Calcium-sensing receptor regulation of PTH-dependent calcium absorption by mouse cortical ascending limbs

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Motoyama, Hiroki I., and Peter A. Friedman. Calcium-sensing receptor regulation of PTH-dependent calcium absorption by mouse cortical ascending limbs. Am J Physiol Renal Physiol 283: F399–F406, 2002.—Resting Ca2+ absorption by mouse cortical ascending limbs (CALS) is passive and proceeds through the paracellular pathway. In contrast, parathyroid hormone (PTH) stimulates active, transcellular Ca2+ absorption (J_{Ca}). The Ca2+-sensing receptor (CaSR) is expressed on basolateral plasma membranes of CALs. In the present study, we tested the hypothesis that activation of the CAL CaSR indirectly inhibits passive Ca2+ transport and directly suppresses PTH-stimulated Ca2+ absorption (J_{Ca}). To test this theory, we measured J_{Ca}, Na absorption (J_{Na}), and transepithelial voltage (V) in single perfused mouse CALs. Net absorption was measured microfluorimetrically in samples collected from tubules perfused and bathed in symmetrical HEPES-buffered solutions or those in which luminal Na+ was reduced from 150 to 50 mM. We first confirmed that Gd3+ activated the CaSR by measuring intracellular Ca2+ concentration ([Ca2+]i) in CALs loaded with fura 2. On stepwise addition of Gd3+ to the bath, [Ca2+]i increased, with a half-maximal rise at 30 μM Gd3+. J_{Ca} and transepithelial voltage (V) were measured in symmetrical Na+-containing solutions. PTH increased J_{Ca} by 100%, and 30 μM Gd3+ inhibited this effect. V was unchanged by either PTH or Gd3+. Similarly, NPS R-467, an organic CaSR agonist, inhibited PTH-stimulated J_{Ca}, without altering V. Neither PTH nor Gd3+ affected J_{Na}. Addition of bumetanide to the luminal perfuse abolished J_{Na}, and V. These results show that CaSR activation directly inhibited PTH-induced transepithelial J_{Ca} and that cellular Ca2+ transport can be dissociated. To test the effect of CaSR activation on passive paracellular Ca2+ transport, J_{Ca} was measured under asymmetrical Na conditions, in which passive Ca2+ transport dominates transepithelial absorption. PTH stimulated J_{Ca} by 24% and was suppressed by Gd3+. In this setting, Gd3+ reduced V by 32%, indicating that CaSR activation inhibited both transepithelial and paracellular Ca2+ transport. We conclude that the CaSR regulates both active transcellular and passive paracellular Ca2+ reabsorption but has no effect on J_{Na} by CALs.

calcium transport; thick ascending limbs; kidney; parathyroid hormone; transepithelial voltage

RENNAL Ca2+ ABSORPTION PROCEEDS throughout the nephron. The majority of filtered Ca2+ is reclaimed by proximal tubules, where it is absorbed largely by passive transport processes and is not subject to specific hormonal regulation. In contrast, downstream Ca2+ absorption in cortical thick ascending limbs (CALS) is mediated by a combination of active and passive absorption (16), and exclusively by active cellular absorption in distal convoluted tubules (DCTs) (10). Moreover, the CAL and DCT are sites where the PTH and PTH-related peptide receptor (PTH1R) is expressed (36, 45, 55). PTH stimulates active Ca2+ transport in both of these tubule segments (2, 16).

Two early observations suggested that renal Ca2+ absorption is regulated, at least in part, by extracellular Ca2+ itself. First, a prodigious literature testifies to the inhibitory effect of hypercalcemia on renal Ca2+ absorption (39). Some of the attenuation is presumably due to reductions in circulating PTH. However, when Ca2+ was acutely raised in thyroparathyroidectomized rats, tubular Ca2+ absorption clearly diminished and was accompanied by increased urinary Ca2+ excretion (41). Furthermore, Ca2+ infusion inhibited distal Ca2+ absorption without affecting Na+ or K+ transport. Therefore, the inhibitory actions of Ca2+ infusion are not likely to be due to reductions of glomerular ultrafiltration (32). Second, raising extracellular Ca2+ selectively inhibited PTH-stimulated cAMP formation in CALs but not in proximal tubules (49), where PTH1Rs are also expressed. These findings are now attributed to the activation by extracellular Ca2+ of the Ca2+-sensing receptor (CaSR).

The CaSR is prominently expressed in CAL and in DCT, although to a lesser extent in the latter (44). In CALs, the CaSR is located on basolateral plasma membranes (44). Extensive studies by Wang et al. (51, 52) established that the CaSR regulates an apical membrane 70-pS K+ channel that is an important determinant of the transepithelial voltage (V) in CALs. In CALs, V is normally oriented with the lumen electronegative with respect to the basolateral surface. Activating the CaSR decreases the K+ current, thereby depolarizing V. The reduction in voltage, in turn, would be expected to result in diminished passive, paracellular Ca2+ absorption (2). Indeed, increasing basolateral Ca2+ inhibited net Ca2+ absorption (J_{Ca})
by single perfused mouse CALs without altering Na\(^+\), Cl\(^-\), K\(^+\), or Mg\(^{2+}\) transport (12). While these findings are entirely consistent with the proposed role of the extracellular CaSR to regulate Ca\(^{2+}\) absorption by CALs, two relevant issues require further examination. First, in studies by Desfleurs et al. (12), CaSR activation was achieved by raising basolateral Ca\(^{2+}\). The CAL, however, is rather permeable to Ca\(^{2+}\) (3), and elevating Ca\(^{2+}\) asymmetrically at the serosal surface would be expected to enhance Ca\(^{2+}\) backflux and result in diminished \(J_{\text{Ca}}^{\text{net}}\) regardless of an effect on the CaSR. Under these conditions, it would be difficult to discriminate a nonspecific effect of diminished driving force from a specific action that could be attributed to the CaSR. Second, these initial studies did not address the possibility that CaSR activation regulates PTH-dependent active Ca\(^{2+}\) absorption. We now show that the CaSR regulates both PTH-dependent, active transcellular Ca\(^{2+}\) absorption and passive paracellular Ca\(^{2+}\) transport but has no effect on Na\(^+\) absorption by CALs.

**MATERIALS AND METHODS**

**Single-tubule microperfusion.** The techniques used for studying Ca\(^{2+}\) absorption by single in vitro microperfused mouse CALs were similar to those described originally by Burg et al. (6, 7) for isolated rabbit nephron segments and to those used previously by this laboratory (16, 17, 19) for the study of transport processes in segments of mouse CAL. Stated briefly, 25- to 30-day-old (~20 g) male ICR white mice (Harlan, Indianapolis, IN) were killed by cervical dislocation and rapid exsanguination. All protocols were approved by an institutional animal care and use committee. The kidneys were removed, and the cortical thick limbs were dissected freehand, without use of collagenase or other enzymatic treatment, from coronal sections of renal cortex immersed in a HEPES-buffered solution containing 5% BSA and maintained at 4°C (see Table 1 for composition). After transfer to a Lucite chamber, tubule segments 0.5–1.0 mm in length were connected to concentric glass pipettes, and perfusion was initiated by hydrostatic pressure. The tubules were perfused at rates of 2–15 nl/min at 25°C; the specific perfusion rate is indicated in each set of experiments and did not vary statistically among groups. Fluid was collected in constant-rate collection chambers. The peritubular bath fluid collection rate, and \(L\) is the tubule length measured with an eyepiece micrometer.

**Intracellular free Ca\(^{2+}\).** Intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\); nM) was determined in single perfused tubules by incubating dissected tubule fragments with 10 \(\mu\)M fura 2-AM for 1 h at 18°C as previously performed (1). The tubule was then transferred to the stage of an inverted microscope and connected to concentric holding and perfusion pipettes as described above. The microscope was fitted with an InCyt Im2 imaging system (Intracellular Imaging, Cincinnati, OH) through the epifluorescence port. Eight to ten cytoplasmic fields were identified along the length of the tubule in which [Ca\(^{2+}\)]\(_i\) was continuously monitored. Calibration was performed using known standards.

**Materials.** Bovine PTH(1-84) [bPTH(1-84)] was obtained from Sigma and were of the highest analytic grade available.

**Statistics.** Data are presented as means ± SE, where \(n\) indicates the number of independent experiments. Effects of experimental treatments were assessed by paired comparisons within experiments and reported as the means ± SE of \(n\) independent experiments. Paired results were compared by ANOVA with posttest repeated measures analyzed by the Bonferroni or Tukey procedure (Instat 3; GraphPad, San Diego, CA). Differences greater than \(P ≤ 0.05\) were assumed to be significant.

**RESULTS**

**Activation of CaSR by Gd\(^{3+}\) in single CALs.** Pilot experiments were conducted to characterize the effects.

### Table 1. Compositions of solutions used for perfusing and bathing single tubules

<table>
<thead>
<tr>
<th>Perfusate</th>
<th>Bath (150 mM Na(^+))</th>
<th>150 mM Na(^+)</th>
<th>50 mM Na(^+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>150</td>
<td>150</td>
<td>50</td>
</tr>
<tr>
<td>K(_2)HPO(_4)/K(_2)HPO(_4)</td>
<td>2.8</td>
<td>2.8</td>
<td>2.8</td>
</tr>
<tr>
<td>CaCl(_2)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>MgCl(_2)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>HEPES</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>d-Glucose</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Urea</td>
<td>0</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

Values are in mM. Solutions were gassed with 100% O\(_2\). pH was adjusted to 7.4 at 37°C and osmolality to 295–300 mosmol/kgH\(_2\)O.

Dialyzed BSA (0.6 mg/dl) was added to all bathing solutions.
and kinetic parameters of Gd$^{3+}$ action on the CaSR. Single mouse CALs were dissected and perfused as described in MATERIALS AND METHODS. However, in this instance, the tubes were loaded with fura 2 and a fluorescence microscope was used that permitted collecting real-time measurements of [Ca$^{2+}$], along the length of the perfused segment. Gd$^{3+}$ was introduced in the serosal bathing solution in a stepwise manner. On addition of 3, 30, or 300 μM Gd$^{3+}$ to the serosal bathing solution in the presence of 1 mM Ca, [Ca$^{2+}$], increased by 50, 80, and 155 nM, respectively. Because half-maximal increases in [Ca$^{2+}$], occurred at 30 μM Gd$^{3+}$, this concentration was used in subsequent studies of the effects of CaSR activation on Ca$^{2+}$ or Na$^{+}$ absorption.

We next measured Ca$^{2+}$ absorption under conditions in which the concentrations of Ca$^{2+}$ and of Na$^{+}$ in the perfusate and bath were identical and in the presence of a spontaneous $V_e$. In this environment, $J_{Na}^{\text{net}}$ averaged 0.10 pmol·s$^{-1}$·cm$^{-1}$ with $V_e$ of 5.9 ± 1.4 mV. This residual rate of Ca$^{2+}$ absorption results from the persistent action of PTH on Ca$^{2+}$ transport that involves an effect on de novo protein synthesis and only slowly dissipates (21, 23). Nonetheless, on addition of 10 nM PTH, $J_{Ca}^{\text{net}}$ increased by 92% (Fig. 1, Table 2) without a change in $V_e$. Further inclusion of 30 μM Gd$^{3+}$ to the bath reduced $J_{Ca}^{\text{net}}$ to values that were indistinguishable from control without altering $V_e$. The perfusion rate was constant between experimental periods. With the assumption of an average internal luminal diameter of 20 μm for CALs from similarly sized mice of the same strain (16, 18, 31), the resting and PTH-stimulated rates of Ca$^{2+}$ absorption are equivalent to those previously reported (16, 18). To confirm that the inhibitory action of Gd$^{3+}$ was referable to activation of the CaSR, the effect of NPS R-467, a type II CaSR agonist, was examined. As summarized in Table 3, the inhibitory action of this organic CaSR agonist on PTH-stimulated Ca$^{2+}$ absorption was comparable to that of Gd$^{3+}$, as displayed in Fig. 1. Thus the present results verify that Gd$^{3+}$, and NPS R-467 inhibit PTH-stimulated $J_{Ca}^{\text{net}}$ but not $V_e$. This set of findings shows that CaSR activation directly inhibited PTH-induced transcellular Ca$^{2+}$ absorption.

$J_{Na}^{\text{net}}$ was measured under the same conditions to determine whether the inhibitory action of Gd$^{3+}$ was specific for Ca$^{2+}$ or caused nonspecific suppression of all absorptive ion transport in the CAL. $V_e$ and tubule perfusion rates (Table 4) were similar in magnitude to those in the previous set of experiments. As shown in Fig. 2 and summarized in Table 4, $J_{Na}^{\text{net}}$ averaged 10.73 pmol·s$^{-1}$·cm$^{-1}$ and was unaffected by addition of either PTH or of Gd$^{3+}$ to the peritubular bathing solution. However, introduction of bumetanide in the tubule perfusion solution as a positive control abolished both $J_{Na}^{\text{net}}$ and $V_e$ (Fig. 2, Table 4). Thus, as in the preceding set of maneuvers where Ca$^{2+}$ absorption was measured, neither PTH nor Gd$^{3+}$ inhibited $V_e$. More importantly, these results show that cellular Ca$^{2+}$ transport can be dissociated from $J_{Na}^{\text{net}}$ by CaSR activation in the CAL.

### Table 2. Effect of PTH and of Gd$^{3+}$ on Ca$^{2+}$ absorption and $V_e$ in CALs determined in the presence of symmetric Na$^{+}$-containing solutions

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PTH</th>
<th>PTH+Gd$^{3+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$J_{Ca}^{\text{net}}$, pmol·s$^{-1}$·cm$^{-1}$</td>
<td>0.103 ± 0.021</td>
<td>0.198 ± 0.042*</td>
<td>0.100 ± 0.034</td>
</tr>
<tr>
<td>$V_e$, mV</td>
<td>5.9 ± 1.4</td>
<td>5.8 ± 1.3</td>
<td>5.0 ± 1.3</td>
</tr>
<tr>
<td>$V_L$, nl/min</td>
<td>3.84 ± 0.37</td>
<td>3.73 ± 0.50</td>
<td>4.14 ± 0.46</td>
</tr>
</tbody>
</table>

Values are means ± SE of 4 independent observations. PTH, parathyroid hormone; CAL, cortical ascending limb; $J_{Ca}^{\text{net}}$, net Ca$^{2+}$ absorption; $V_e$, transepithelial voltage; $V_L$, fluid collection rate. Tubules were perfused and bathed in external solutions containing 150 mM Na$^{+}$. Bovine PTH(1–84) was added to the bath at a final concentration of 10 nM. GdCl$_3$ was added to give a final concentration of 30 μM. $V_e$ was determined from the volume of the collected sample and the duration of the sample collection period. *P < 0.05 vs. control.

### Table 3. Effect of PTH and of NPS R–467 on Ca$^{2+}$ absorption and $V_e$

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PTH</th>
<th>PTH+NPS R–467</th>
</tr>
</thead>
<tbody>
<tr>
<td>$J_{Na}^{\text{net}}$, pmol·s$^{-1}$·cm$^{-1}$</td>
<td>0.129 ± 0.026</td>
<td>0.179 ± 0.037*</td>
<td>0.121 ± 0.023</td>
</tr>
<tr>
<td>$V_e$, mV</td>
<td>9.4 ± 1.2</td>
<td>8.0 ± 1.0</td>
<td>7.7 ± 1.0</td>
</tr>
<tr>
<td>$V_L$, nl/min</td>
<td>3.97 ± 0.3</td>
<td>3.68 ± 0.15</td>
<td>3.97 ± 0.3</td>
</tr>
</tbody>
</table>

Values are means ± SE of 4 independent observations. Tubules were perfused and bathed in symmetrical external solutions containing 150 mM Na$^{+}$ as described in Table 2. Bovine PTH(1–84) was added to the bath at a final concentration of 10 nM. NPS R-467 was added at a final concentration of 10 μM. *P < 0.01 vs. control.

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The final set of experiments was undertaken to examine the effects of Gd$^{3+}$ on Ca$^{2+}$ absorption under conditions that favor a large passive absorption of Ca$^{2+}$. This was achieved by imposing asymmetric Na$^{+}$-containing solutions to create a highly positive lumen-to-bath diffusion potential (3). In this situation, $V_e$ averaged 27 ± 0.44 mV (Table 5). As predicted, resting $J_{\text{net}}^{\text{Ca}}$ was considerably greater than that at the spontaneous $V_e$ of 6 mV (0.203 vs. 0.103 pmol·s$^{-1}$·cm$^{-1}$, respectively). Moreover, in the presence of this large driving force, passive Ca$^{2+}$ absorption constitutes the bulk of transepithelial Ca$^{2+}$ absorption, i.e., paracellular + cellular. Thus it is not surprising that PTH exerted only a modest stimulatory effect on $J_{\text{net}}^{\text{Ca}}$ (Fig. 3, Table 5). This increase in $J_{\text{net}}^{\text{Ca}}$ was not accompanied by a change in $V_e$. However, addition of Gd$^{3+}$ now caused $J_{\text{net}}^{\text{Ca}}$ to decrease below control levels (Fig. 3) and caused a significant decrease in $V_e$ (Table 5).

### DISCUSSION

The experiments described here were intended to determine the action of CaSR activation on hormone-stimulated transcellular Ca$^{2+}$ absorption. We now report that CaSR activation with trivalent Gd$^{3+}$ or with the calcimimetic NPS R-467 inhibits PTH-stimulated active Ca$^{2+}$ absorption. CaSR activation also suppressed passive paracellular Ca$^{2+}$ absorption, thereby providing independent confirmation of the findings obtained by Desfleurs et al. (12). The effects of CaSR activation were specific for Ca$^{2+}$ absorption and had no effect on Na$^{+}$ transport.

Table 5. Effect of PTH and Gd$^{3+}$ on Ca$^{2+}$ absorption and $V_e$ in CAL in the presence of an imposed salt dilution voltage

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PTH</th>
<th>PTH + Gd</th>
</tr>
</thead>
<tbody>
<tr>
<td>$J_{\text{net}}^{\text{Ca}}$, pmol·s$^{-1}$·cm$^{-1}$</td>
<td>0.203 ± 0.012</td>
<td>0.243 ± 0.012$^*$</td>
<td>0.120 ± 0.00$^*$</td>
</tr>
<tr>
<td>$V_e$, mV</td>
<td>27 ± 0.4</td>
<td>27 ± 0.3</td>
<td>19 ± 1.8$^+$</td>
</tr>
<tr>
<td>$V_L$, nl/min</td>
<td>3.37 ± 0.23</td>
<td>3.67 ± 0.26</td>
<td>3.53 ± 0.42</td>
</tr>
</tbody>
</table>

Values are means ± SE of 3 independent observations. $^*$ $P < 0.05$, $^+$ $P < 0.01$ vs. antecedent conditions, respectively.

Ligands that mimic or potentiate the actions of extracellular Ca$^{2+}$ on the CaSR are termed calcimimetics. They are grouped into two categories. Type I calcimimetics are full agonists and include Ca$^{2+}$ and a...
variety of other inorganic and organic polycations (37). Type II calcimimetics are phenylalkylamine derivatives that allosterically modulate the CaSR. The initial set of experiments described here evaluated the effect of CaSR activation on PTH-dependent Ca\textsuperscript{2+} absorption. The pharmacology of CaSR activation in thick limbs exhibits substantial species variability. For example, Gd\textsuperscript{3+}, neomycin, and elevated extracellular Ca\textsuperscript{2+} evoked strong increases in [Ca\textsuperscript{2+}]i in the mouse CAL (40), whereas in the rabbit only Ca\textsuperscript{2+} exhibited such an effect (12). In the rat, most type I calcimimetics elicited CaSR-mobilized Ca\textsuperscript{2+} release in the CAL (9, 14). Gd\textsuperscript{3+} was chosen as the prototype of a non-Ca\textsuperscript{2+}-selective CaSR agonist so as to maintain equal concentrations of Ca\textsuperscript{2+} at both apical and basolateral surfaces, thereby avoiding a transepithelial Ca\textsuperscript{2+} gradient that would alter passive Ca\textsuperscript{2+} diffusion, and because it is the most universal non-Ca\textsuperscript{2+} CaSR ligand. Pilot studies were performed that identified the concentration of Gd\textsuperscript{3+} that elicited a half-maximal activation of the CaSR, as reflected by the rise in [Ca\textsuperscript{2+}]i in mouse CAL. The concentration of Gd\textsuperscript{3+} so determined was 30 \mu M, consistent with that reported in other tissues (37).

Under symmetrical perfusion and bathing conditions, J\textsubscript{Ca net} averaged 0.103 pmol s\textsuperscript{-1} cm\textsuperscript{-1} and the average V\textsubscript{e} was 5.9 mV (Table 2). Both of these parameters are consistent with those reported in other studies (16, 19). Addition of PTH to the serosal bath increased J\textsubscript{Ca net} by nearly 100% to 0.198 pmol s\textsuperscript{-1} cm\textsuperscript{-1} without an accompanying change in V\textsubscript{e}. On addition of Gd\textsuperscript{3+}, J\textsubscript{Ca net} returned to control levels, again without a change in V\textsubscript{e}. Thus within experimental error, the inhibitory effect of Gd\textsuperscript{3+} on PTH-dependent Ca\textsuperscript{2+} transport occurred in the absence of a change in electro motive driving force; i.e., the reduction in J\textsubscript{Ca net} was due to inhibition of active, transcellular Ca\textsuperscript{2+} absorption and not to a change in passive Ca\textsuperscript{2+} movement. A small effect on passive Ca\textsuperscript{2+} cannot be entirely excluded but would be less than the limit of detection of a significant change in V\textsubscript{e}, ~1 mV.

The inhibitory effects of Gd\textsuperscript{3+} on PTH-stimulated Ca\textsuperscript{2+} absorption were reproduced with an unrelated organic compound, NPS R-467, a type II calcimimetic (Table 3). This finding supports the view that the inhibitory effects observed with Gd\textsuperscript{3+} are likely due to its activation of the CaSR and attendant release of Ca\textsuperscript{2+} and were not due to blockade of ATP-permeable channels (46), Ca\textsuperscript{2+}-selective K\textsuperscript{+} channels, the nonselective Ca\textsuperscript{2+}-permeable cation channel polycystin-2 (25), the Ca\textsuperscript{2+}-selective Trp3 channel (38), or other nonselective cation channels (15, 54) or mechanosensitive channels (27). It should be noted that although the solutions containing Gd\textsuperscript{3+} were prepared to a final concentration of 30 \mu M, the free Gd\textsuperscript{3+} concentration was likely to be substantially lower. The reason for this is that trivalent phosphate anions in the extracellular bathing solution avidly bind free Gd\textsuperscript{3+} (8).

To determine whether the inhibitory effect of CaSR activation in the CAL was specific for Ca\textsuperscript{2+} transport and to verify the conclusion that there was no effect on passive driving forces, we examined the effects of PTH alone or in combination with Gd\textsuperscript{3+} on Na\textsuperscript{+} absorption in the same extracellular fluid environment as before. In contrast to the action on Ca\textsuperscript{2+} absorption, PTH had no discernable effect on J\textsubscript{Na net} (Fig. 3, Table 5). Again, there was no significant change in V\textsubscript{e}. Moreover, whereas CaSR activation with Gd\textsuperscript{3+} inhibited PTH-stimulated Ca\textsuperscript{2+} absorption, Gd\textsuperscript{3+} had no effect either on Na\textsuperscript{+} absorption or on V\textsubscript{e}. This finding supports the view that the regulatory influence of the CaSR is specific to Ca\textsuperscript{2+} absorption in the CAL and strengthens the conclusion that CaSR activation in the CAL inhibits PTH-stimulated active Ca\textsuperscript{2+} absorption. It does not speak to the possibility that CaSR activation in the MAL may modulate vasopressin-stimulated Na\textsuperscript{+} transport (29).

The mechanism whereby CaSR activation inhibits PTH-dependent Ca\textsuperscript{2+} absorption has not been examined. Several possible pathways may be involved. Activation of the CaSR results in G-protein-dependent stimulation of phospholipase C with attendant inositol trisphosphate formation and rapid but transient release of Ca\textsuperscript{2+} from intracellular stores. Other CaSR signaling pathways, including activation of G\textsubscript{i}, phospholipase A\textsubscript{2}, phospholipase D, and mitogen-activated protein kinase, have been described but are less well characterized (4, 34). Because PTH stimulation of Ca\textsuperscript{2+} transport in CAL cells and in DCTs requires activation of protein kinase A (20), it is attractive to speculate that the negative regulatory effect of G\textsubscript{i} blocks the stimulatory influence on G\textsubscript{s}, thereby abrogating the action of PTH.

Because neither PTH nor Gd\textsuperscript{3+} affected J\textsubscript{Na net}, the tubule perfusate was changed to one containing bumetanide at the end of the experiment to serve as a positive
control. In each instance, bumetanide abolished $V_e$ and $J_{\text{Na}}^e$ (Fig. 3, Table 5). The absence of an effect of PTH on $J_{\text{Net}}^\text{Na}$ as described here is consistent with earlier reports, in which cAMP had no effect on the equivalent electrical flux in mouse CALs (17), and there was an absence of an effect on $V_e$ in rabbit CAL (2, 33, 48). However, de Rouffignac et al. (11) found that PTH increased $V_e$ and, hence, passive paracellular Na$^+$ absorption. Furthermore, under symmetrical perfusion conditions, and when $V_e$ was abolished by the inclusion of furosemide in the luminal perfusate, PTH had no stimulatory effect on Ca$^{2+}$ absorption. This latter result, and the discrepancy with the present and earlier findings, can be partially reconciled on the basis of electrophysiological effects of furosemide, which depolarizes the apical membrane voltage of CALs and decreases intracellular Cl$^-$ activity (26). In this setting, the stimulatory effect of PTH on Ca$^{2+}$ entry, which requires membrane hyperpolarization, is negated and cellular Ca$^{2+}$ transport is abolished (24). However, we have no explanation for the finding that PTH increased $V_e$.

The final set of studies examined the influence of CaSR activation on Ca$^{2+}$ absorption under conditions in which transepithelial Ca$^{2+}$ transport is dominated by its passive movement. This was accomplished by imposing a salt-dilution voltage across the tubule by reducing the Na$^+$ concentration of the luminal fluid (Table 1). In the presence of the high Na$^+$-to-Cl$^-$ perme-selectivity ratio of the paracellular pathway of the CAL, this strongly enhances the magnitude of the lumen-positive $V_e$ (3, 28, 30). By reducing luminal Na$^+$ to 50 mM, $V_e$ increased to 27 mV and was accompanied by a substantial augmentation of passive Ca$^{2+}$ absorption (Fig. 3, Table 5) compared with that observed under symmetrical Na$^+$ conditions (Fig. 1, Table 2). Addition of PTH elicited only a modest enhancement of $J_{\text{Ca}}^e$. Notably, Gd$^{3+}$ now reduced $J_{\text{Net}}^\text{Ca}$ below control levels with an accompanying inhibition of $V_e$ below control (Table 5). This finding is consistent with the view that CaSR activation inhibits passive Ca$^{2+}$ movement and provides independent corroboration of the conclusion reached by Desfleurs et al. (12).

When the luminal Na$^+$ concentration was reduced to 50 mM but the serosal bathing solution contained 150 mM Na$^+$ (Table 1), Gd$^{3+}$ reduced $V_e$ by 30% (Table 5). This effect may have several explanations. The imposition of asymmetrical external solutions is likely to promote cell swelling with a variety of compensatory sequelae. Hypotonicity inhibits the functional activity of the apical Na$^+$-K$^+-2Cl$\text{"} co-transporter (22). The inhibitory effect of Gd$^{3+}$ may be due to blockade of swelling-sensitive mechanosensitive channels that are activated under these conditions (50). However, there may well be additional or alternative molecular targets of Gd$^{3+}$ action. Apical membrane K$^+$ current is low under conditions of symmetrical external Na$^+$, but its activity and sensitivity to blockade by Gd$^{3+}$ (52) may be augmented as part of a regulatory volume decrease. A related possibility is that dilution of the luminal fluid causes some reduction in the NaCl concentration within the lateral intercellular space (LIS). Such a decrease in ionic strength may modify the sensitivity of the CaSR as described by Quinn et al. (42). However, in a study of the effect of luminal dilution on dilation of the LIS (35), an even greater dilution of the luminal fluid (to 70 mosM) than imposed here resulted in a 10% change in the volume of the LIS. If the magnitude of the change of ionic strength of the fluid in the paracellular pathway was of the same magnitude, it is unlikely to have affected the CaSR sensitivity. Finally, the asymmetrical conditions employed here may directly affect the behavior of basolateral membrane Cl$^-$ channels as shown for such channels reconstituted in bilayer membranes (43). However, it is difficult to predict, on the one hand, the effect of asymmetrical external conditions on intracellular Cl$^-$ activity, especially in view of the finding that trans, i.e., cytosolic, Cl$^-$ gates basolateral Cl$^-$ channels derived from MAL but not CALs (53), on the other.

In the context of Ca$^{2+}$ absorption along the entire length of the thick ascending limb, transport is a combination of passive paracellular movement and active cellular absorption. The latter process would be restricted to those segments and species in which PTH receptors are expressed. Because PTH receptors are generally limited to the CAL, it is likely that the majority of Ca$^{2+}$ absorption proceeds through a passive mechanism. This is compatible with the view that changes in Ca$^{2+}$ absorption in thick limbs proceed in parallel with those of Na$^+$. When Na$^+$ absorption by thick limbs is decreased by loop diuretics such as furosemide, the increases in Na$^+$ excretion are accompanied by enhanced Ca$^{2+}$ excretion. Similarly, in Bartter’s syndrome Na$^+$ wasting is attended by conspicuous Ca$^{2+}$ losses (13, 47). These clinical observations underscore the view that Ca$^{2+}$ absorption by thick limbs is driven secondarily to that of Na$^+$. By the end of the CAL, the tubular Na$^+$ concentration is markedly reduced, thus resembling the asymmetrical external environment applied here. In this situation, activation of the CaSR would be expected to reduce Ca$^{2+}$ absorption and contribute to its excretion. Such receptor activation arises during hypercalcemia, in which reduction of renal Ca$^{2+}$ reabsorption would represent a purposeful compensatory response. The results described herein suggest that CaSR activation would be expected to dampen both passive and active Ca$^{2+}$ transport, thereby promoting greater Ca$^{2+}$ excretion in an attempt to restore extracellular Ca$^{2+}$ to normal levels.

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