Calcium-Sensing Receptor Regulation of PTH-Inhibitable Proximal Tubule Phosphate Transport

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Abstract:

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Inorganic phosphate (Pi) is absorbed by proximal tubules through a cellular pathway that is inhibited by parathyroid hormone (PTH). The calcium-sensing receptor (CaSR) is expressed on apical membranes of proximal tubules. In the present studies, we determined the effect of luminal and/or basolateral PTH on phosphate absorption and tested the hypothesis that CaSR activation blocks PTH-inhibitable phosphate absorption. Single proximal S3 tubules were dissected from the kidneys of mice and studied by the Burg technique. Tubules were bathed with DMEM culture media supplemented with 6% BSA and perfused with an ultrafiltrate prepared from the bathing solution. [33P] and FITC-inulin were added to the luminal perfusate to measure phosphate absorption (JPi) and fluid absorption (Jv), respectively. JPi averaged 2.9 pmol min⁻¹ mm⁻¹ under control conditions and decreased by 20% upon addition of serosal PTH. PTH had no effect on Jv. Inclusion of PTH in the luminal perfusate reduced JPi to 2.1 pmol min⁻¹ mm⁻¹. Combined addition of PTH to perfusate and bathing solutions reduced JPi to 1.5 pmol min⁻¹ mm⁻¹ without affecting Jv. Indirect immunofluorescence studies revealed abundant PTH receptor (PTH1R) expression on brush-border membranes, with lower amounts on basolateral membranes. CaSRs were localized primarily, but not exclusively to brush-border membranes. CaSR activation with luminal Gd³⁺ abolished the inhibitory action of PTH on JPi. Addition of Gd³⁺ to the serosal bathing solution had no effect on PTH-sensitive JPi. Gd³⁺ did not affect basal, i.e., PTH-
independent $J_{Pi}$. $\text{Gd}^{3+}$ had no effect on $J_v$ when added to lumen or bath. Dopamine-inhibitable $J_{Pi}$ was not affected by $\text{Gd}^{3+}$. Experiments with proximal-like OK cells showed that elevated extracellular $\text{Ca}^{2+}$ or NPS R467, a type II calcimimetic, inhibited PTH action on Pi uptake. In conclusion, PTH Type 1 receptors are expressed on apical and basolateral membranes of mouse proximal tubules. Stimulating apical or basolateral PTH1R inhibits phosphate absorption. CaSR activation specifically regulates PTH-suppressible phosphate absorption.
Renal phosphate reabsorption occurs mostly in proximal tubules through a cellular pathway. Type IIa Na-Pi cotransporters accounts for about 70% of brush-border membrane (BBM) Na-Pi cotransporter activity and is the target of parathyroid hormone (PTH) action (6). PTH inhibits Pi absorption by internalizing the apical brush-border membrane type IIa Na-Pi cotransporter (45). Functional Type 1 PTH receptors (PTH1R) are located in both basolateral and, perhaps, on luminal membranes of proximal tubules (2). PTH internalizes the apical NaPi-IIa cotransporter in mouse kidney when added either to mucosal or basolateral surfaces of proximal straight tubules (64). Although PTH sequestered the Na-Pi cotransporter when applied to the luminal side of proximal tubules, the expression and functional role of apical PTH1Rs on phosphate absorption by intact segments of proximal tubules is not clear. The first goal of the present studies was to determine the effect of luminal PTH, alone or in combination with basolateral PTH, on phosphate absorption by single mouse proximal straight tubules and to localize the PTH1R.

Extracellular calcium influences renal phosphate absorption. Most studies report that elevating plasma calcium increases Pi absorption (1, 39, 54). Such an effect could arise indirectly by suppressing PTH release, or directly. Direct effects of calcium on proximal tubule Pi absorption have been examined only in a limited number of studies. In the first, eliminating luminal calcium decreased Pi absorption (24). Such an action must be interpreted cautiously since calcium removal would be expected to increase Pi permeability of tight junctions (43) with attendant backflux from the peritubular fluid and reduction of net absorption. In a second study, increasing luminal calcium enhanced Pi absorption (59). Finally, effects of extracellular calcium on proximal Na-Pi cotransport
have been described (48). In these latter studies, elevation of calcium increased Pi absorption and vice versa. These findings suggest that calcium directly affects renal Pi absorption. The mechanism responsible for this action is unknown. Calcium could modify renal Pi transport by directly modulating luminal membrane Na-Pi cotransport. Alternatively, calcium could modify the action of PTH on retrieval of apical membrane Na-Pi cotransporter. The actions of calcium on PTH-dependent Pi transport have not been investigated. The calcium-sensing receptor (CaSR) is expressed on apical membranes of proximal tubules (56). However, the physiological function of proximal tubule CaSRs is unknown. The second objective of the present studies was to test the hypothesis that CaSR activation attenuates PTH-suppressible Pi absorption. Since hypercalcemia alters glomerular ultrafiltration and filtered load, which in turn might influence renal phosphate reabsorption, we analyzed the effects of CaSR on phosphate absorption by single mouse proximal tubules that were microperfused in vitro.
MATERIALS AND METHODS

*Single tubule microperfusion.* The techniques used for studying phosphate absorption by single in vitro microperfused mouse proximal tubules were similar to those described originally by Burg et al. (13) for isolated rabbit nephron segments and to those used previously by this laboratory (42) for the study of transport processes in segments of the mouse nephron. Stated briefly, outbred 25-30-day-old (~25 g) male ICR (Institute of Cancer Research) white mice (Harlan, Indianapolis, IN) were killed by cervical dislocation and rapid exsanguination. All procedures were approved by an institutional animal care and use committee (IACUC protocol 11977). The kidneys were removed, and proximal straight S3 tubules were dissected freehand, without use of collagenase or other enzymatic treatment, from coronal sections of renal cortex immersed in a HEPES-buffered DMEM media (Sigma D-2902) containing 6% BSA and maintained at 4 °C. 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. Tubules were perfused at average rates of 10-15 nl/min at 37 °C; a rate that is consistent with reported values for single nephron GFR in the mouse (41). The specific perfusion rates in individual periods varied from 8.7-19.3 nl/min in control periods, and 8.5-22.3 nl/min in experimental periods. Perfusion rates are indicated for each set of experiments and did not vary statistically among groups. Fluid was collected in constant-bore pipettes under water-saturated mineral oil or tetradecane colored with oil red O. The rate of tubule perfusion was calculated from the collection rate corrected for fluid absorption.
**Perfusion and bathing solutions.** Tubules were bathed with phenol red-free DMEM culture media (measured composition, mM: NaCl, 140; KCl, 5.0; NaH2PO4, 1.0; CaCl2, 1.8; MgSO4, 0.8; D-Glucose, 5.5 HEPES, 10) supplemented with 6% BSA. The perfusion was solution was prepared as an ultrafiltrate (Amicon 52 Ultrafiltration Cell, PM10 membrane; Bedford, MA) from the bathing solution. [33P] and FITC-inulin (Sigma, 150 mg%) were added to the luminal perfusate to measure phosphate absorption (Jp) and fluid absorption (Jv), respectively. All solutions were adjusted to pH 7.4 and 290-300 mosmol/kg H2O and were equilibrated with 100% O2. The perfusion chamber was bubbled with 100% O2. After 3 10-min control periods, 100 nM human PTH(1-34) was added to the bath and after 20 minutes, 3 experimental samples were collected. In other experiments, PTH or the CaSR agonist GdCl3 was added to the luminal perfusate. Dopamine was prepared freshly for each experiment in a stock solution of 200 mM sodium metabisulfite to prevent oxidation to norepinephrine, which is antiphosphaturic (30). The stock solution was diluted 1,000-fold to give a final concentration of 1 µM dopamine in the tubular perfusion solution. Sodium metabisulfite was added alone during control periods.

**Fluid absorption.** Changes of the concentration of FITC-inulin (Sigma) were used to measure the rate of fluid absorption (40). The concentration of FITC-inulin in 15-nl samples of perfusate and collected tubular fluid were determined by a fluorimetric procedure using a NanoFlo fluorimeter (69) (WPI, Sarasota, FL). The rate of fluid absorption (Jv, nl min⁻¹ mm⁻¹) was calculated according to the standard equation:

\[
J_v = ([In]_o/[In]_L - 1)^* V_L / L \quad (1)
\]
where $[\text{In}]_0$ and $[\text{In}]_L$ are the concentrations of FITC-inulin in the collected and perfused fluid samples, respectively, $V_L$ is the fluid collection rate, and $L$ (mm) is the tubule length measured with an eyepiece micrometer.

**Phosphate absorption.** The rate of phosphate absorption was measured with $^{33}\text{P}$orthophosphoric acid (ICN). In all studies, $^{33}\text{P}$ was present in the luminal perfusate at a concentration greater than 20 cpm nl$^{-1}$. Radioactivity of 20-nl collected samples was measured in duplicate by beta emission spectrometry (Beckman Coulter LS 6500, Fullerton, CA). The lumen-to-bath absorption of phosphate was calculated as the difference between the amount perfused and the amount collected, corrected for fluid absorption according to the following equation:

$$J_{\text{Pi}}^{l,b} = \frac{V_i C_i^* - V_o C_o^*}{L} \times \frac{[\text{PO}_4]^i}{C_i^*}$$

(2)

where $J_{\text{Pi}}^{l,b}$ is the unidirectional lumen-to-bath phosphate flux (pmol min$^{-1}$ mm$^{-1}$), $V_i$ and $V_o$ (nl min$^{-1}$) are the fluid perfusion and collection rates, respectively, $L$ (mm) is the length of the perfused segment as determined by an eyepiece micrometer, $C_i^*$ and $C_o^*$ (cpm nl$^{-1}$) is the concentration of $^{33}\text{P}$ in the perfusion and collected fluid samples, and $[\text{PO}_4]$ is the molar concentration of inorganic phosphate in the perfusion fluid.

**Cell Culture.** OK/E cells were grown on 25 cm$^2$ Falcon dishes (Becton Dickenson, Franklin Lakes, NJ) in a 50:50 mix of DMEM/F12 (10-092, Mediatech, Herndon, VA) with 15 mM HEPES, 2.5 mM L-glutamine, and supplemented with 5% FBS (BioWhittaker, Walkersville, MD), and 1% penicillin-streptomycin-neomycin (PSN) antibiotic mixture (Invitrogen Life Technologies, Carlsbad, CA) in a humidified
atmosphere of 95% air-5% CO₂ at 37 °C. Unless stated otherwise, cells were switched to serum-free DMEM/F12 media 16 h before use.

**Phosphate uptake.** Confluent cells in 12-well plates were serum-starved overnight in antibiotic-free media. CaSR activation was accomplished by augmenting the concentration of calcium to that indicated, or by the inclusion of NPS 467 to serum-free media in the presence or absence of hPTH(1-34), 100 nM, for 2 hr. Cells were then washed 3× with a Na-containing buffer (in mM: NaCl, 140; KCl, 4.8; MgSO₄, 1.2, KH₂PO₄, 0.1, HEPES, 10) or Na-free buffer, where n-methyl-d-glucamine (NMDG) isosmotically replaced Na. Measurement of phosphate uptake was initiated by adding buffer containing 4 μCi/ml [³²P]-orthophosphate for 10 min to triplicate wells. Uptake is linear over this time frame. Uptake was stopped by washing 3× with ice-cold NMDG media. The cells were extracted overnight with 0.5% Triton X-100 or 1 N NaOH and then counted by beta-scintillation spectrometry. Na-dependent Pi uptake was calculated by subtracting uptake in the absence of Na from that in the presence of Na. Na-independent uptake represents ≤5% of Na-dependent uptake.

**PTH1R immunofluorescence.** Mice were anesthetized with sodium pentobarbital (Nembutal; 0.1 ml of a 50 mg/ml solution per 100g body weight, IP), and were perfused through the left ventricle first with phosphate buffered saline (PBS - 10 mM sodium phosphate buffer containing 0.9% NaCl, pH 7.4) and then with paraformaldehyde-lysine-periodate fixative (PLP) for 5 minutes as previously described (9). Kidneys were removed and slices were fixed by immersion in PLP at 4 °C overnight. Tissues were washed 3 times in PBS and stored until use in the same buffer containing 0.02% sodium azide.
For immunocytochemical staining, cryostat sections were cut at a thickness of 4 µm. Tissues were cryoprotected by immersion in 0.9 M (30%) sucrose in PBS for at least 1h prior to freezing in liquid nitrogen and sectioning with a Reichert Frigocut cryostat. Sections were picked up on Fisher Superfrost Plus charged glass slides. Sections were rehydrated in PBS and then treated for 4 min with 0.1% sodium dodecyl sulfate (SDS), an antigen retrieval technique, as previously described (10). They were incubated with PBS containing 1% BSA for 15 min and then incubated for 1h at room temperature with a polyclonal rabbit anti-rat antipeptide antibody (20 µg/ml) (PRB-630P, rat pep IV) targeted to the extracellular sequence (CTLDEAERLTEEELH) of the PTH1R was obtained from Covance (Berkeley, CA). We also localized the CaSR in proximal S3 tubules using a well-characterized CaSR-specific polyclonal antibody (#2; (15)) kindly provided by Dr. Dolores Shoback (UCSF). Sections were washed 2 x 5 min in high salt PBS (PBS containing 2.7% NaCl) and 1 x 5 min in PBS to reduce non-specific staining, and the secondary donkey anti-rabbit antibody conjugated to CY3 (Jackson Immunologicals) was applied for 1h at room temperature at a dilution of 1:800. The sections were again washed in high salt PBS and in PBS before mounting in Vectashield anti-fade solution diluted 1:1 in Tris buffer pH 9.0. Sections were examined using a Nikon Eclipse 800 epifluorescence microscope and images were captured with a Hamamatsu Orca CCD camera and IP Lab acquisition software (Scanalytics).

Materials. Human PTH(1-34)NH₂ [hPTH(1-34)] was synthesized as described (22). NPS R-467 and S-467 were obtained from NPS Pharmaceuticals, Salt Lake City, UT. All other reagents were purchased 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. Paired results were compared by ANOVA with post-test repeated measures analyzed by the Bonferroni or Tukey procedure (Instat 3; GraphPad, San Diego, CA). The relationship of perfusion rate and \( J_{Pi} \) was analyzed by linear regression analysis (Instat). Differences greater than \( P = 0.05 \) were assumed to be significant.
RESULTS

*Basolateral addition of PTH inhibits phosphate but not fluid absorption.* Single mouse S3 proximal tubules were dissected and perfused as described in MATERIALS AND METHODS. The perfusion rate was constant between control and experimental periods (14.42 ± 0.42 vs. 13.67 ± 0.52). Under control conditions $J_{Pi}$ averaged 2.9 pmol min$^{-1}$ mm$^{-1}$ (Fig. 1A). Lines connect results for 11 independent experiments, where, after adding 100 nM PTH to the basolateral bathing, $J_{Pi}$ significantly decreased by $\approx$20%. Separate experiments showed no effect of PTH on phosphate backflux from bath-to-lumen, as reported for rabbit proximal tubules (16). Fluid absorption was measured concurrently in the same experiments. The results shown in Fig. 1B indicate that basolateral PTH had no effect on fluid absorption.

*PTH1R localization and effects of apical PTH.* Studies were initiated to localize the PTH1R in proximal tubules. Receptor localization was determined using an anti-peptide antibody directed against an epitope in the extracellular domain of the PTH receptor. Fig. 2 shows that in mouse S3 proximal tubules, the PTH receptor is intensely expressed on brush-border membranes. Only slight staining of basolateral membranes is apparent. Controls were performed by incubating sections in the absence of the primary antibody (secondary alone) and by exposing sections to antibody that had been pre-absorbed with a 10-fold excess (200 µg/ml) of the immunizing PTH IV peptide overnight at 4 °C. No staining was seen in the absence of the primary antibody. All proximal tubule staining was abolished by the peptide pre-incubation (results not shown). Strong PTH1R staining was present in thick ascending limbs of Henle (TAL), where receptors were expressed at both apical and basolateral membranes.
We next tested the effect of luminal or combined luminal plus basolateral PTH on phosphate and fluid absorption. The results are shown in Fig. 3. Inclusion of 100 nM PTH in the luminal perfusate inhibited phosphate transport by 20% compared to controls. Combined addition of PTH to perfusate and bathing solutions caused a further reduction of J_{Pi} to 1.5 pmol min^{-1} mm^{-1} (Fig 3A). This represents a total reduction of phosphate absorption by 50%. Fig. 3B shows that PTH had no effect significant effect on fluid absorption when added to lumen and bath in the above experiments. However, there was a slight decline of fluid absorption in 3 of 4 observations.

Flow dependence of phosphate absorption. Proximal tubule solute and fluid absorption have been noted to be flow dependent (28). Therefore, we analyzed the relationship between perfusion rate and phosphate absorption. As shown in Fig. 4, under control conditions, i.e., in the absence of PTH, J_{Pi} increased in parallel with the perfusion rate over the range of 8-22 nl/min (r = 0.48, P<0.01). These results indicate that the rate of phosphate absorption in proximal tubules is related to the rate of phosphate delivery. Upon addition of PTH to the basolateral bathing solution, J_{Pi} decreased and, as expected, phosphate absorption no longer correlated with perfusion rate (r = 0.19, NS). Under the same conditions, there was no statistically significant relation between perfusion rate and the magnitude of fluid absorption.

CaSR activation blunts PTH-inhibitable phosphate absorption. Recent studies show that the CaSR is expressed on brush-border membranes of proximal tubules (56, 57). We confirmed that the CaSR is intensely expressed on brush-border membranes of S3 proximal tubules of the mouse kidney (Fig. 5). However, expression was also detectable along lateral cell membranes and, in some instances, on basal surfaces. The
physiologic role of the CaSR in proximal tubules is unknown. We theorized that CaSR activation regulates PTH-dependent proximal tubule phosphate absorption, much as it does calcium absorption by cortical ascending limbs (42). Therefore, in the next set of experiments we tested the effect of CaSR activation on PTH-sensitive phosphate absorption. Gd\(^{3+}\), a CaSR agonist, was used to activate the CaSR. Previous studies established that half-maximal CaSR activation occurred at 30 µM Gd\(^{3+}\), which corresponds closely to its reported EC\(_{50}\) (47, 55). Accordingly, this concentration was used to study the effects of CaSR activation on phosphate absorption. As shown in Fig. 6A, addition of 30 µM Gd\(^{3+}\) to the luminal perfusate abolished the inhibitory action of basolateral PTH on phosphate absorption. As a control, Gd\(^{3+}\) was added to the serosal bathing solution. In this situation, Gd\(^{3+}\) did not block the inhibitory effect of PTH on phosphate transport (Fig. 6B). These findings are consistent with the view that apical membrane calcium receptors regulate PTH-sensitive phosphate absorption by proximal tubules. Furthermore, Gd\(^{3+}\) had no effect on fluid absorption when added to either lumen or bath (data not shown).

**Specificity of CaSR activation for PTH-sensitive phosphate transport.** Proximal tubular phosphate absorption is regulated not only by PTH, but also by dopamine (4, 19). Addition of 1 µM dopamine to the basolateral solution bathing S3 proximal tubules inhibited \(J_{pi}\) by 28% \((P<0.01)\) (Fig. 7A). To determine if CaSR activation is specific for PTH or also affected dopamine inhibitable phosphate absorption, we used the same protocol described above. Inclusion of 30 µM Gd\(^{3+}\) in the luminal perfusate did not affect resting phosphate absorption (2.55 vs. 2.57 pmol min\(^{-1}\) mm\(^{-1}\)). Furthermore, in contrast
to its inhibitory effect on PTH-dependent phosphate absorption, Gd$^{3+}$ did not diminish dopamine-sensitive phosphate transport (Fig. 7B).

**Characterization of CaSR activation on PTH-sensitive phosphate uptake by OK/E cells.** To evaluate further the effect of CaSR activation on phosphate transport, we examined the action of CaSR activation on basal and PTH-inhibitable phosphate uptake by opossum kidney epithelial (OK/E) cells. Fig. 8 shows three aspects of phosphate uptake by these cells. First, raising extracellular calcium from 1 to 10 mM had no effect of phosphate uptake. Thus, basal phosphate transport is not subject to regulation by the CaSR. Second, at an ambient concentration of 1 mM calcium, PTH inhibited phosphate uptake in a concentration-dependent manner. At 0.1 nM, PTH reduced phosphate uptake by 28% and at 10 nM, PTH uptake was inhibited by 53%. Third, elevation of calcium from 1 to 10 mM blocked the inhibitory effect of PTH on phosphate accumulation. These results show that CaSR activation regulates the PTH-sensitive component of phosphate transport in OK cells.

The specificity of the effects of elevated extracellular calcium for the CaSR were tested by using NPS 467, an organic compound that is a specific calcium receptor-activating ligand (66). The results are shown in Fig. 9. PTH reduced phosphate uptake by 60%. However, in the presence of NPS R-467, the active form of the compound, PTH action was significantly reduced. NPS S-467, the less active optical isomer was used as a negative control, did not affect PTH-inhibitable phosphate uptake.
DISCUSSION

The present study examined the interactions between PTH and CaSR activation on phosphate absorption. Many factors, including dietary phosphate intake, PTH, and vitamin D, affect phosphate absorption by proximal tubules (20, 23, 35, 44). PTH is the principal short-term regulator of phosphate transport by proximal tubules. Its inhibitory effects are mediated by sequestering the brush-border membrane type IIA Na-Pi cotransporter (45). Considerable information, albeit primarily indirect, suggests that changes of extracellular calcium also affect the rate or magnitude of renal phosphate transport. Most studies report that elevating plasma calcium decreases renal Pi excretion (1, 39, 54). The mechanism responsible for this effect is unknown. Calcium could modify renal Pi transport by directly affecting Na-Pi cotransporter-mediated entry across luminal membranes. Alternatively, calcium could block the action of PTH on retrieval of apical membrane Na-Pi cotransporters. Indirect effects of hypercalcemia on renal phosphate reabsorption might entail alterations of glomerular ultrafiltration and filtered load, or affect PTH secretion from parathyroid glands. The present studies were initiated to test the hypothesis that the regulatory effect of extracellular calcium on proximal tubule phosphate absorption is mediated by the CaSR. We used a combination of isolated single proximal tubules and a cell culture model to avoid complicating systemic effects of raising extracellular calcium or of changes of GFR. The results show that CaSR activation directly blunts PTH-sensitive phosphate absorption by proximal tubules, and inhibits PTH-dependent phosphate uptake by OK/E proximal-like kidney cells.
Basolateral PTH inhibited phosphate absorption by S3 proximal tubules. This is the first investigation of phosphate absorption by single mouse tubules. The inhibitory effect of PTH on $J_{Pi}$ is consistent with earlier studies of phosphate absorption by rabbit proximal tubules (16). In the rabbit, PTH suppressed phosphate absorption only in proximal straight tubules but not convoluted proximal tubules. In the rabbit proximal tubule, basal phosphate absorption ($2.6 \text{ pmol min}^{-1} \text{ mm}^{-1}$) was equivalent to that reported here ($2.9 \text{ pmol min}^{-1} \text{ mm}^{-1}$). However, inhibition of phosphate transport by basolateral PTH application in rabbit proximal straight tubules was somewhat greater (28%) than in the mouse (20%). PTH had no inhibitory effect on fluid absorption in rabbit proximal straight tubules when they were perfused with a low-bicarbonate containing solution. PTH did not alter phosphate backflux in the rabbit (16), the dog (26) or the rat (26). Likewise, PTH did not alter the rate of phosphate backflux in mouse S3 proximal tubules. Thus, qualitatively and quantitatively, the rate of resting and PTH-sensitive proximal tubule phosphate absorption is comparable in the mouse and rabbit. However, the absolute magnitude of PTH-suppressible phosphate transport in these perfused tubule experiments was roughly half that reported in studies of the effect of PTH on phosphate absorption in intact dogs or rats (5, 67). The present findings may explain the difference in the magnitude of the PTH inhibition. In the whole animal studies, PTH action was assessed by comparing tubular phosphate absorption in euparathyroid with thyroparathyroidectomized animals. It is now reasonably clear that PTH receptors are expressed on both luminal and basolateral membranes of the proximal tubules (Fig. 2) (2) and that PTH promotes NaPi2 internalization in proximal tubules when added to either luminal or basolateral solution (64). Since full-length PTH
is 84 amino acids, with a nominal size of under 10,000 D, it will be filtered at the glomerulus and enter the lumen of proximal tubules. Hence, we suggest that in intact animals, the inhibitory action of PTH on proximal phosphate absorption is exerted from both membrane surfaces. Consistent with this suggestion, systemic administration of a parathyroid extract inhibited fractional proximal tubular phosphate reabsorption by some 40% (67). In these instances, the magnitude of the effect of parathyroidectomy or of systemic PTH infusion on proximal phosphate transport compares favorably to that here, where PTH was added to both the luminal perfusate and peritubular bathing solution.

Recent studies using a polyclonal antiserum raised to an extracellular epitope of the type 1 PTH receptor (PTH1R) described expression at both basolateral and luminal membranes of mouse proximal tubules (2). The results shown in Fig. 2 indicate that the PTH1R is abundantly expressed along the luminal brush-border membrane of S3 mouse proximal tubules. The prominent brush-border localization described here contrasts to the work of Amizuka, where the PTH1R was primarily expressed at basolateral membranes of proximal tubules. Consistent with an apical membrane PTH1R localization, Traebert et al. reported that when added to luminal or basolateral surfaces of perfused mouse proximal straight tubules, 1 µM PTH(1-34) effectively induced NaPi-IIa sequestration (64). We sought to extend these findings by examining the effect of luminal PTH on phosphate absorption in the same, i.e., S3 proximal tubule segment. We used 100 nM PTH(1-34) because in prior work this was the lowest concentration that elicited maximal cAMP stimulation in mouse proximal tubule cells (21). It has been noted that circulating levels of PTH in mice are in the picomolar range
The normal circulating PTH levels in mice are in the picomolar range (37, 62), while the EC$_{50}$ for the PTH receptor is about 1 nM. The reason for this disparity is not entirely understood but may be explained by the possibility that PTH accumulates at the receptor to higher levels than measured in circulation (52). The high in vivo efficacy of PTH may reflect preferential pharmacokinetic and pharmacodynamic properties that result in higher and protracted concentrations of the drug at its target sites in bone and kidney. Alternatively, the extensive amphiphilic helical conformation of the ligand binding site of the PTH1R in vivo may not be represented in the cell culture lines that are used for in vitro characterization of EC50s.

The present results illustrate that PTH reduced phosphate absorption by 30% when added to the luminal perfusate. Combined addition of PTH to both perfusate and basolateral bathing solution further inhibited the magnitude of phosphate absorption to a total of 50%. Over the range of perfusion rates of 8-22 nl/ml, phosphate absorption varied with the rate of tubule perfusion (Fig. 4). This finding indicates that the rate of phosphate absorption by proximal tubules is related to the magnitude of phosphate delivery. Phosphate infusion increased the rate of phosphate reabsorption by proximal convoluted tubules in phosphate-deprived rats (3). In rabbit proximal tubules, however, the rate of phosphate absorption is limited only by the transport capacity of the tubule rather than by the delivery rate (17).

In contrast to proximal tubules and cortical collecting ducts, where the CaSR is expressed on luminal plasma membranes, in thick ascending limbs of Henle’s loop the CaSR is primarily located on basolateral plasma membranes (56). In previous studies we showed that activation of the CaSR in mouse cortical ascending limbs inhibited
PTH-stimulated calcium transport (42). CaSR activation in cortical collecting ducts regulates vasopressin-induced water permeability (60). Such an action would diminish the potential for calcium precipitation and renal stone formation. Little is known of the physiological role of the CaSR on proximal tubule solute or fluid absorption. A recent preliminary report suggests that CaSR activation increases 1,25(OH)₂vitamin D₃ synthesis through activation of the CaSR (8). Because of its important function in regulating renal mineral ion homeostasis, we hypothesized that the proximal CaSR regulates PTH-dependent phosphate absorption. We directly analyzed the effects of CaSR activation on phosphate absorption by single mouse proximal tubules that were microperfused in vitro. For these studies, we used Gd³⁺ to activate the CaSR. Gd³⁺ is a potent type I CaSR agonist, with an EC₅₀ of 30-50 µM (47). Using Gd³⁺ permitted us to avoid millimolar elevations of calcium at one membrane surface or the other, which would directly alter fluid absorption by virtue of its osmotic action. We found that luminal, but not serosal, addition of Gd³⁺ blocked the inhibitory action of PTH on phosphate absorption. Gd³⁺ had no effect on basal, i.e., PTH-independent phosphate transport. Previous work by Rouse and Suki demonstrated that calcium did not affect basal phosphate transport in rabbit S3 proximal tubules (59). These observations are consistent with the predominant apical localization of the CaSR and suggest that the effects of Gd³⁺ on phosphate absorption were mediated by CaSR activation. Gd³⁺ had no effect on fluid absorption when added to either lumen or bath. In contrast, a preliminary report described an inhibitory affect of Gd³⁺ on fluid absorption by proximal tubules but at Gd³⁺ concentrations (200 µM) considerably greater than employed here (50). The mechanism of this effect is not clear but may be related to a “pre-conditioning
action of angiotensin II on Na\textsuperscript{+}K\textsuperscript{+}-ATPase activity (S.C. Hebert). The present results are in agreement with the view that CaSR activation regulates phosphate absorption without detectably or directly altering sodium transport, and therefore fluid absorption.

Dopamine also regulates renal phosphate transport (46). The inhibitory effect of dopamine is mediated by D\textsubscript{1} and D\textsubscript{2} dopamine receptors expressed in the proximal nephron (32). Dopamine inhibits phosphate absorption by rabbit and rat proximal renal tubules (31, 33). In the present investigation, application of dopamine to the basolateral bathing solution inhibited J\textsubscript{Pi} by 28%, somewhat greater than that we observed with basolateral addition of PTH (20%). In contrast, dopamine inhibited phosphate absorption less effectively than PTH in the rat (29). However, in the latter studies the animals were placed on a low-phosphate diet. Other experimental parameters also differ appreciably from the present report.

More importantly, whereas CaSR activation blocked the inhibitory effect of PTH on phosphate absorption, CaSR activation did not curtail the inhibitory action of dopamine on phosphate transport. Thus, CaSR activation specifically inhibits PTH and not dopamine-sensitive phosphate absorption. Again, CaSR activation had no effect on basal phosphate transport.

The inhibitory effects of CaSR activation on PTH-inhibitable phosphate transport were further examined in OK cells, where it is possible to increase calcium without complications due to asymmetric osmotic effects. CaSRs were activated by raising extracellular Ca\textsuperscript{2+} and by the addition of an unrelated organic compound, NPS R-467, a type II calcimimetic. Elevating extracellular Ca\textsuperscript{2+} from 1 to 10 mM had no effect on resting phosphate uptake (Fig. 8), similar to the absence of an effect of Gd\textsuperscript{3+} on basal
phosphate transport in the perfused tubule experiments. However, increasing Ca\textsuperscript{2+} abolished PTH-inhibitable phosphate transport at 0.1 or 10 nM PTH. This finding supports the view that the inhibitory effects observed with Gd\textsuperscript{3+} are likely attributable to CaSR activation and were not due to blockade of ATP-permeable channels (58), Ca\textsuperscript{2+}-sensitive K\textsuperscript{+} channels, the nonselective Ca\textsuperscript{2+}-permeable cation channel polycystin-2 (25), the Ca\textsuperscript{2+}-selective Trp3 channel (49), or other nonselective cation channels (18, 68), or mechanosensitive channels (27). It should be noted that although the solutions containing Gd\textsuperscript{3+} were prepared to a final concentration of 30 µM, the free Gd\textsuperscript{3+} concentration was likely to be substantially lower because trivalent phosphate anions in the extracellular bathing solution avidly bind free Gd\textsuperscript{3+} (14).

The mechanism whereby CaSR activation blocks PTH-inhibitable phosphate absorption has not been examined. Several possible signaling pathways may be involved. Activation of the CaSR most commonly results in G-protein-dependent stimulation of phospholipase C with attendant inositol trisphosphate formation and a transient release of Ca\textsuperscript{2+} from intracellular stores (12). Other CaSR signaling pathways, including activation of Gi, phospholipase A2, phospholipase D, and mitogen-activated protein kinase, have been described but are less well characterized (11, 34).

Mice with targeted inactivation of the CaSR rescued by crossing with PTH-null mice (36) or with Gcm2-null mice (65) do not exhibit statistically significant differences in plasma phosphate. The situation with humans harboring activating CaSR mutations is more complex. Individuals with hypocalcemic hypercalciuria with high serum phosphate have been reported (51, 63). This suggests that CaSR activation and inactivation may have different effects on PTH-sensitive renal phosphate transport. Alternatively, humans
and mice may respond differently. It is also quite likely that PTH is not the only peptide hormone regulating proximal nephron phosphate transport. Recent work has clearly identified important roles of FGF23 and FRP4 in renal phosphate absorption (7, 38, 53, 61). Taken together, these findings suggest that CaSR-independent regulation of phosphate homeostasis provides additional levels of defense against significant alterations of extracellular inorganic phosphate.

The observations reported here show that activation of the apical membrane CaSR specifically attenuates PTH-suppressible Pi absorption in proximal tubules and phosphate uptake by OK/E cells, a model for the proximal tubule. This effect may represent part of a multi-tiered hormone and cytokine regulation of renal phosphate transport that involves not only PTH and dopamine, but also FGF23 and FRP4 (38).
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REFERENCES


61. **Shimada T, Mizutani S, Muto T, Yoneya T, Hino R, Takeda S, Takeuchi Y, Fujita T, Fukumoto S, and Yamashita T.** Cloning and characterization of FGF23 as a


FIGURE LEGENDS

Fig. 1 Basolateral effects of PTH on phosphate and fluid absorption. A: PTH inhibits phosphate absorption ($J_{Pi}$). Lines connect the results of 11 independent experiments, in which each observation represents the average of 2-3 collection periods under the indicated condition. After the control period, 100 nM hPTH(1-34) was added to basolateral bathing solution. Numbers show the mean ± SEM for each group. The paired difference was highly statistically different. B: Basolateral PTH does not alter the fluid absorption ($J_v$). $J_v$ was measured concurrently in paired determinations with $J_{Pi}$.

Fig. 2 Immunocytochemical localization of PTHR1 in mouse kidney. A: Staining was seen in the apical brush border (arrows) and at the basolateral pole of proximal tubule S3 segments (S3). Considerably stronger staining was present in thick ascending limbs of Henle (TAL), where both apical and basolateral receptors were detectable. This section is from the outer stripe of the outer medulla. B: At higher magnification, apical (asterisks) and basolateral (arrows) was often seen in a punctate pattern in proximal tubule S3 segments in the outer stripe of the outer medulla. Bar = 40 microns (A) and 20 microns (B).

Fig. 3 Combined action of luminal plus basolateral PTH. A: Luminal PTH further inhibited $J_{Pi}$. Addition of PTH to the luminal perfusate alone decreased $J_{Pi}$ by 20%. Combined addition of 100 nM PTH(1-34) to the luminal perfusate and serosal bathing solution further reduced $J_{Pi}$ to 1.5 pmol min$^{-1}$ mm$^{-1}$ ($P<0.05$). B: Combined addition of PTH to luminal perfusate and basolateral bathing solution does not affect $J_v$. The control period consisted of PTH (100 nM) in the luminal
perfusate. Further addition of PTH to the basolateral bathing solution had no effect on $J_v$.

**Fig. 4** PTH inhibits flow-dependent phosphate absorption. Each point represents a single sample collected during control periods or after addition of PTH. Phosphate absorption exhibits a significant positive regression with perfusion rate during control periods. ($R=0.48$, $P<0.001$). This relation is suppressed upon addition of PTH group ($R=0.19$, NS).

**Fig. 5** Immunocytochemical localization of CaSR in mouse S3 proximal tubules. CaSr immunofluorescence showed strong apical localization in the apical region of proximal tubules (S3 segment). Weaker basolateral staining was also present. Bar = 20 microns.

**Fig. 6** CaSR activation blocks the inhibitory effect of PTH on $J_{Pi}$.  
*A*: 30 µM Gd$^{3+}$ was included in the luminal perfusate during the control periods. 100 nM hPTH(1-34) was added to basolateral bathing solution. The data show that luminal Gd$^{3+}$ blocked PTH-suppressible $J_{Pi}$ ($P >0.05$).  
*B*: Basolateral Gd$^{3+}$ did not affect the inhibitory action of PTH on $J_{Pi}$. 30 µM Gd$^{3+}$ was included in the basolateral bathing solution during control periods. After the control periods, basolateral addition of 100 nM PTH(1-34) prompted a significant inhibition of $J_{Pi}$ ($P <0.05$).

**Fig. 7** CaSR activation does not interfere with dopamine-inhibitable phosphate absorption. 
*A*: Basolateral addition of dopamine ($10^{-6}$ M) inhibited $J_{Pi}$ by 28% ($P<0.01$).  
*B*: Luminal Gd$^{3+}$ (30 µM) had no inhibitory action on dopamine-sensitive $J_{Pi}$. 

Fig. 8 Calcium inhibited PTH-sensitive phosphate uptake by OK/E cells. Raising extracellular calcium from 1 to 10 mM had no effect of phosphate uptake. Calcium exerted a concentration-dependent inhibition of PTH-sensitive phosphate uptake. At 1 mM calcium, PTH-inhibitable Pi uptake was reduced at 0.1 and at 10 nM PTH. Further elevation of calcium to 10 mM abolished the inhibitory effect of PTH on phosphate uptake at both low- and high-dose PTH.

Fig. 9. NPS R 467 blocked PTH-inhibitable phosphate uptake by OK/E cells. PTH (10 nM) reduced phosphate uptake by 60%. In the presence of NPS R 467 (3 µM), PTH action was significantly reduced. NPS S467 was used as a negative control and did not alter PTH inhibition of phosphate uptake.
Fig. 1

A

B

$J_{p}$ pmol min$^{-1}$ mm$^{-1}$

$J_{v}$ nl min$^{-1}$ mm$^{-1}$

Control

PTH

2.93 ± 0.38

2.37 ± 0.34

P < 0.01

1.45 ± 0.19

1.41 ± 0.15

NS
Fig. 3

**A**

- **J_{Pi, pmol min^{-1} mm^{-1}}**
- **Apical**
  - 2.15 ± 0.59
- **Apical + Basolateral**
  - 1.54 ± 0.55

**B**

- **J_{v, nl min^{-1} mm^{-1}}**
- **Apical**
  - 2.0
- **Apical + Basolateral**
  - 0.5

*P < 0.05*
Fig. 4

The graph shows the relationship between perfusion rate and Pi flux (J\textsubscript{Pi}), with data points indicating control and PTH conditions. The graph includes a line of best fit for each condition, visually demonstrating the relationship between the two variables.
Fig. 5
Fig. 6
Fig. 7
Fig. 8
**Fig. 9**

Bar chart showing the P₃ uptake, % Maximum for different conditions: Control, PTH, PTH + NPS R₄₆₇, and PTH + NPS S₄₆₇. The chart indicates statistical significance with ** symbols.