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Extracellular Ca\(^{2+}\) regulates the stimulation of Na\(^{+}\) transport in A6 renal epithelia

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Jans, Danny, Jeannine Simaels, Els Larivière, Paul Steels, and Willy Van Diessche. Extracellular Ca\(^{2+}\) regulates the stimulation of Na\(^{+}\) transport in A6 renal epithelia. Am J Physiol Renal Physiol 287: F840–F849, 2004; 10.1152/ajprenal.00388.2003.—We investigated the involvement of intracellular and extracellular Ca\(^{2+}\) in the stimulation of Na\(^{+}\) transport during hypotonic treatment of A6 renal epithelia. A sudden osmotic decrease elicits a biphasic stimulation of Na\(^{+}\) transport, recorded as increase in amiloride-sensitive short-circuit current (I\(_{sc}\)) from 3.4 ± 0.4 to 24.0 ± 1.3 μA/cm\(^2\) (n = 6). Changes in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) were prevented by blocking basolateral Ca\(^{2+}\) entry with Mg\(^{2+}\) and emptying the intracellular Ca\(^{2+}\) stores before the hypotonic challenge. This treatment did not noticeably affect the hypertonicity-induced stimulation of I\(_{sc}\). However, the absence of extracellular Ca\(^{2+}\) severely attenuated Na\(^{+}\) transport stimulation by the hypotonic shock, and I\(_{sc}\) merely increased from 2.2 ± 0.3 to 4.8 ± 0.7 μA/cm\(^2\). Interestingly, several agonists of the Ca\(^{2+}\)-sensing receptor, Mg\(^{2+}\) (2 mM), Gd\(^{3+}\) (0.1 mM), neomycin (0.1 mM), and spermine (1 mM) were able to substitute for extracellular Ca\(^{2+}\). When added to the basolateral solution, these agents restored the stimulatory effect of the hypotonic solutions on I\(_{sc}\). In the absence of extracellular Ca\(^{2+}\) to levels that were comparable to control conditions. None of the above-mentioned agonists induced a change in [Ca\(^{2+}\)]\(_i\). Quinacrine, an inhibitor of PLA\(_2\), overruled the effect of the agonists on Na\(^{+}\) transport. In conclusion, we suggest that a Ca\(^{2+}\)-sensing receptor in A6 epithelium mediates the stimulation of Na\(^{+}\) transport without the interference of changes in [Ca\(^{2+}\)]\(_i\).

intracellular calcium concentration; magnesium; phospholipase A\(_2\); osmolality

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The activation mechanism that elevates Na\(^{+}\) transport in the renal epithelial A6 cell line in response to changes in osmolality has been the subject of many studies performed in different laboratories, including the team of Wills et al. (7, 19, 38), Marunaka et al. (20–23), and our own group (12). Although already a half-century ago the general concept of Na\(^{+}\) transport in tight epithelia was modeled by Ussing and co-workers (13, 35), the stimulation of Na\(^{+}\) transport by hypotonicity remains poorly understood.

According to the two-membrane hypothesis, tight epithelia, such as those found in the distal tubule of the kidney and in the distal colon, transport Na\(^{+}\) transcellularly in a two-step process (13, 35). Na\(^{+}\) reabsorption is enabled by the Na\(^{+}\)-conductive apical membrane that provides passive entry of Na\(^{+}\) through the amiloride-sensitive epithelial Na\(^{+}\) channel (ENaC) driven by 1) the negative intracellular potential established by the K\(^{+}\)-conductive basolateral membrane and 2) by the activity of the Na\(^{+}\)-K\(^{+}\)-ATPase in the basolateral membrane that is responsible for active extrusion of Na\(^{+}\) from the cell. It has been suggested that upregulation of Na\(^{+}\) pump activity is the initial trigger to boost Na\(^{+}\) transport in the A6 epithelium (20). Within this concept, cell swelling activates tyrosine kinases that upregulate a basolateral Cl\(^{-}\) conductance that stimulates the Na\(^{+}\)-K\(^{+}\)-ATPase, a prerequisite to increase Na\(^{+}\) transport in the A6 epithelium (20). On the other hand, there is more consensus that apical Na\(^{+}\) entry constitutes the rate-limiting step for Na\(^{+}\) transport and, conceivably, for its regulation by hypotonicity as well. Consequently, many investigators have explored several factors that could regulate ENaC activity (32). One cellular component that is well known to modulate ion channel activity is intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)). Recently, it has been reasoned that neural precursor cell-expressed, developmentally downregulated protein (Nedd4) reduces Na\(^{+}\) transport activity. The ubiquitin ligase lowers the number of functional Na\(^{+}\) channels in the apical membrane by catalyzing the conjugation of β-ENaC to ubiquitin, thereby introducing its degradation by the proteasome. Such a concept was indirectly proven to be effective in A6 epithelia (18). Nedd4 translocates from the cytosol to the apical plasma membrane after binding Ca\(^{2+}\) to its C2 domain, allowing a connection of the C2 domain to annexin, thereby enabling interaction of the WW domain of Nedd4 with the PY motif in β-ENaC (26). On the other hand, inactivation of Nedd4 is mediated by phosphorylation, catalyzed by activated serum and glucocorticoid-inducible kinase isofrom 1 (SGK1) (8). In recent years, it has become evident that SGK1 acts as a point of convergence in the stimulation of epithelial Na\(^{+}\) transport in response to hormones, such as aldosterone and insulin (25), but also to other factors, such as hypotonicity, as demonstrated recently in A6 epithelia (33). Nevertheless, insulin and aldosterone elevate [Ca\(^{2+}\)]\(_i\), which is thought to augment the expression of ENaC at the apical membrane (30). Due to a number of conflicting findings, general agreement on whether [Ca\(^{2+}\)]\(_i\) actually enhances or diminishes Na\(^{+}\) transport activity is still lacking. Measurements of [Ca\(^{2+}\)]\(_i\) changes in fura
2-loaded A6 epithelia showed a biphasic increase in \([\text{Ca}^{2+}]_i\), when polarized A6 cells were subjected to a sudden decrease in osmolality (11). We related the initial rapid phase of the \([\text{Ca}^{2+}]_i\) increase to the release of ATP across the basolateral membrane that occurred as a response to the sudden decrease in osmolality (11). A linear relationship was observed among the degree of cell swelling, the maximal rate of ATP release, and the initial change in \([\text{Ca}^{2+}]_i\) (11). However, suppressing the initial phase of the rise in \([\text{Ca}^{2+}]_i\) by emptying intracellular \(\text{Ca}^{2+}\) stores did not change the stimulation of \(\text{Na}^+\) transport by the decrease in osmolality. A complete suppression of the changes in \([\text{Ca}^{2+}]_i\) during hypotonicity obtained by including Mg\(^{2+}\) in the basolateral bath to block \(\text{Ca}^{2+}\) entry had no effect on the rise of \(\text{Na}^+\) transport in these conditions.

Besides its blocking effect on \(\text{Ca}^{2+}\) influx, basolateral Mg\(^{2+}\) also enhances the stimulation of \(\text{Na}^+\) transport by hypotonic treatment. We reported this stimulatory effect of Mg\(^{2+}\) on \(\text{Na}^+\) transport in a previous paper and suggested the presence of a \(\text{Ca}^{2+}\)-sensing receptor at the basolateral border (11). In this report, we further explore the putative \(\text{Ca}^{2+}\)-sensing receptor at the basolateral membrane of the A6 epithelium. A proper evaluation of the effect of extracellular \(\text{Ca}^{2+}\) requires removal of the divalent cation from the bath, a maneuver that causes the loss of epithelial integrity (15). This problem can be at least partially prevented by treating the tissues with the PKC inhibitor H-7 (6, 14, 16, 17). Removal of extracellular \(\text{Ca}^{2+}\) in the presence of H-7 severely attenuated the activation of \(\text{Na}^+\) transport during a hyposmotic shock. Interestingly, several agonists of the \(\text{Ca}^{2+}\)-sensing receptor (4), i.e., Mg\(^{2+}\), Go\(^{1+}\), neomycin, and spermine, were able to replace \(\text{Ca}^{2+}\) and stimulate \(\text{Na}^+\) transport during hypotonic conditions in the absence of extracellular \(\text{Ca}^{2+}\). The present observations thus confirm our previous suggestions and point to the existence of a \(\text{Ca}^{2+}\)-sensing receptor in the basolateral membrane of A6 cells that is involved in the stimulation of \(\text{Na}^+\) transport.

**MATERIALS AND METHODS**

**Cell culture.** The epithelial A6 cell line is derived from the distal nephron of the African clawed toad *Xenopus laevis*. The cells were a kind gift of Dr. J. P. Johnson (University of Pittsburgh, Pittsburgh, PA). All experiments were carried out in A6 epithelial monolayers that were allowed to polarize on permeable supports (pore size 0.2 μm; Anopore, Nunc Intermed, Roskilde, Denmark). Cell cultures were kept at 28°C in a humidified incubator inflated with 1% CO\(_2\). The growth medium was renewed twice weekly and consisted of a 1:1 mixture of Leibovitz’s L-15 and Ham’s F-12 media, supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO), 3.8 mM L-glutamine, 2.6 mM NaHCO\(_3\), 95 IU/ml penicillin, and 95 μg/ml streptomycin. Cells were seeded at a density of 2.5 × 10\(^5\)/cm\(^2\). The monolayers for the \([\text{Ca}^{2+}]_i\) measurements were used between days 5 and 9 of culturing, whereas those for electrophysiological recordings remained in culture for 8–15 days.

**Solutions and chemicals.** Hypotonic solutions (140 mosmol/kgH\(_2\)O) contained (in mM) 70 Na\(^+\), 2.5 K\(^+\), 2.5 HCO\(_3^-\), 1 Ca\(^{2+}\), and 72 Cl\(^-\) (pH 8.0). Hypotonic \(\text{Ca}^{2+}\)-free solutions contained (in mM) 71 Na\(^+\), 2.5 K\(^+\), 5 HEPES, 2 EGTA, and 68.5 Cl\(^-\) (pH 7.4). Isosmotic solutions (260 mosmol/kgH\(_2\)O) were prepared by adding 65 mM NaCl. Solutions at 200 mosmol/kgH\(_2\)O contained (in mM) 102 Na\(^+\), 2.5 K\(^+\), 2.5 HCO\(_3^-\), 1 Ca\(^{2+}\), and 104 Cl\(^-\). A hypotonic shock was elicited by a sudden decrease in basolateral osmolality, whereas the osmolality of the apical perfusate was lowered to 140 mosmol/kgH\(_2\)O at least 30 min in advance. The water impermeability of the apical membrane of the A6 cells allows this procedure. Experiments with solutions at 200 mosmol/kgH\(_2\)O were bilaterally equiosmolar all the time.

Mg\(^{2+}\) was added as a chloride salt and, in case its concentration exceeded 2 mM, equiosmolar NaCl was omitted. Similar steps were taken when 10 mM CaCl\(_2\) was included in the medium. Neomycin, H-7, spermine, and quinacrine were purchased from Sigma.

**Measurements of \([\text{Ca}^{2+}]_i\).** A6 cells, grown on permeable supports for at least 5 days, were incubated with an apical solution containing 10 μM fura 2-AM (Sigma) and 0.2 g/l pluronic acid (F-127, Molecular Probes, Eugene, OR) for 120 min at 28°C in 1% CO\(_2\). The excess dye was washed off gently before the monolayer was placed in an Ussing-type chamber and mounted on the stage of an inverted fluorescence microscope equipped with a ×40 objective (Zeiss LD, Achronplan, Carl Zeiss, Jena, Germany). The apical surface of the epithelium faced toward the objective of the microscope. Both the apical and basolateral surfaces of the monolayer were perfused independently at room temperature. Tissues were excited with light that alternated between 340 and 380 nm. The fluorescence emission at each wavelength was filtered through a band-pass filter centered at 510 nm (6 nm) and detected by photon counting using a photomultiplier tube (Hamamatsu H3400–04, Hamamatsu Photonics, Shizouka, Japan). Photon counts of the emission at each of the excitation wavelengths were corrected for autofluorescence, using signals from monolayers (n = 10) not exposed to the dye. The ratio of corrected fluorescence excited at 340 nm to that excited at 380 nm (i.e., R = I\(_{340}/I_{380}\)) was then used to estimate \([\text{Ca}^{2+}]_i\),

\[
[\text{Ca}^{2+}]_i = \frac{R - R_{\text{min}}}{R_{\text{max}} - R}
\]

where R\(_{\text{max}}\) and R\(_{\text{min}}\) are the corrected fluorescence ratios in the presence of neomycin (5 μM) under saturating and \(\text{Ca}^{2+}\)-free conditions, respectively; K\(_d\) is the dissociation constant of fura 2 for \(\text{Ca}^{2+}\) (9).

**RESULTS**

**Effect of the hypotonic shock on short-circuit current, trans-epithelial conductance, and \([\text{Ca}^{2+}]_i\).** The hypotonic shock was introduced by a sudden decrease in basolateral osmolality from 260 to 140 mosmol/kgH\(_2\)O through the removal of 65 mM NaCl. Figure 1A illustrates the biphasic increases in short-circuit current (\(I_s\)) and transepithelial conductance (\(G_T\)). Similar results are obtained when extracellular osmolality is reduced by removing sucrose from the solutions. The exposure time required to obtain a stable response of \(I_s\) and \(G_T\) was ~60 min. During this time period, \(I_{\text{sc}}\) increased from 3.4 ± 0.4 to 24.0 ± 1.3 μA/cm\(^2\), whereas \(G_T\) was augmented from 0.19 ± 0.05 to 0.33 ± 0.04 mS/cm\(^2\). We measured the amiloride-sensitive part of \(I_s\) in both isosmotic and hypotonic condi-
Effects of removing extracellular Ca\textsuperscript{2+} on \(I_{sc}\) and \(G_T\). The more pronounced stimulation of \(I_{sc}\) in the experiments in which Ca\textsuperscript{2+} stores were depleted and Mg\textsuperscript{2+} blocked basolateral Ca\textsuperscript{2+} entry suppressed the hypotonicity-induced [Ca\textsuperscript{2+}]i changes, than the [Ca\textsuperscript{2+}]i for the \(I_{sc}\) increase during hyposmotic conditions.

We also monitored the effect of the hypotonic shock on [Ca\textsuperscript{2+}]i. During isosmotic conditions, the mean value for [Ca\textsuperscript{2+}]i was 245 ± 12 nM (n = 10). Lowering the osmolality of the apical perfusate to 140 mosmol/kgH\textsubscript{2}O did not result in noticeable changes in [Ca\textsuperscript{2+}]i for up to 30 min (data not shown). The sudden change in hyposmotic solutions at the basolateral border elicited a transient biphasic increase in [Ca\textsuperscript{2+}]i, as illustrated in Fig. 1B. The underlying mechanisms involved in the [Ca\textsuperscript{2+}]i changes during hypotonicity were explored in a previous study (11). In summary, the first phase of the [Ca\textsuperscript{2+}]i changes is caused by the release of Ca\textsuperscript{2+} from intracellular stores, whereas the second phase depends on Ca\textsuperscript{2+} entry from the extracellular solution across the basolateral membrane. Both phases are independent of each other, and blocking one of them does not affect the other. Emptying the intracellular Ca\textsuperscript{2+} stores through the application of three successive basolateral treatments with ATP (0.5 mM), before the hypotonic shock, suppresses the first phase of the [Ca\textsuperscript{2+}]i changes (11). The second phase of the [Ca\textsuperscript{2+}]i changes is abolished by including 2 mM Mg\textsuperscript{2+} in the basolateral solution (11). Figure 2B illustrates that the combination of emptying intracellular Ca\textsuperscript{2+} stores and blocking basolateral Ca\textsuperscript{2+} entry suppresses the hypotonicity-induced [Ca\textsuperscript{2+}]i changes completely. We used this approach to verify the relationship between the [Ca\textsuperscript{2+}]i changes and the stimulation of Na\textsuperscript{+} transport during hypotonicity. Figure 2A demonstrates that the magnitude of the responses of \(I_{sc}\) and \(G_T\) to hypotonicity is not influenced by the inhibition of [Ca\textsuperscript{2+}]i changes. On the contrary, hypotonicity increased \(I_{sc}\) from 1.7 ± 0.2 to 27.0 ± 2.1 µA/cm\textsuperscript{2} and \(G_T\) from 0.10 ± 0.02 to 0.34 ± 0.03 mS/cm\textsuperscript{2} (n = 3). It is noteworthy that the increase in \(I_{sc}\) is larger in this series of experiments, i.e., 14.9 times the control value, than the augmentation recorded in control conditions, i.e., 6.1 times the control value. Importantly, the above observations indicate that the [Ca\textsuperscript{2+}]i changes during the lowered osmotic conditions are not related to the stimulation of Na\textsuperscript{+} transport.

**Fig. 1.** Effects of lowering basolateral osmolality on short-circuit current (\(I_{sc}\)), transepithelial conductance (\(G_T\)), and intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]i). A: responses of \(I_{sc}\) and \(G_T\) (n = 6). [Ami], amiloride concentration; ap, apical. B: response of [Ca\textsuperscript{2+}]i (n = 10). bl, Basolateral. Monolayers were perfused with NaCl-Ringer solutions on the apical and basolateral sides. During the indicated time period, basolateral osmolality was reduced from 260 to 140 mosmol/kgH\textsubscript{2}O by removing 65 mM NaCl. During hypotonicity, apical and basolateral solutions had an identical composition. Solid lines represent the mean values recorded during \(n\) experiments. Dashed lines are means ± SE.
entry could be related to the basolateral effect of Mg$^{2+}$ on Na$^{+}$ transport previously reported (12). In this study, we hypothesized that Mg$^{2+}$ exerted its effect on a Ca$^{2+}$-sensing receptor in the basolateral membrane. To further explore this idea, we intended to evaluate the effect of extracellular Ca$^{2+}$ on the stimulation of $I_{Na}$ by reducing osmolality. Because the presence of Ca$^{2+}$ in the basolateral solution is crucial for epithelial integrity (14), we included the nonspecific protein kinase inhibitor H-7 (50 μM) in the perfusion solutions. At the concentration used in our experiments, H-7 has been described as blocking PKC (6) and preventing excessive increases in $G_{T}$ on removal of extracellular Ca$^{2+}$ (14). Because PKC has been demonstrated to modulate $I_{Na}$ (1, 3), we first evaluated the effects of the blocker on $I_{sc}$ and $G_{T}$.

Figure 3 shows the effects of the hyposmotic shock on $I_{sc}$ and $G_{T}$ in four different conditions. In the presence of extracellular Ca$^{2+}$, but in the absence of extracellular Mg$^{2+}$ and H-7 (solid lines), hypotonicity increased $I_{sc}$ from 3.4 ± 0.3 to 24.9 ± 0.5 μA/cm$^2$ and $G_{T}$ from 0.38 ± 0.02 to 0.77 ± 0.04 mS/cm$^2$ ($n = 4$). The responses of $I_{sc}$ and $G_{T}$ were not markedly altered by applying H-7 to Ca$^{2+}$-containing solutions (dashed lines); their values increased from 2.7 ± 0.3 to 19.1 ± 0.5 μA/cm$^2$ and from 0.27 ± 0.02 to 0.35 ± 0.03 mS/cm$^2$, respectively ($n = 4$). However, a conspicuous inhibition of the activation of Na$^{+}$ transport was observed in conditions of zero Ca$^{2+}$: the hyposmotic shock increased $I_{sc}$ merely from 2.2 ± 0.3 to 4.8 ± 0.7 μA/cm$^2$. The record of $G_{T}$ in Fig. 3 shows that the opening of the paracellular pathway that is observed after removal of Ca$^{2+}$ from the bath was markedly retarded by the H-7 treatment. The $G_{T}$ values remained within limits that still enabled reliable recording of $I_{sc}$. In the absence of H-7, removing Ca$^{2+}$ from the extracellular solutions dramatically increases $G_{T}$ to levels that do not allow for trustworthy electrophysiological recordings (dashed-dotted line in $G_{T}$).

Effect of Ca$^{2+}$-sensing receptor agonists in the absence of extracellular Ca$^{2+}$. The above experiments demonstrate the requirement of extracellular Ca$^{2+}$ for $I_{sc}$ stimulation by hyposmotic shock. Because of the stimulatory effect of Mg$^{2+}$ on $I_{sc}$, we considered that Mg$^{2+}$ could function as a substitute for Ca$^{2+}$ in stimulating Na$^{+}$ transport in response to hypotonicity. Figure 4A illustrates the effects of 2 mM Mg$^{2+}$ on $I_{sc}$ and $G_{T}$ during hyposmotic shock in the absence of extracellular Ca$^{2+}$ and with H-7 in the perfusion solutions. The lack of a stimu-
latory effect of hypotonic solutions on $I_{sc}$ in Ca$^{2+}$-free solutions is overruled by addition of Mg$^{2+}$ to the basolateral bath. With Mg$^{2+}$ the rise in $I_{sc}$ was from 3.4 ± 0.2 to 25.7 ± 0.4 $\mu$A/cm$^2$, whereas $G_T$ was augmented from 0.15 ± 0.01 to 0.51 ± 0.03 mS/cm$^2$ ($n = 4$). This augmentation in $G_T$ only partly covers an increase in apical conductance but also reflects an increase in paracellular conductance. It should be noted that in Ca$^{2+}$-free solutions the integrity of the epithelium was better preserved in the presence of Mg$^{2+}$. Thus far, we observe a stimulation of $I_{sc}$ in hypotonic solutions only in the presence

Fig. 3. Effects of extracellular Ca$^{2+}$ removal on $I_{sc}$ and $G_T$ during hypotonic shock. The hypotonic shock was induced through the removal of 65 mM NaCl from the basolateral bath. Solid line, control experiments performed in Ca$^{2+}$-containing bathing media; dashed line, in the presence of extracellular Ca$^{2+}$ and protein kinase inhibitor H-7; dotted line, bilateral extracellular Ca$^{2+}$-free conditions and with H-7; short-dashed-dotted line in $G_T$, bilateral extracellular Ca$^{2+}$-free conditions and without H-7. H-7 (50 $\mu$M) was applied 30 min before the hypotonic shock was induced. Ca$^{2+}$ was bilaterally removed at the time when hypotonicity was induced ($n = 6$).

Fig. 4. Effect of agonists of the Ca$^{2+}$-sensing receptor on hypotonic stimulation of $I_{sc}$ and $G_T$ in the absence of extracellular Ca$^{2+}$. A: 2 mM Mg$^{2+}$ (dashed line). B: 1 mM spermine (dotted-dashed line) and 0.1 mM neomycin (dashed line). H-7 (50 $\mu$M) was added 30 min before the hypotonic shock was induced. Extracellular Ca$^{2+}$ was removed when the hypotonic shock was applied. Agonists were present in the basolateral bath for the entire duration of the experiment.
of one of the divalent cations, Ca\(^{2+}\) and Mg\(^{2+}\). This kind of behavior is reminiscent of a receptor-like mechanism to be involved in the stimulation of Na\(^{+}\) transport during hyposmotic conditions. The sensitivity to both divalent cations has led to the molecular identification of the Ca\(^{2+}\)-sensing receptor that was originally cloned from the bovine parathyroid gland (4). This finding established Ca\(^{2+}\) as a first messenger in these cells involved in the secretion of parathyroid hormone (4). The Ca\(^{2+}\)-sensing receptor lacks specificity and is also sensitive to the divalent cation Mg\(^{2+}\), to the trivalent cation lanthanides Gd\(^{3+}\) and La\(^{3+}\), and to polyvalent compounds, such as neomycin and spermine. We tested a number of these cations to create an agonist profile for the putative Ca\(^{2+}\)-sensing receptor in A6 cells involved in the stimulation of Na\(^{+}\) transport elicited by hypotonicity in Ca\(^{2+}\)-free conditions. As illustrated in Fig. 4B, the presence of either neomycin or spermine in the basolateral bath is sufficient to stimulate Na\(^{+}\) transport during hypotonicity in the absence of extracellular Ca\(^{2+}\). In the presence of 1 mM spermine, hypotonicity stimulated \(I_{sc}\) and \(G_T\) from 3.3 ± 0.1 to 19.2 ± 0.4 μA/cm\(^2\) and 0.15 ± 0.02 to 0.55 ± 0.05 mS/cm\(^2\) (n = 4), respectively. With 0.1 mM neomycin in the basolateral perfusate during hyposmotic shock, \(I_{sc}\) and \(G_T\) increased from 4.9 ± 0.1 to 18.7 ± 0.5 μA/cm\(^2\) and 0.16 ± 0.03 to 0.52 ± 0.06 mS/cm\(^2\) (n = 4), respectively. Pilot experiments with Gd\(^{3+}\) (0.1 mM) also showed that the lanthanide was able to take over the role of Ca\(^{2+}\). Other divalent cations, such as Ni\(^{2+}\), Cd\(^{2+}\), or Zn\(^{2+}\), were unable to replace Ca\(^{2+}\) in the stimulation of \(I_{sc}\) in response to hypotonic shock.

Additive effects of Ca\(^{2+}\) and Mg\(^{2+}\) on \(I_{sc}\) at constant osmolarity. So far, our experiments underlined the requirement of at least one of the agonists of the Ca\(^{2+}\)-sensing receptor for the activation of \(I_{Na}\) during hyposmotic shock. Activation of the Ca\(^{2+}\)-sensing receptor by extracellular Ca\(^{2+}\) shows positive cooperativity (29). Although the molecular mechanism is undefined, it has been suggested to result from multiple Ca\(^{2+}\) binding sites in the extracellular domain of the receptor. To test positive cooperativity between the agonists of the putative Ca\(^{2+}\)-sensing receptor in A6 cells, we monitored Na\(^{+}\) transport at constant lowered osmolality. Figure 5 illustrates an experiment in which cells were perfused with 200 mosmol/kgH\(_2\)O solutions that contained 1 mM Ca\(^{2+}\). The addition of 10 mM Ca\(^{2+}\) to the basolateral bath increased \(I_{sc}\) from 5.5 ± 0.2 to 10.2 ± 0.3 μA/cm\(^2\) (n = 4), corresponding to a 9% increase in \(G_T\). The effect of 10 mM Mg\(^{2+}\) was more pronounced and resulted in an increase in \(I_{sc}\) from 7.1 ± 0.2 to 15.8 ± 0.3 μA/cm\(^2\) (n = 4), matching a rise in \(G_T\) of 26%. These data suggest that the receptor is more sensitive to Mg\(^{2+}\) than to Ca\(^{2+}\). This also becomes apparent in experiments in which 1 mM Mg\(^{2+}\) was added to solutions that contained 10 mM Ca\(^{2+}\). The additional Mg\(^{2+}\) elevated \(I_{sc}\) from 6.1 ± 0.1 to 13.6 ± 0.3 μA/cm\(^2\) (n = 4), parallel to a 33% increase in \(G_T\). This demonstrates the positive cooperativity between Ca\(^{2+}\) and Mg\(^{2+}\) in stimulating Na\(^{+}\) transport. A similar behavior could be observed for neomycin and spermine in relation to Ca\(^{2+}\) (data not shown).

**Signaling pathway.** Because Na\(^{+}\) transport can be stimulated in the absence of changes in [Ca\(^{2+}\)], (Fig. 2B), it is quite likely that the activation of the putative Ca\(^{2+}\)-sensing receptor in A6 cells does not augment [Ca\(^{2+}\)]. To verify this hypothesis, we recorded the effect of the agonists on [Ca\(^{2+}\)] in response to elevating Ca\(^{2+}\) (Fig. 6A). Figure 6A shows that the addition of 10 mM Ca\(^{2+}\) to 200 mosmol/kgH\(_2\)O solutions increased [Ca\(^{2+}\)] from 370 ± 9 to 580 ± 12 nM (n = 4). However, Mg\(^{2+}\), neomycin, and spermine were all unable to increase [Ca\(^{2+}\)], but rather induced a slow decrease in [Ca\(^{2+}\)] (Fig. 6B). Interestingly, the [Ca\(^{2+}\)] rise observed on increasing Ca\(^{2+}\) in the basolateral bath declined rapidly by adding 1 mM Mg\(^{2+}\) to the solution. This demonstrates that the increase in [Ca\(^{2+}\)] observed in response to elevating Ca\(^{2+}\) in the basolateral perfusion solution is due to Ca\(^{2+}\) entry through the Mg\(^{2+}\)-blockable basolateral Ca\(^{2+}\) entry channels, which are gated to the open state by the reduction in osmolality in solutions of 200 mosmol/kgH\(_2\)O (11), and not via the activation of the Ca\(^{2+}\)-sensing receptor. Indeed, [Ca\(^{2+}\)], was
not increased when 10 mM Ca\(^{2+}\) was added to isosmotic solutions.

The absence of a rise in [Ca\(^{2+}\)]\(_i\) on addition of the receptor agonists suggests that PLC is not activated downstream of the receptor, as is the case for the receptor cloned from bovine parathyroid (4). To test whether the activation of PLA\(_2\) was involved, we evaluated the activity of the agonists in the presence of quinacrine, a common inhibitor of this enzyme. Application of quinacrine (100 \(\mu M\)) at the basolateral border abolished the increase in \(I_{sc}\) completely in response to the hyposmotic shock (Fig. 7). A similar lack of activity of the agonists in stimulating Na\(^{+}/H^+\) transport at constant osmolality could be observed in the presence of quinacrine (data not shown). These results suggest that the stimulation of Na\(^+\) transport by activating the Ca\(^{2+}\)-sensing receptor in A6 epithelia requires the activity of PLA\(_2\).

**DISCUSSION**

The data presented in this paper relate to the initial trigger for the stimulation of Na\(^+\) transport in the renal epithelial cell line A6 in response to a hyposmotic shock. We observed that extracellular Ca\(^{2+}\) functions as an essential component in the basolateral perfusion solution for increasing the rate of Na\(^+\) transport during the hyposmotic challenge. Our findings point to the presence of a Ca\(^{2+}\)-sensing receptor in the basolateral membrane of the A6 epithelium. This suggestion is supported by 1) the requirement for extracellular Ca\(^{2+}\) to stimulate Na\(^+\) transport by hypotonicity and 2) the ability of agonists of the Ca\(^{2+}\)-sensing receptor, i.e., Mg\(^{2+}\), Gd\(^{3+}\), neomycin, and spermine, to replace extracellular Ca\(^{2+}\) at the basolateral membrane and to restore the stimulatory effect of the hyposmotic solutions on Na\(^+\) transport in Ca\(^{2+}\)-free conditions. The activation process has the following properties: 1) the receptor is more sensitive to Mg\(^{2+}\) than it is to Ca\(^{2+}\); 2) receptor activation with the agonists also stimulates Na\(^+\) transport at constant osmolality; and 3) downstream signaling does not require a change in [Ca\(^{2+}\)]\(_i\), but rather activates PLA\(_2\). Unfortunately, molecular identity for the suggested Ca\(^{2+}\)-sensing receptor is at this moment still lacking. So far, attempts that we made with RT-PCR screening of total mRNA were not successful. Failure to achieve a positive outcome in the RT-PCR screening can be attributed to the fact that primer design was based on the Ca\(^{2+}\)-sensing receptor cloned from the gastric mucosa of the amphibian *Necturus maculosus* (5). The *Necturus* Ca\(^{2+}\)-sensing receptor is closely homologous to that of the rat (31), and this receptor signals...
through increases in \([\text{Ca}^{2+}]\). Activation of the putative \(\text{Ca}^{2+}\)-sensing receptor in A6 epithelia does not cause an increase in \([\text{Ca}^{2+}]\). The elevation in \([\text{Ca}^{2+}]\), that is observed during hypotonicity in the A6 cells is related to the stimulation of P2 purinergic receptors in the basolateral membrane, because of ATP release across this border during cell swelling (11). We observed a rise in \([\text{Ca}^{2+}]\), that takes place on increasing the extracellular \(\text{Ca}^{2+}\) concentration when A6 epithelia are perfused with solutions of 200 mosmol/kg H2O. However, this \([\text{Ca}^{2+}]\), increase is caused by \(\text{Ca}^{2+}\) entry across the basolateral membrane and is blocked by Mg2+ in the basolateral bath. Thus the rise in \([\text{Ca}^{2+}]\), on increasing the \(\text{Ca}^{2+}\) concentration in the basolateral bath is not related to the activation of the \(\text{Ca}^{2+}\)-sensing receptor but to the opening of basolateral Mg2+-blockable \(\text{Ca}^{2+}\) entry channels in solutions of lowered osmolality. Addition of agonists of the \(\text{Ca}^{2+}\)-sensing receptor had no effect on \([\text{Ca}^{2+}]\), in A6 epithelia in isosmotnic conditions. Palmer and Frindt (24) studied \(\text{Na}^{+}\) channels in the cortical collecting tubule of the rat and did not observe a direct effect of \(\text{Ca}^{2+}\) on the channel, but they noticed a decrease in ENaC activity on increasing cytosolic \(\text{Ca}^{2+}\) concentration by the use of the \(\text{Ca}^{2+}\)-ionophore ionomycin. Ishikawa et al. (10) observed a similar downregulation of the activity of rat ENaC, expressed in Madin-Darby canine kidney cells, when ionomycin was used to elevate \([\text{Ca}^{2+}]\). Recently, Awayda et al. (2) reported a lack of ENaC activity when using BAPTA to chelate intracellular \(\text{Ca}^{2+}\) and mentioned a possible role of changes in \([\text{Ca}^{2+}]\) for stimulating \(\text{Na}^{+}\) transport in A6 epithelia via either the cellular trafficking machinery or a second messenger, such as PLC. Our observations do not support the idea that activation of PLC regulates ENaC activity through changes in \([\text{Ca}^{2+}]\). Nevertheless, our observations do not exclude a role for \(\text{Ca}^{2+}\) in regulating the activity of the \(\text{Na}^{+}\) channel. It has been observed that reloading of intracellular \(\text{Ca}^{2+}\) stores can take place without an apparent change in \([\text{Ca}^{2+}]\) (27). It is reasonable that local changes in \([\text{Ca}^{2+}]\) exert important regulatory effects on protein conformation and operation.

The \(\text{Ca}^{2+}\)-sensing receptor was originally cloned from chief cells of the bovine parathyroid gland, where increases in extracellular \(\text{Ca}^{2+}\) and Mg2+ concentration diminish the secretion of parathyroid hormone by the cell via a PLC-mediated increase in \([\text{Ca}^{2+}]\) (4). Meanwhile, a \(\text{Ca}^{2+}\)-sensing receptor has been cloned from various cells sharing some of the properties with the original. Although \(\text{Ca}^{2+}\) is the physiological regulator of this receptor, it can also sense and respond to other multivalent cations. The latter include the polyamine spermine and the aminoglycosidic antibiotic neomycin, which at physiological pH possess four and five positive charges, respectively, due to the presence of primary and secondary amine moieties. Agonist binding occurs within the large extracellular domain rather than in a pocket defined by the seven transmembrane helices, as is characteristic of most G protein-coupled receptors. Interestingly, the extracellular domain of the \(\text{Ca}^{2+}\)-sensing receptor and the NMDA receptor channel share limited homology, allowing all \(\text{Ca}^{2+}\)-sensing receptor agonists mentioned here to modulate NMDA channel function. Sensitivity to cationic agonists might involve surface charge-shielding effects. Within this concept, a reduction in ionic strength was described to be a potent activator of the receptor (28). It should be noted that stimulation of \(\text{Na}^{+}\) transport can take place by decreasing the osmolality of the basolateral solution without a change in ionic strength of the solution, for instance, by taking out sucrose. In addition, keeping the osmolality constant by replacing the membrane-impermeant solute sucrose with the membrane-permeant solutes ureum or glycerol will stimulate \(\text{Na}^{+}\) transport to comparable levels in this epithelium. This is conceivable with the intracellular tonicity functioning as the actual trigger for activation of the receptor in A6 cells. The suggested \(\text{Ca}^{2+}\)-sensing receptor that we describe shares the properties of the agonist profile with the original, despite the fact that we observed a higher sensitivity of the receptor for Mg2+ than for \(\text{Ca}^{2+}\).

Stimulation of the receptor by the agonists was inhibited in the presence of quinacrine, suggesting the involvement of PLA2, downstream of the receptor in A6 epithelia. This finding links our observations to the description of the involvement of PLA2 in the stimulation of \(\text{Na}^{+}\) transport (19, 39). Recently, it has been reported for A6 cells that activation of PLA2 at the basolateral border stimulated \(\text{Na}^{+}\) transport, whereas apical activation of the enzyme had the adverse effect (39). Interestingly, the authors suggested a tonic activity of the enzyme, an effect that may result from the continuous presence of \(\text{Ca}^{2+}\) at the basolateral membrane causing a constitutive activity of the receptor. A \(\text{Ca}^{2+}\)-sensing receptor that signals via PLA2 without changing \([\text{Ca}^{2+}]\), has also been reported in the thick ascending limb of the kidney (37). Because activation of \(\text{Na}^{+}\) transport in A6 epithelia by hypotonicity has been demonstrated to be mediated by SGK1 (33), a link between PLA2 and activation of SGK1 would be a next challenge. Possible pathways may involve K-Ras2A, which

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**Fig. 7.** Use of the PLA2 inhibitor quinacrine during hyposmotic shock. Hyposmotic shock was introduced during the indicated time period. The PLA2 inhibitor quinacrine (100 μM) was present in the basolateral bath from the beginning of the experiment. CTRL, control.
was recognized as an early aldosterone-induced protein in A6 cells, or the activation of phosphatidylinositol 3-kinase, which recently has been recognized to be involved in hypertonic stimulation of Na\(^+\) transport in A6 epithelia (33).

In summary, we have substantial evidence for a Ca\(^{2+}\)-sensing receptor in A6 epithelia that mediates stimulation of Na\(^+\) transport during hypotonic shock and at steady osmotic conditions. The lack of a molecular relationship to the cloned Ca\(^{2+}\)-sensing receptor impedes an effective functional evaluation, and hence its possible role in the collecting duct remains difficult to evaluate. Nevertheless, the presence of the putative Ca\(^{2+}\)-sensing receptor that we describe in this paper may represent an important mechanism for the distal nephron to supplement the previous finding of an extracellular Ca\(^{2+}\)-sensing receptor in the apical membranes of cells in the inner medullary collecting duct (34). During periods of diuresis when the renal tubules are filled with primary urine of low tonicity, water leaks toward the interstitial space even in the absence of antidiuretic hormone. Both systems mentioned above may cooperate to prevent urine concentration. Lowered intracellular tonicity of the principal cells of the cortical collecting duct, modeled by the A6 epithelium, may stimulate Ca\(^{2+}\)-sensing receptor-mediated Na\(^+\) transport in this part of the nephron, whereas lowered tonicity in the lumen of the inner medullary sections activates the apical Ca\(^{2+}\)-sensing receptor to diminish local water reabsorption.

GRANTS

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REFERENCES


