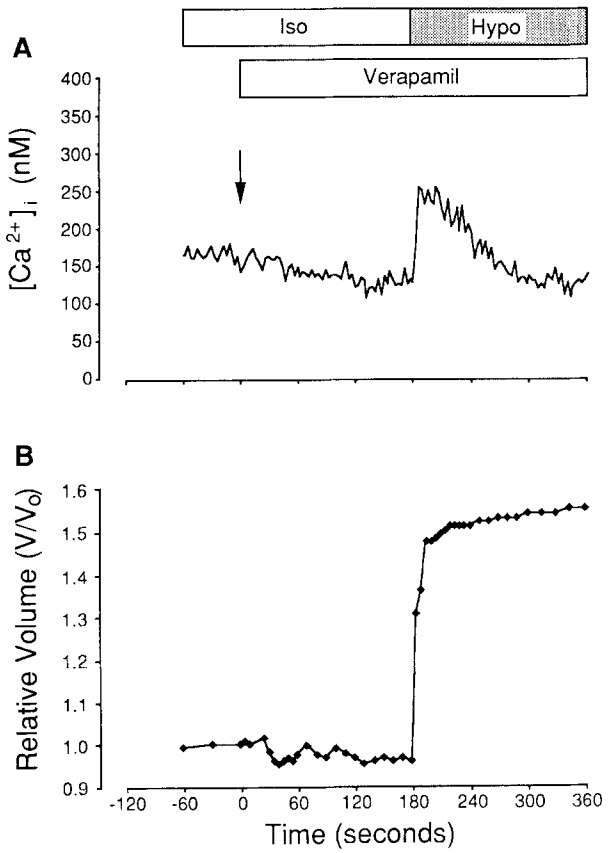

Fig. 3. Swelling-activated Ca^{2+} release and Ca^{2+} entry in tubules pre-exposed to verapamil. (*A*) $[Ca^{2+}]_i$, and (*B*) relative cell volume. Pre-incubation in isotonic solution with 10 μM verapamil led to $[Ca^{2+}]_i$ reduction and volume fluctuations, as in Fig. 1. Subsequently, swelling in hypotonic solution (*Hypo*) with 1 mM Ca^{2+} in the continued presence of verapamil led to a transient increase in $[Ca^{2+}]_i$, due to verapamil-insensitive release from intracellular Ca^{2+} stores, followed by a fall in $[Ca^{2+}]_i$ back to the preswelling baseline. RVD was completely blocked under these conditions.

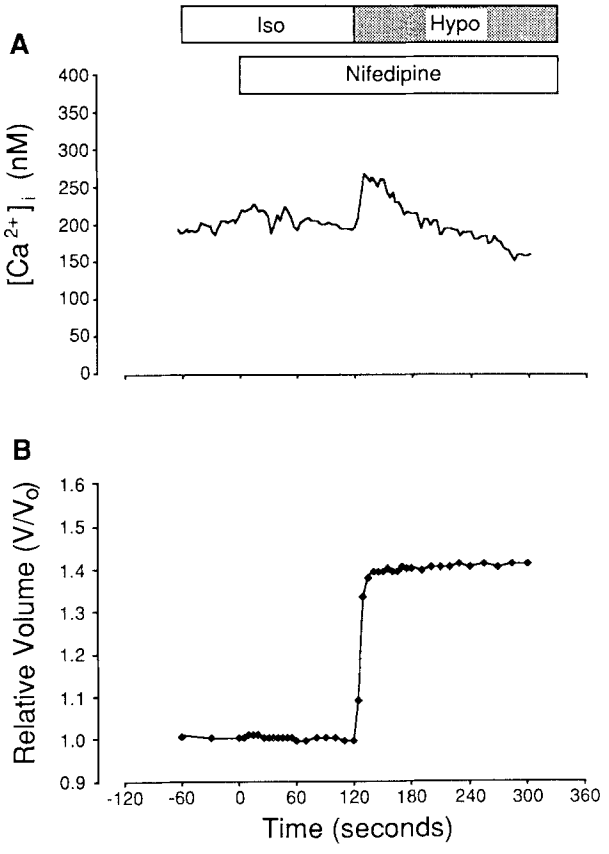

Fig. 4. Swelling-activated Ca^{2+} release and Ca^{2+} entry in tubules pre-exposed to nifedipine. (*A*) $[Ca^{2+}]_i$ and (*B*) relative cell volume. Pre-incubation in isotonic solution with 10 μM nifedipine had no effects on $[Ca^{2+}]_i$ or cell volume. Subsequently, swelling in hypotonic solution (*Hypo*) with the continued presence of nifedipine led to a transient increase in $[Ca^{2+}]_i$, due to nifedipine-insensitive release of stored Ca^{2+}, followed by a fall back to the baseline $[Ca^{2+}]_i$ before nifedipine treatment, which is equal to the baseline after isotonic nifedipine treatment. On average, the $[Ca^{2+}]_i$ plateau did not fall significantly below the pre-swelling level (Table 2). RVD was completely blocked under these conditions.

K^+ and Cl^-. As KCl leaves the cell, osmotically obliged water follows, allowing a return toward preswelling volume.

The mechanism leading to activation of the ion conductances in RVD is poorly understood, but is highly dependent upon Ca^{2+} in many tissues. In the PST, previous studies have shown that RVD is inhibited in the absence of extracellular calcium, and that the Ca^{2+} channel blockers lanthanum, nifedipine, nitrendipine, and verapamil mimic the effect of low bath $[Ca^{2+}]$ [11]. The effects of these Ca^{2+} channel blockers are likely due to inhibition of Ca^{2+} channels, and not other channels involved in RVD, since it has been shown previously that the actions of verapamil on RVD can be overcome with the Ca^{2+} ionophore, ionomycin. These effects imply that Ca^{2+} entry occurs from the external milieu into the cell interior. Accordingly, it was found that swelling in the presence of normal calcium is associated with an increase in intracellular free calcium concentration ($[Ca^{2+}]_i$) [11]. Subsequent studies have shown that the increase in $[Ca^{2+}]_i$ observed in hypotonic conditions is characterized by a biphasic response [13]. Cell swelling initially leads to a transient increase in $[Ca^{2+}]_i$ due to release of Ca^{2+} from intracellular stores. This appears to be a low capacity system, since $[Ca^{2+}]_i$ falls back to the baseline rapidly when $[Ca^{2+}]_o$ is low. In the presence of normal $[Ca^{2+}]_o$, the transient increase is followed by maintenance of a sustained increase in $[Ca^{2+}]_i$ above the baseline. This sustained phase is due to Ca^{2+} entry across the plasma membrane. Apparent high rates

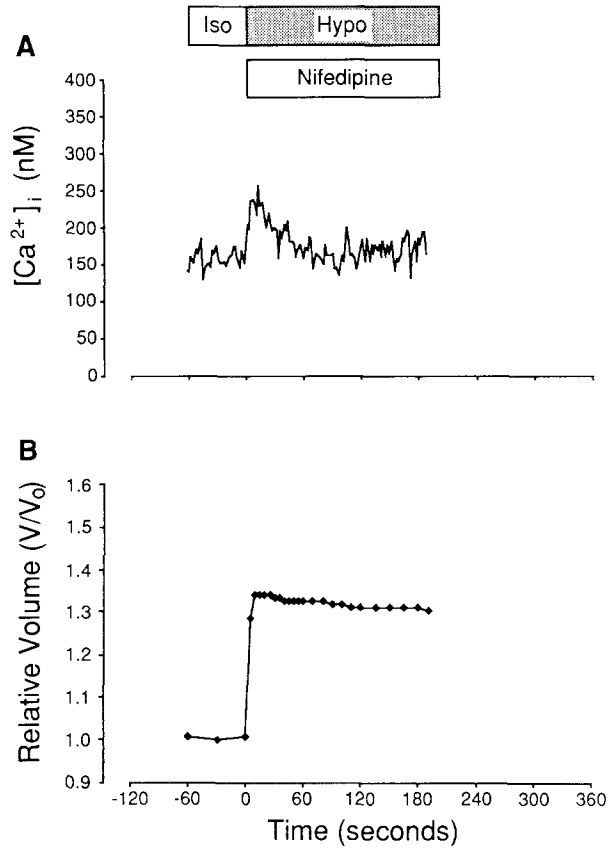

Fig. 5. Swelling-activated Ca^{2+} release and Ca^{2+} entry in tubules not pre-incubated with nifedipine. (*A*) $[Ca^{2+}]_i$, and (*B*) relative cell volume. Swelling in *Hypo* with 10 μM nifedipine without pre-incubation led to a plateau phase that was significantly less than in control swells in the absence of inhibitor, but was significantly greater (on average) than the pre-swelling baseline. RVD was partially inhibited by nifedipine without pre-incubation.

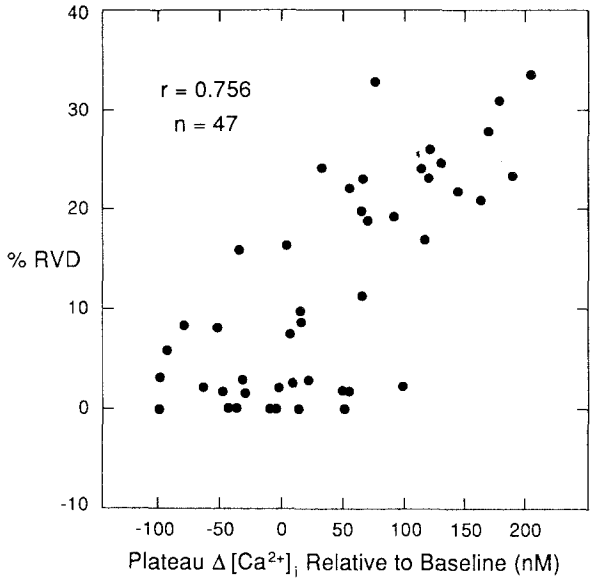

Fig. 6. Relationship between the 3-min plateau calcium concentration during swelling and RVD with the extent of volume regulation (%RVD). Data were pooled from experimental swelling periods (Ca^{2+} channel blockers and low Ca^{2+}) and control swelling periods in the presence of 1 mM Ca^{2+} without inhibitors. The data are presented as the %RVD *vs.* the change in $[Ca^{2+}]_i$ during the plateau phase (Plateau $\Delta[Ca^{2+}]_i$ relative to the isotonic (1 mM Ca^{2+}) baseline. The correlation coefficient for this relationship is $r = 0.756$ ($p \ll 0.01$). The regression equation is

$$\%RVD = 0.124\ (\text{nM Plateau } \Delta[Ca^{2+}]_i) + 9.8$$

($SEM_{(X)} = 1.96$, $SEM_{(Y)} = 11.84$).

of Ca^{2+} influx occur via this pathway, which are responsible for the activation of the ion conductances by which RVD is achieved.

The objective of the present study was to determine the pathways by which intracellular calcium concentration is regulated both under baseline, stead-state conditions and during anisotonic cell volume regulation. To this end, experiments were performed using simultaneous measurements of $[Ca^{2+}]_i$ and cell volume.

Resting Ca^{2+} Entry Pathways in the Isotonic State

Proximal straight tubule cells, like other cells, tightly regulate $[Ca^{2+}]_i$. These cells control $[Ca^{2+}]_i$ by the extrusion actions of a Ca^{2+}-ATPase and a Na^+/Ca^{2+} exchanger in the plasma membrane along with mechanisms of sequestration into and release from intracellular stores [16]. Experiments in the present study indicate the operation of a Ca^{2+} permeability in the plasma membrane during baseline conditions, as well. This conclusion is based upon the sensitivity of $[Ca^{2+}]_i$ to reduction in the driving force for Ca^{2+} entry. Isotonic reduction of bath $[Ca^{2+}]$ from 1 mM to 1 μM leads to an immediate fall in $[Ca^{2+}]_i$ (Fig. 1*A*). This effect was mimicked by isotonic exposure to verapamil (Fig. 1*C*), but not by exposure to the dihydropyridine (DHP) nifedipine (Fig. 1*E*). Treatments that reduced $[Ca^{2+}]_i$ (low Ca^{2+} and verapamil) also caused changes in cell volume, indicating that cell volume is partially regulated by Ca^{2+} even in the isotonic state.

The above experiments indicate that the plasma membrane of PST under steady-state conditions is characterized by a Ca^{2+} permeability, probably a Ca^{2+} channel, that is verapamil-sensitive, but DHP-insensitive. Similar data have been put forth in a few cases. For instance, reduction of bath $[Ca^{2+}]$ led to a decrease in $[Ca^{2+}]_i$ in primary cultures of proximal tubule cells [1, 4, 5, 8, 18], mouse renal juxtaglomerular cells [9], gonadotropes [17], and toad urinary

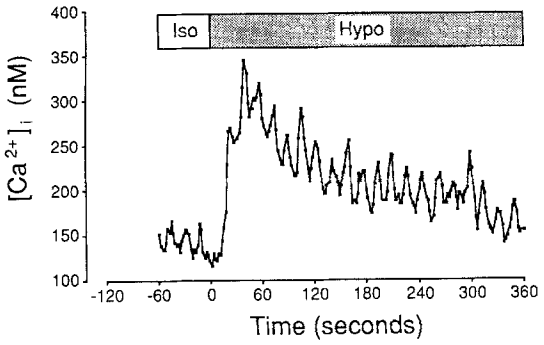

Fig. 7. Fluctuations in $[Ca^{2-}]_i$ occur under both isotonic and hypotonic conditions. Individual data points are shown in this figure to indicate that the frequency of measurement was well within the frequency of the fluctuations. The slow fluctuations in cell volume were not related to these high-frequency $[Ca^{2+}]_i$ fluctuations, but may be related to fluctuations in $[Ca^{2+}]_i$ of a longer period.

bladder cells [19]. The baseline Ca^{2+} permeability in osteosarcoma cells [21] was sensitive to verapamil, nicardipine (another DHP), and diltiazem. Both nifedipine and verapamil led to a decrease in $[Ca^{2+}]_i$ in mesangial cells [22]. In contrast, 3 min incubation with 25 μM verapamil did not reduce $[Ca^{2+}]_i$ in a suspension of canine proximal tubule cells [4].

Cell Swelling-Activated Ca^{2+} Entry Pathway

In osteosarcoma cells [20], swelling-induced Ca^{2+} entry was inhibited by verapamil, lanthanum, and nicardipine. Swelling-induced increases in $[Ca^{2+}]_i$ in toad urinary bladder cells were inhibited completely by 10 μM verapamil after 15 min pre-incubation with the drug [19]. In this same study, nitrendipine (1 μM) partially inhibited the rise in $[Ca^{2+}]_i$. However, the actions of Ca^{2+} channel inhibitors in both of these two studies were on the *peak* $[Ca^{2+}]_i$ levels, not the *plateau* levels. Sustained rates of Ca^{2+} entry leading to a plateau phase of increased $[Ca^{2+}]_i$ were not shown in either case.

The sustained phase of the swelling-induced increase in $[Ca^{2+}]_i$ is absent in PST cells swollen in low Ca^{2+} conditions (Fig. 2*A*) or in the presence of verapamil (Fig. 3*A*). Following the initial transient increase in $[Ca^{2+}]_i$ induced by Ca^{2+} release, $[Ca^{2+}]_i$ fell to the baseline, even when the pre-swelling baseline was already lowered by pre-incubation in verapamil or low Ca^{2+} conditions. This reduction below the original isotonic baseline after completion of the transient may be due to the continued operation of Ca^{2+} extrusion by the plasma membrane Ca^{2+}-ATPase [14]. In contrast to the actions of verapamil or low Ca^{2+}, swelling in the presence of nifedipine led to a plateau phase that was equal to the pre-swelling baseline for tubules pre-incubated with the drug and only slightly above the baseline for tubules not pre-incubated. The improvement of nifedipine blockade of the increase in $[Ca^{2+}]_i$ (and volume regulation) with pre-incubation is probably due to enhanced time for binding of the drug to its active site during pre-incubation. Thus, nifedipine inhibited Ca^{2+} entry relative to control swells in the presence of 1 mM Ca^{2+}. However, the 3-min plateau $[Ca^{2+}]_i$ in hypotonic solution was not below the baseline, as it was with swelling in low Ca^{2+} conditions or in the presence of verapamil. Some Ca^{2+} entry must have been continuing to occur in the presence of nifedipine, since $[Ca^{2+}]_i$ remained at the pre-swelling level. It appears, therefore, that nifedipine is not capable of blocking all components of Ca^{2+} entry that occur during swelling, but is capable of blocking the swelling-induced portion of Ca^{2+} entry and, hence, RVD. The swelling-activated Ca^{2+} entry is, therefore, DHP-sensitive and verapamil-sensitive. Similar dependence of stimulated Ca^{2+} entry on sustained operation of DHP-sensitive Ca^{2+} channels has been shown in several cells, including rat anterior pituitary gonadotropes [17].

Two Ca^{2+} Channels in PST?

Based on the results of the present study, it seems reasonable to conclude that DHP-sensitive Ca^{2+} channels may be responsible for the swelling-activated Ca^{2+} entry observed in PST cells. In contrast, DHP-insensitive Ca^{2+} channels may be involved in baseline Ca^{2+} entry, which occurs in both the isotonic state and the hypotonic state. The data presented herein on the effects of Ca^{2+} channel blockers are consistent with the operation of two separate Ca^{2+} permeability pathways, since the actions of verapamil and nifedipine are different. Verapamil inhibits both baseline Ca^{2+} entry and swelling-induced Ca^{2+} entry. Nifedipine inhibits swelling-activated Ca^{2+} entry only, so that baseline Ca^{2+} entry continues to occur in swollen cells in the presence of nifedipine. Under nifedipine treatment, $[Ca^{2+}]_i$ did not fall below baseline in the late phase of the $[Ca^{2+}]_i$ response to swelling, in spite of the effective block of swelling-activated Ca^{2+} entry, indicating the operation of two parallel pathways. Verapamil is known to block Ca^{2+} channels that are DHP-sensitive in other cells as well as Ca^{2+} channels that are DHP-insensitive [10], while nifedipine appears to block only DHP-sensitive, voltage-activated Ca^{2+} channels of the *L*- or long-lasting type [7].

It appears, therefore, that cell swelling induces

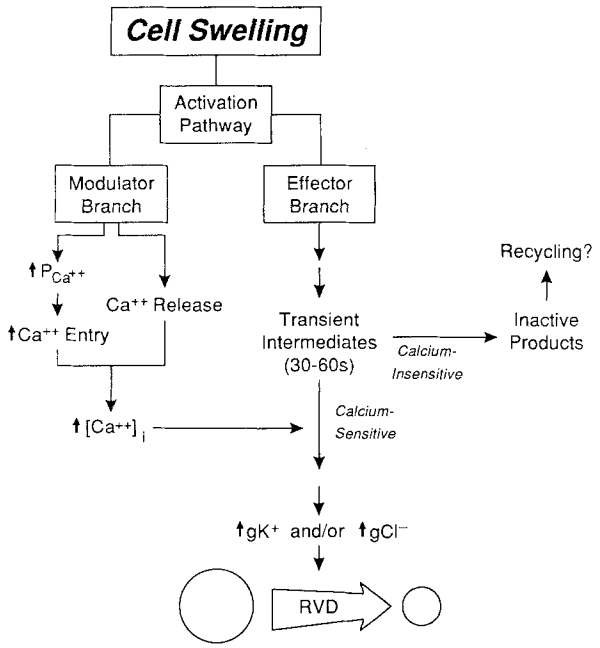

Fig. 8. Model of the mechanism of activation of RVD in proximal straight tubule. The model incorporates the calcium dependence, mechanism of generation of the intracellular calcium signal, and the temporal aspect of calcium's action (the calcium window), as derived from the experiments in this study and the accompanying study [13]. *See* text for details.

Ca^{2+} entry via the activation of a swelling-activated Ca^{2+} channel, and that swelling-induced Ca^{2+} entry is supplemented by continued constitutive or endogenous Ca^{2+} entry at a low rate even during RVD. Since cell volume regulation is inhibited fully by nifedipine after a short pre-incubation, it is clear that Ca^{2+} entry by the supplemental endogenous pathway is not sufficient to support RVD. Rather, it appears that the Ca^{2+} influx which occurs through the DHP-sensitive, swelling-activated Ca^{2+} channel is responsible for supplying Ca^{2+} to activate the machinery that leads to RVD.

Model of the Mechanism of Activation of RVD

The data presented in this study are consistent with a mechanism wherein cell swelling leads to RVD via a bifurcated activation pathway composed of a modulator branch and an effector branch (Fig. 8). Activation of the modulator branch causes the permeability of the basolateral plasma membrane to calcium to increase, supposedly through the opening of swelling-activated DHP-sensitive Ca^{2+} channels. Simultaneously, a transient release of Ca^{2+} from intracellular stores is also activated by swelling. In the presence of sufficient extracellular calcium, Ca^{2+} entry occurs through the swelling-activated Ca^{2+} channels. The combined actions of Ca^{2+} entry and Ca^{2+} release cause a rise in $[Ca^{2+}]_i$. The Ca^{2+} release mechanism is short lived, while the Ca^{2+} entry mechanism supports sustained rates of Ca^{2+} influx over several minutes. Concurrently, the effector pathway is initiated (Fig. 8) which leads to the production of certain "transient intermediates" of presently undetermined character. In the absence of sustained elevation of $[Ca^{2+}]_i$, these intermediates enter an escape pathway by conversion into inactive products which may eventually be recycled. In the presence of elevated $[Ca^{2+}]_i$, the intermediates combine with Ca^{2+} to activate an effector which, through an unknown number of steps, causes the observed increase in K^+ and/or Cl^- conductances, allowing RVD to occur. It is the activation of this proposed effector branch that lends temporal dependence to the activation of RVD and the occurrence of a "calcium window," as described previously [11] and in the accompanying paper [13].

The calcium window phenomenon [11, 13] may be a function of timing between the modulator and effector branches of the activation pathway. If the modulator branch is delayed over 60 sec by lack of sufficient Ca^{2+} entry, the transient intermediates of the effector branch may enter the Ca^{2+}-insensitive escape pathway and thus may not be available for use when $[Ca^{2+}]_i$ is raised after this time. However, alternative explanations are possible. Hence, while the modulator branch of this model has been delineated in the present study, the components of the effector branch and the transient nature of the calcium window phenomenon remain largely unknown.

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