Parathyroid hormone inhibits sodium-phosphate co-transport in proximal renal tubule cells through activation of several kinases. We tested the hypothesis that the activity of these kinases was coordinated by an A kinase anchoring protein (AKAP) by demonstrating that the type II sodium-phosphate cotransporter (NaPi-4) physically associated with an AKAP and that this association was necessary for regulation of phosphate transport by parathyroid hormone. Immunoprecipitation with anti-NaPi-4 antiserum and glutathione S-transferase pull-down with GST-NaPi-4 showed that NaPi-4 associated with AKAP79, protein kinase A catalytic and regulatory subunits, and the parathyroid hormone receptor in opossum kidney cells. When the regulatory subunit of protein kinase A was uncoupled from the AKAP by a competitive peptide, parathyroid hormone lost the ability to inhibit phosphate transport. This result was confirmed by co-transfecting HEK293 cells with the sodium-phosphate cotransporter and wild type AKAP, a mutant AKAP79, or the empty vector. 8-Bromo-cAMP was able to inhibit phosphate transport in cells expressing the wild type AKAP79 but not empty vector or mutant AKAP79. We conclude that parathyroid hormone inhibits proximal renal tubule sodium-phosphate cotransport through a signaling complex dependent upon an AKAP.

Phosphate balance is maintained primarily by regulation of sodium-dependent phosphate reabsorption by type II sodium-phosphate cotransporters in the proximal renal tubule (1–4). Parathyroid hormone (PTH) inhibits the function and expression of type II sodium-phosphate cotransporters through activation of several signal transduction pathways (5–7). Stimulation of PTH receptors leads to activation of protein kinase A (PKA) through coupling to the stimulatory guanine nucleotide regulatory protein Gs. Simultaneously, PTH receptor stimulation activates protein kinase C (PKC) through coupling to Gq. Direct activation of either PKA or PKC causes inhibition of sodium-dependent phosphate transport in renal proximal tubules, similar to the effect of PTH itself. The significance of this dual signaling is not clear; nor are the mechanisms for coordinating the two pathways.

Activation of similar signaling pathways by other stimuli such as dopamine does not result in identical regulation of sodium-phosphate cotransport or cotransporter expression (8). The mechanism for this agonist-specific effect on phosphate despite activation of ostensibly similar second messengers is not understood. Several mechanisms to explain agonist-specific functional effects have been proposed, including activation of different enzyme isoforms; coupling to different G proteins, or activation of different unrecognized signals. Recently, interest has focused on the role of scaffolding and anchoring proteins as a means to compartmentalize and individualize agonist effect on intracellular processes (9–11). A kinase anchoring proteins (AKAPs) are a family of proteins that express a well conserved sequence that binds to the regulatory subunit of PKA (PKA RII) (12–15). Each AKAP also expresses a sequence that allows it to interact with a specific subcellular position and so directs signal transduction to a unique locale. Some AKAPs, such as AKAP79 and gravin, bind multiple kinases and phosphatases (16–20). AKAPs play a role in the regulation of multiple transport proteins, including sodium channels (21), potassium channels (22), and calcium channels (23–25). Recently, Klussmann et al. (26), using a competing peptide to block AKAP binding to PKA regulatory subunits, demonstrated that forskolin activated PKA but could not stimulate translocation of water channels to the apical membrane. Thus, tethering of PKA to a specific cellular site by an AKAP was critical to the ability of hormone-activated PKA to regulate water transport. Lamprecht et al. (27) reported that ezrin, an AKAP (28), regulates cAMP inhibition of NHE3, the sodium-hydrogen exchanger, in opossum kidney (OK) cells.

These findings suggested to us the possibility that PTH regulated the expression and function of the sodium-phosphate cotransporter through a signaling complex assembled by an AKAP. We tested this hypothesis by examining the physical association of the sodium-phosphate cotransporter with an AKAP and other signal transduction proteins. We also examined the effect of disruption of the AKAP-PKA binding on PTH regulation of sodium-phosphate cotransport. These studies were performed in OK cells, a continuous cell line derived from the Virginia opossum that exhibits several characteristics of mammalian renal proximal tubule including a polarized morphology, apical expression of sodium-phosphate cotransporters (NaPi-4), and regulation of phosphate uptake by PTH, PKA, and PKC.

MATERIALS AND METHODS

Wild type OK cells were a generous gift of Dr. Steve Scheiman (Syracuse Health Science Center, Syracuse, NY). ARAP79/150 affinity-purified polyclonal antibody and recombinant PKA RII protein were a generous gift from Dr. Christine Loh (ICOS Corp., Bothell, WA).
Dr. Heini Murer and Dr. Jung Biber (University of Zurich, Zurich, Switzerland) generously provided the cDNA for NaPi-4. Dr. John D. Scott (Oregon Health Science University, Portland, OR) kindly provided the wild type and dominant negative mutant AKAP79 cDNA constructs (25).

Cell Culture—OK cells were grown to confluence in monolayers in 175-cm² flasks in culture medium consisting of Eagle’s medium with Earle’s salts (Invitrogen) supplemented with 10% heat inactivated fetal calf serum, 4 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin, pH 7.4, as previously described (30). The cells from passages 82–88 were used for experiments at 100% confluence.

Immunoprecipitation—The OK cell plates were washed with Hank’s balanced saline solution twice and once with 50 mM mannitol, 5 mM Tris-HCl, pH 7.4. The cells were lysed with 50 mM Tris-HCl buffer, pH 7.4, and the lysate (100 μg of protein) was incubated with preimmune serum for 1 h at room temperature on a rotator. 10 μl of Protein A-Sepharose beads were added and incubated for another 1 h at room temperature. The sample was centrifuged for 5 min at 5000 rpm in a microcentrifuge. The pellet was discarded, and the supernatant was incubated with 10 μl of antigen serum against NaPi-4 (30) or antibody against AKAP79/150 overnight at 4 °C. 10 μl of Protein A-Sepharose beads were added and incubated on a rotator for 1 h at 4 °C. The samples were centrifuged for 5 min at 5000 rpm. The pellet was washed with Hank’s balanced saline solution three times and subjected to SDS-PAGE.

Immunoprecipitation Using Seize-X Beads—The anti-NaPi-4 antibody (antibody) was immobilized on Protein A-Sepharose beads using the Seize X Protein A immunoprecipitation kit (Pierce) according to the manufacturer’s protocol. Briefly, 0.4 ml of the ImmunoPure plus Immobilized Protein A-Sepharose beads were washed twice with binding/wash buffer (0.14 M NaCl, 0.008 mM Na2PO4, 0.002 mM potassium phosphate, 0.01 mM KCl, pH 7.4). 0.4 ml of 1:1 diluted (in binding buffer) antiserum was added to the beads and incubated on a rotator for 1 h at room temperature. The beads were washed three times with 0.5 ml of binding buffer, and 0.4 ml of the binding buffer was added. 2 ml of the disuccinimidyl suberate was dissolved in 80 μl of Me2SO, and 25 μl of disuccinimidyl suberate reagent was added to the beads and incubated on a rotator for 1 h at room temperature. The beads were centrifuged and washed three times with 0.5 ml of quenching buffer (25 mM Tris-HCl, 0.15 mM NaCl, pH 7.2). The beads were then washed with elution buffer (Pierce) until no protein was detected at 280 nm in the eluent. The beads were then washed with the quenching buffer twice. OK cells were lysed in immunoprecipitation lysis buffer containing 20 mM Tris-HCl, pH 7.4, 157 mM NaCl, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 20 mM NaF, 2 mM dithiorethiol, and 0.1 mM sodium vanadate, pH 7.4, and 5 μl of γ-32P]ATP (1 mCi/ml) in the presence of absence of 0.5 mM CaCl2. A positive control contained basic protein and 2 μl of active catalytic unit of PFK in 30 μl of kinase buffer and a negative control (positive control plus 2 μl of the PFK inhibitor TTYADFIA6STGRTRGNHAIH) were run simultaneously with the test samples. The reaction was stopped by adding 1× Laemmli buffer and heating the samples at 95 °C for 5 min. The samples were run on 10% SDS-PAGE, and phosphorylation was detected by autoradiography.

Preparation of GST and GST-NaPi-4—Glutathione-Sepharose Beads—GST-NaPi-4 full-length or deletion mutant (DM-4) was shuttled from NaPi-4 pCR2.1 plasmid into the EcoRI site of GST-pGEX-3. The ligated GST-pGEX3-NaPi-4 cDNA was transformed into E. coli DH5α. Positive colonies were selected by restriction enzyme mapping and were propagated in E. coli BL21 (Stratagene). The plasmid constructs were confirmed by sequencing. The GST-pGEX-3 NaPi-4 or GST-pGEX3-DM-4 plasmid was transformed into E. coli BL21 (Stratagene), and the expression and purification of GST and NaPi-4 or GST-DM-4 fusion protein was performed as previously described by Zu et al. (31). Briefly, a fresh overnight culture of E. coli BL21 transformed with either GST-pGEX-3, recombinant GST-pGEX-3NaPi-4, or GST-pGEX3-DM-4 was diluted 1:10 in 2× YT medium containing ampicillin (100 μg/ml) and grown at 37 °C. After this reached an absorption of 0.6 at 600 nm, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM, and the bacterial growth was continued for 4 h at 37 °C. Cells were harvested, washed once with phosphate-buffered saline, and lysed in phosphate-buffered saline containing 0.5% Triton X-100 and 50 μM phenylmethylsulfonyl fluoride on ice. The samples were centrifuged at 10,000 × g for 15 min at 4 °C. Samples (10 ml) of bacterial supernatant were rocked for 30 min at 4 °C with 800 μl of glutathione-Sepharose beads previously washed three times by and resuspended in phosphate-buffered saline with 0.5% Triton X-100. The beads were washed 8–10 times with 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% (v/v) EDTA, and 1% (v/v) KC1 and resuspended in phosphate-buffered saline with 0.5% Triton X-100.

GST Pull-down Assay—OK cells grown on six-well plates (Corning) were washed with Hank’s balanced saline solution and lysed with 200 μl of immunoprecipitation lysis buffer containing 20 mM Tris-HCI, pH 7.4, 150 mM NaCl, 1% (v/v) Triton X-100, 0.5% (v/v) Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 20 mM sodium orthovanadate, 20 mM NaF, 5 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 21 μg/ml aprotinin, and 5 μg/ml leupeptin. The lysates were preincubated with GST-glutathione-Sepharose beads for 2 h at 4 °C. Following this preincubation step, GST-NaPi-4, GST vector, or glutathione-Sepharose beads were added to the lysates and incubated at 4 °C overnight on a rotator. The beads were washed nine times with Krebs Plus buffer, and 40 μl of 2× Laemmli buffer was added to each tube. The samples were boiled for 5 min and then subjected to 10% SDS-PAGE. Proteins were transferred onto nitrocellulose for immunoblot analysis.

Transfection—HEK293 cells were maintained in DMEM (Cellgro, Herndon, VA) supplemented with 10% fetal calf serum and 1% penicillin and streptomycin. The cells were split a day prior to transfection at 80–90% confluence such that the cells were 60–80% confluent. HEK293 cells were transfected with the indicated plasmids using LipofectAMINE (Invitrogen) according to the manufacturer’s protocol. The cells were washed with OPTI medium 24 h before treatment with 8-Br-cAMP.

Phosphate Uptake—Phosphate transport was measured by determination of radiolabeled phosphate uptake as previously described (30). Each assay was performed in triplicate and averaged, and the mean was considered as a single data point.

Statistics—Data are shown as mean ± S.E. The n values shown represent the number of separate experiments. Each experiment was done in triplicate. p value is calculated using SigmaStat software utili-
RESULTS

Association of NaPi-4 and an AKAP—To determine whether any AKAPs associated with NaPi-4, we immunoprecipitated OK cell membranes with antiserum to NaPi-4 and performed immunoblot analysis for AKAP79/150 and AKAP149 (Fig. 1). The AKAP79/150 antibody identified bands at 79 and 60 kDa. AKAP149 monoclonal antibody did not identify a band. Ezrin antibody inconsistently and only faintly identified a band at the 79-kDa position (data not shown). Immunoblots of OK cell lysates and crude membrane preparations for AKAP79/150 are shown in Fig. 2. Only very faint bands are seen in the whole cell lysates; however, the crude membranes exhibited strong bands at 60 kDa and a 79/80-kDa doublet. We therefore focused our attention on AKAP79. To determine which of the bands identified by the AKAP79/150 polyclonal antibody in NaPi-4 immunoprecipitates were AKAPs, we performed an RII overlay assay on the immunoprecipitated proteins in the presence and absence of a competing peptide, DLIEEAASRVSQVAA-KAGAY, or a nonfunctional analogue, DLIEEAASRPVSQVAA-KAGAY (Fig. 3). In this assay, the immunoblot membrane is incubated in buffer containing the regulatory subunit of PKA (RII), which should bind any AKAP, followed by immunoblot for RII. The RII overlay assay identified the 79-kDa protein (left lane). The addition of the competing peptide virtually abolished RII binding (middle lane), whereas the addition of the inactive analogue had no effect on RII binding (right lane). The RII overlay assay also demonstrated significant nonspecific binding as noted by the heavy lower molecular weight bands. The intensity of these bands was not diminished by either the competing peptide or the nonfunctional analogue. There was an additional band identified at about 150 kDa by the RII overlay assay; however, the intensity of this band was diminished by both the competing peptide and the nonfunctional analogue. These findings suggest that in the NaPi-4 immunoprecipitates, only the 79-kDa band is specifically an RII-binding protein (i.e. an AKAP).

To exclude the possibility of a nonspecific interaction between the polyclonal antiserum and the OK cell membrane proteins, we immunoprecipitated OK cell membranes with pre-immune anti-NaPi-4 serum, immune serum, and immune serum preincubated overnight with cognate peptide. Subsequent immunoblot with the ICOS AKAP79/150 antibody demonstrated that the preimmune serum did not immunoprecipitate AKAP79 and that preincubation of the immune serum with peptide markedly decreased the ability to immunoprecipitate AKAP79 (Fig. 4). Note in the accompanying panel that preincubation of the immune serum with immunizing peptide markedly diminished the ability of the antiserum to immunoprecipitate NaPi-4. To confirm the AKAP79/NaPi-4 association, we immunoprecipitated OK cell membranes with the ICOS 79/150 antibody and blotted for NaPi-4. We detected NaPi-4 in the ICOS antibody immunoprecipitates (Fig. 5). Immunoprecipitation with both NaPi-4 and AKAP79/150 antibody resulted in several nonspecific bands, especially at molecular sizes of 50 kDa or less. We therefore repeated the NaPi-4 immunoprecipitation using antibody covalently linked to beads. Using this technique, as shown in Fig. 6, we confirmed the fact that NaPi-4 antibody immunoprecipitates an AKAP recognized by AKAP79 antibody.

Identification of Proteins That Co-immunoprecipitate with NaPi-4—We next determined whether NaPi-4 and the AKAP associated with other cellular signaling components. We rea-
experiments.

Blots are representative of three separate AKAP79 (immunoprecipitated with AKAP79/150 antiserum preincubated with right lane AKAP79/150 antiserum (clearly present as a broad band in cells immunoprecipitated with NaPi-4 antisera). Preimmune serum likewise does not immunoprecipitate NaPi-4 (middle lane). NaPi-4 in immunoprecipitates runs as a broad band between 90 and 110 kDa (middle lane).

The proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane, and blotted for AKAP79/150. The arrow indicates the AKAP79/150 band. Immunoprecipitation with preimmune serum did not show an AKAP79/150 band. Immunoprecipitation with NaPi-4 antisera blotted positively for AKAP79/150. The intensity of the AKAP79/150 band was markedly decreased in immunoprecipitates of NaPi-4 antisera preincubated with peptide. Blots are representative of three separate experiments. The top panel demonstrates that preincubation of NaPi-4 antisera with immunizing peptide significantly decreases the ability of NaPi-4 antisera to immunoprecipitate NaPi-4 (far right lane). Preimmune serum likewise does not immunoprecipitate NaPi-4 (far left lane). NaPi-4 in immunoprecipitates runs as a broad band between 90 and 110 kDa (middle lane).

The ability of NaPi-4 antibody to immunoprecipitate the PTH receptor and AKAP79/150 suggests that all three proteins can be found in renal brush border membranes (BBM). Therefore, we prepared BBM from OK cells grown on permeable supports and blotted for NaPi-4, the PTH receptor, and AKAP79/150. As shown in Fig. 10, there was about 6–8-fold enrichment of NaPi-4 expression and the PTH receptor in the BBM when compared with expression in crude cell preparations; however, there was no difference in AKAP79/150 expression between lysates and BBM.

We corroborated the association of NaPi-4 with AKAP79/150 and the PTH receptor by performing GST pull-down assay on OK cell lysates using a full-length NaPi-4-GST fusion protein linked to glutathione-Sepharose beads. Immunoblot analysis of the separated proteins from the GST pull-down assay confirmed the presence of AKAP79/150 and the PTH receptor (Fig. 11). We confirmed that the band identified by the AKAP79/150 antibody was an AKAP by an RII overlay assay, demonstrating RII binding of the same band. Similar analysis of pull-downs with GST vector alone failed to reveal any of the proteins. We performed a pull-down with a NaPi-4-GST fusion protein, where the C-terminal of the NaPi-4 was deleted (DM-4). Pull-downs using DM-4 showed the expected absence of staining for NaPi-4 using the C-terminal NaPi-4 antisera. Immunoblots using an N-terminal antibody confirmed the actual presence of NaPi-4 in the pull-downs (data not shown). The C-terminal deletion mutant GST pull-downs showed a marked decrease in the amount of AKAP79 and a corresponding decrease in the intensity of the RII overlay assay. These data corroborate our immunoprecipitation data, demonstrating a physical association between NaPi-4, PKA RII, the PTH receptor, and an AKAP similar to the human AKAP79/150. They also suggest that the C terminus of NaPi-4 is required for full binding of the AKAP.

Functional Significance of the AKAP-NaPi-4 Association—To determine whether the AKAP-NaPi-4 association was of functional significance, we uncoupled PKA from AKAPs by incubating OK cells with the blocking peptide stearated at its N terminus or with an inactive stearated analogue for 30 min at 37 °C. The cells were then treated with either vehicle or with 10−7 m PTH (1–34) for 2 h, followed by measurement of radiolabeled phosphate uptake. As shown in Fig. 12, PTH decreased 32P uptake in OK cells. Pretreatment with competing peptide inhibited the ability of PTH to decrease the 32P uptake, whereas pretreatment with the inactive analogue did not.
To confirm that AKAP79 (or a similar AKAP) is involved in the regulation of NaPi-4, we co-transfected HEK293 cells with NaPi-4 cDNA and with the wild type or mutant AKAP79 or vector alone (Fig. 13). 48 h post-transfection, the cells were treated with vehicle or 10^{-4} M 8-Br-cAMP, and phosphate uptake was measured (Fig. 14). Control HEK293 cells exhibited a phosphate uptake of 5.3 ± 1.0 pmol/min/mg protein. Cells transfected with NaPi-4 showed a 20% increase in basal phosphate uptake (6.4 ± 1.2 pmol/min/mg protein). Cells transfected with NaPi-4 plus wild type AKAP showed a 35% increase in basal phosphate uptake (7.3 ± 1.7 pmol/min/mg protein), whereas cells transfected with NaPi-4 plus the mutant AKAP showed no increase in basal phosphate uptake. In cells transfected with NaPi-4 alone, 8-Br-cAMP decreased Pi uptake by 38%, compared with cells transfected with vector (13%). In cells transfected with wild-type AKAP79 alone, 8-Br-cAMP increased P_i uptake by 14%, whereas in cells transfected with mutant AKAP79, 8-Br-cAMP decreased P_i uptake by 23%. However, when the cells were co-transfected with NaPi-4 and wild type AKAP79, 8-Br-cAMP inhibited P_i uptake by 63%. 8-Br-cAMP inhibited P_i uptake by only 8% in cells co-transfected with NaPi-4 and the dominant negative AKAP mutant.

DISCUSSION

Renal proximal tubule phosphate transport is regulated by many physiologic stimuli through multiple signaling pathways. Dietary phosphate deprivation, insulin, and growth factors increase phosphate reabsorption, whereas dietary phosphate excess, PTH, and dopamine decrease phosphate reabsorption. Under typical physiologic conditions, dietary ingestion of phos-
phosphate exceeds demands, necessitating net renal excretion of phosphate. PTH, a major regulator of renal phosphate homeostasis, inhibits phosphate transport through activation of PKA and PKC, resulting in acute and chronic decrease in apical membrane expression of type II sodium-phosphate cotransporters. Other activators of PKC such as epinephrine and angiotensin II can actually increase, not decrease, phosphate reabsorption in this tubule segment. The complex nature of this regulatory process suggested that PTH-specific phosphaturia may be accomplished by compartmentalization of PTH-stimulated signaling pathways with target substrates. An AKAP seemed a likely candidate for this function, as several AKAPs have been reported to bind multiple kinases and phosphatases. Additionally, although both PKA and PKC can mimic the phosphaturic action of PTH, several studies have suggested that the role of PKA is more important than that of PKC. We reasoned that an AKAP could bind PKA and possibly PKC and phosphatases in close proximity to the OK cell type II sodium-phosphate cotransporter, thus ensuring specificity of action.

Our studies support our hypothesis that PTH regulation of sodium-phosphate cotransport is dependent on an AKAP. First, we demonstrated that NaPi-4 is physically associated with an AKAP. Immunoprecipitation of OK cell lysates with polyclonal NaPi-4 antiserum yielded the presence of an AKAP recognized by polyclonal antibody directed against AKAP79/150. Immunoprecipitation with either preimmune serum or antiserum preincubated with NaPi-4 peptide failed to demonstrate the AKAP79/150 band. This same band also stained positively in an RII overlay assay, confirming that the protein recognized by AKAP79/150 antibody also could bind the regulatory subunit of PKA, a cardinal feature of AKAPs.

We next identified other components of this signaling complex. We demonstrated the presence of the catalytic subunit of PKA along with the regulatory subunit in NaPi-4 immunoprecipitates with the AKAP and PTH receptor. RII overlay confirmed that the protein stained positive for the AKAP79/150 antibody is an AKAP. Ezrin stained positive in GST-vector, GST-NaPi-4, and GST-DM4 beads, indicating that its association is nonspecific to NaPi-4. Blots are representative of three different experiments.
AKAP79/150 with PKA is the critical interaction; however, the competing peptide blocks all AKAP/PKA interactions and is therefore not specific for AKAP79/150. The transfection experiments, however, confirm that AKAP79 or a similar AKAP could serve in this capacity.

These data raise several questions. First, association of the PTH receptor with NaPi-4 suggests that PTH receptors are localized on the apical membrane of proximal tubule. Classical teaching holds that PTH receptors have a basolateral localization; however, studies suggest the presence of PTH receptors on apical membranes. Traebert and colleagues (33) have shown in microperfused proximal renal tubules that apical perfusion with PTH inhibits phosphate transport similar to basolateral incubation with PTH. It is likely then that PTH receptors are present on both membranes. Our data suggest that the PKA-stimulated and PKC-stimulated actions of PTH may be to some extent spatially separated. PTH regulates the activity of the Na-K-ATPase, a protein that is confined to the basolateral membrane and indirectly influences sodium-phosphate cotransport through regulation of intracellular sodium concentration. PTH, acting on apical receptors through activation of PKA, might function primarily to regulate NaPi-4 trafficking in and out of the apical membrane, whereas PTH activation of basolateral receptors might function to inhibit Na-K-ATPase through activation of both PKA and PKC.

Our data suggest that the AKAP/PKA association is essential for PTH inhibition of phosphate transport but do not address the mechanism by which this is accomplished. Type II sodium-phosphate cotransporters are constantly shuttled through a one-way path from synthesis, insertion into the apical membrane, removal into a lysosome, and degradation. Regulation of the number of active transporters is accomplished by altering either the rate or the bulk flow of proteins through this pathway. Acute changes in transporter number occur by increasing the rate of insertion or removal from the apical membrane. Chronic changes either increase or decrease the overall quantity of proteins by changing the rate of synthesis. This theorized hypothesis for sodium-phosphate cotransporter trafficking is supported by several pieces of data (34–40). Sodium-phosphate cotransporters have been identified on endosomal vesicles (38). Pretreatment of OK cells with colchicine, which blocks microtubule-dependent processes (39), or C3 exotoxin, which inactivates Rho, a small molecular weight GTPase implicated in membrane trafficking (40), partially blocks the ability of PTH to inhibit phosphate transport. Inhibition of the lysosomal degradative pathways in OK cells by leupeptin increases total cellular expression of cotransporters (36, 37). In the presence of leupeptin, PTH inhibits phosphate transport but does not decrease transporter expression. Confocal imaging reveals that the transporters have been removed from the apical membrane into a subapical compartment. These transporters cannot be reinserted into the apical membrane for reuse, indicating that PTH initiates an irreversible chain of events leading to protein destruction.

Uncoupling AKAP from PKA could impair normal base-line turnover of NaPi-4 by interfering with targeting of the sodium-phosphate cotransporter into the apical membrane. This explanation seems unlikely, since the basal rate of phosphate transport was neither increased nor decreased in OK cells incubated with the competing peptide, suggesting that the number of transporters did not change. On the other hand, basal phosphate uptake was higher in the HEK293 cells transfected with NaPi-4 and wild-type AKAP79 when compared with cells transfected with NaPi-4 alone or with NaPi-4 and mutant AKAP79. This observation does suggest a potential role for the AKAP in membrane targeting. The AKAP could bring the PTH
receptor in close proximity to PKA, facilitating the ability of PTH to activate PKA. The transfection experiments in HEK293 cells, where NaPi-4 transport was inhibited by cAMP and not by PTH, however, suggests that the AKAP/PKA RII interaction is critical for a more downstream step. Co-transfection of the dominant negative AKAP79, which is unable to bind PKA RII, blocked inhibition of phosphate transport by 8-Br-cAMP, a direct activator of PKA not requiring PTH receptor. Another possibility is that AKAP/PKA RII dissociation could prevent PKA from phosphorylating an as yet unidentified substrate necessary for regulation of NaPi-4. Data presented here show that PKA is capable of phosphorylating three substrates in NaPi-4 immunoprecipitates. In previous experiments, we have demonstrated that PKA does not directly phosphorylate NaPi-4. Jankowski et al. (41) have demonstrated that the type II sodium-phosphate cotransporter exists as a phosphoprotein and that treatment of OK cells with PTH resulted in a decrease in NaPi-4 phosphorylation. Thus, it is very unlikely that any of the three phosphorylated substrates are NaPi-4, and the targets for AKAP-directed PKA phosphorylation remain unknown.

We do not know what regulates the assembly or disassembly of this signaling complex. Under some clinical conditions, such as dietary phosphate deprivation, the phosphaturic action of PTH is inhibited. This effect of diet could potentially be mediated by AKAP/PKA dissociation. PTH itself could regulate AKAP expression or localization.

In summary, we have produced evidence that PTH regulation of type II sodium-phosphate cotransporters in proximal renal tubule cells is dependent on the integrity of a signaling complex composed of the PTH receptor, PKA regulatory and catalytic subunits, a phosphatase, an AKAP, and the type II sodium-phosphate cotransporter. The role of this complex in the regulation of phosphate homeostasis remains to be elucidated.

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