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Parathyroid Hormone and Parathyroid Hormone-related Peptide Inhibit the Apical Na^+/H^+ Exchanger NHE-3 Isoform in Renal Cells (OK) via a Dual Signaling Cascade Involving Protein Kinase A and C*

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Parathyroid hormone (PTH) and parathyroid hormone-related peptide (PTHRP) interact with a common G protein-coupled receptor and stimulate production of diverse second messengers (*i.e.* cAMP, diacylglycerol, and inositol 1,4,5-trisphosphate) that varies depending on the target cell. In renal proximal tubule OK cells, PTH inhibits the activity of the apical membrane Na^+/H^+ exchanger, although it is unclear whether the signal is transmitted through protein kinase A (PKA) and/or protein kinase C (PKC). To delineate the signaling circuitry, a series of synthetic PTH and PTHRP fragments were used that stimulate the adenylate cyclase-cAMP-PKA and/or phospholipase C-diacylglycerol-PKC pathways. Human PTH-(1–34) and PTHRP-(1–34) stimulated adenylate cyclase and PKC activity, whereas the PTH analogues, PTH-(3–34), PTH-(28–42), and PTH-(28–48), selectively enhanced only PKC activity. However, each peptide fragment inhibited Na^+/H^+ exchanger activity by 40–50%, suggesting that PKC and possibly PKA were capable of transducing the PTH/PTHRP signal to the transporter. This was corroborated when forskolin and phorbol 12-myristate 13-acetate (PMA), direct agonists of adenylate cyclase and PKC, respectively, both inhibited the Na^+/H^+ exchanger. The specific PKA antagonist, H-89, abolished the forskolin-mediated suppression of Na^+/H^+ exchanger activity, but did not prevent the inhibitory effects of PTH-(1–34) or PMA. In comparison, the potent PKC inhibitor, chelerythrine chloride, prevented the inhibition of Na^+/H^+ exchanger activity mediated by PTH-(28–48) and PMA but did not avert the negative regulation caused by PTH-(1–34) or forskolin. However, inhibition of both PKA and PKC prevented PTH-(1–34)-mediated suppression of Na^+/H^+ exchanger activity, indicating that PTH-(1–34) acted through both signaling pathways. In addition, Northern blot analysis revealed the presence of only the NHE-3 isoform of the Na^+/H^+ exchanger in OK cells. In summary, these results demonstrated that NHE-3 is expressed in OK cells and that activation of the PTH receptor can stimulate both the PKA and PKC pathways, each of which can independently lead to inhibition of NHE-3 activity.

The electroneutral transport of extracellular Na^+ in exchange for intracellular H^+ is mediated by the Na^+/H^+ exchanger (NHE).[1] This transporter is involved in the regulation of intracellular pH (pH_i), maintenance of cell volume, transepithelial Na^+ reabsorption, and pH_i-mediated cellular signaling associated with cell growth and differentiation (for review, see Ref. 1). Recently several mammalian isoforms of the Na^+/H^+ exchanger have been identified and characterized by cDNA cloning (NHE-1 to -4) (2–5) and chromosomal mapping (NHE-5) (6). Based on available data, NHE-1 is an amiloride-sensitive exchanger that is expressed in most cells and functions primarily in the regulation of pH_i and cell volume (2, 7, 8). In polarized renal (9) and intestinal (10, 11) epithelial cells, NHE-1 is localized to the basolateral membrane. Likewise, NHE-2, which has a slightly reduced sensitively to amiloride analogues (12), also has a wide tissue distribution (3) and exhibits similar functional characteristics (8). However, its membrane targeting in polarized renal epithelial cells is controversial, with studies reporting localization to the basolateral (13) or apical (14) membranes. In contrast, NHE-3 has a substantially lower affinity for amiloride (7) and is found exclusively on the apical membranes of renal (15) and intestinal (11) epithelia. Consequently this isoform is most likely involved in transepithelial Na^+ reabsorption. Last, NHE-4 is found primarily in stomach and to a much lesser extent in other tissues (2). At present, characterization of its functional properties is rather limited (16) due to its poor expression in heterologous cell systems.

Parathyroid hormone (PTH) inhibits *in vivo* renal proximal tubular Na^+, HCO_3^- and phosphate reabsorption by inhibiting the apically located Na^+/H^+ exchanger and Na^+/phosphate cotransporters (reviewed in Refs. 17 and 18). Similar results (19–21) have also been obtained in the opossum kidney (OK) cell line, which exhibits many of the characteristics of renal proximal tubule cells (22). The signaling mechanism to account for the effects of PTH in target tissues has only recently been investigated. PTH (an 84-amino acid peptide) and the recently identified factor, PTH-related peptide (PTHRP) (a 139–173-amino acid peptide, depending on the species) (23), interact with a common heterotrimeric G protein-coupled receptor (24).

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[1] The abbreviations uses are: NHE, Na^+/H^+ exchanger; PTH, parathyroid hormone; PTHRP, parathyroid hormone-related peptide; PKA and PKC, protein kinase A and C; PMA, phorbol 12-myristate 13-acetate; αMEM, α minimum essential medium.

Interestingly, only 8 of the *N*-terminal 13 amino acids between these two peptides are identical, whereas the remaining sequences are unique. Nonetheless, both can bind and activate the receptor which subsequently stimulates the adenylate cyclase and/or phospholipase C signaling pathways (24). In OK cells, the inhibitory effect of PTH on apical Na^+/H^+ exchanger activity can also be mimicked by forskolin and phorbol esters, pharmacological agonists of the PKA and PKC pathways, respectively (20, 25). Assembling these data together, it has been inferred that the mechanism by which PTH inhibits the OK apical Na^+/H^+ exchanger is through independent activation of PKA and PKC (20, 25). While this is a plausible interpretation, more direct evidence demonstrating this linkage is lacking. Other signaling circuitry could account for these observations. For example, it is quite possible that only one of these two pathways predominates in the PTH $\rightarrow$ Na^+/H^+ exchanger signaling cascade. This has been observed in the SV-40 transformed murine renal cortical proximal tubule (MCT) cell line, where PTH, forskolin, and PMA inhibited the apical Na^+/H^+ exchanger (26). However, PTH was found not to elevate $cAMP_i$ levels but rather to utilize only the phospholipase C-PKC pathway. Aside from this scenario, other signaling routes may also exist. It is conceivable that PTH could activate both pathways which subsequently "talk" with each other such that only one pathway ultimately leads to inhibition of the exchanger. Cross-talk between the PKA and PKC pathways has been documented in some cell systems where activation of PKC stimulates adenylate cyclase type II activity and ultimately PKA (27, 28). Indeed, there is some evidence that PMA may act as a weak activator of $cAMP_i$ production in OK cells (29). Last, Na^+/H^+ exchanger activity in human embryonic kidney (HEK) 293 cells can be modulated by a G protein ($G\alpha_{13}$) that is not linked to either adenylate cyclase or phospholipase C (30). Whether this pathway is also coupled to the PTH receptor in OK cells is unknown. Hence, the potential exists for multiple signaling routes that can be activated by PTH or PTHRP, with the predominant effect being dependent on the exact cellular complement of receptor-associated G proteins and their downstream effectors.

Therefore, in the present study, we wished to examine in greater detail the hypothesis that PTH or PTHRP inhibits the apical membrane Na^+/H^+ exchanger by both the PKA and PKC pathways in the OK cell model system. This was accomplished as follows: (i) by using a series of synthetic peptide fragments of PTH and PTHRP that selectively activate adenylate cyclase and/or phospholipase C and (ii) by using other downstream activators (*i.e.* forskolin and PMA) and selective inhibitors of the PKA and PKC pathways. Second, we wished to establish which isoform(s) of the Na^+/H^+ exchanger is(are) present in these cells. Our results demonstrated that only the NHE-3 isoform is expressed in OK cells and that PTH and PTHRP inhibit NHE-3 via two distinct signaling pathways that act independently of each other.

EXPERIMENTAL PROCEDURES

Materials—Phosphatidyl-L-serine, diolein, dithiothreitol, phenylmethylsulfonyl fluoride, EDTA, EGTA, and leupeptin were obtained from Sigma. α minimum essential medium (αMEM), fetal bovine serum, trypsin-EDTA, and Geneticin were purchased from Life Technologies, Inc. Dowex AG50 WX4 (200–400 mesh) and neutral chromatographic alumina WN-3 were from Bio-Rad. DEAE-Sephacel was purchased from Pharmacia Biotech Inc. Carrier-free $^{22}NaCl$ (5 mCi/ml), and [γ-^{32}P]ATP (0.5 mCi/ml) were obtained from DuPont NEN. The different human PTH and PTHRP synthetic fragments were kind gifts of Dr. K. Muller (Ciba-Geigy, Basel, Switzerland). Forskolin, 1,9-dideoxyforskolin, PMA, 4α-PMA, H-89, and chelerythrine chloride were purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). All other chemicals were from Fisher and British Drug House.

Tissue Culture—The OK cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. The Chinese hamster ovary cell line devoid of endogenous NHE activity (AP-1) was transfected with cDNAs containing the entire coding region of rat NHE-1, NHE-2, or NHE-3 as described previously (3, 7). Rat NHE-4 was heterologously expressed in a Chinese hamster lung cell line (PS120) that also lacked endogenous NHE activity and was called PSCN4-4 (generously provided by Dr. Eugene Chang; University of Chicago) (16). The transfected cells were grown in αMEM supplemented with 10% fetal bovine serum and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone). Cells were incubated in a humidified atmosphere of 95% air, 5% CO_2 at 37 °C.

Adenylate Cyclase Activity—Adenylate cyclase activity was determined using a method based on incorporation of [^{3}H]adenine into ATP and its conversion to [^{3}H]cAMP (31). Prior to each assay, confluent cells were incubated overnight in serum-free medium. The assay was initiated by adding [^{3}H]adenine (4×10^5 cpm) per tissue culture well and incubating for 2 h. Following this incubation period, the medium was aspirated and the cells were washed twice with 1 ml of αMEM without serum. Then, 0.5 ml of previously prepared solutions containing PTH or PTHRP synthetic fragments, forskolin, or PMA in αMEM supplemented with 1 mM isobutylmethylxanthine (to prevent breakdown of cAMP by phosphodiesterases) were added to each well, and cells were incubated for a period of 15 min. The medium of each well was then aspirated in the same order of application and 0.5 ml of ice-cold 10% trichloroacetic acid was added to each well to stop the reaction and extract the [^{3}H]cAMP. Twenty μl of a buffer consisting of 10 mM ATP, 2% SDS, 50 mM Tris-HCl (pH 7.5), 1 mM radioinert cAMP (added as carrier) and 3000 cpm of [^{14}C]cAMP (added as tracer to account for procedural losses) was then added to each well along with 0.5 ml of water. The solutions were then chromatographed on Dowex and alumina columns for the separation of [^{3}H]cAMP and [^{14}C]cAMP from ATP, ADP, and AMP. Fractions from the alumina columns containing the labeled cAMP were mixed with 14 ml of scintillation fluid and quantitated in a β-counter. Adenylate cyclase activity was expressed as [^{3}H]cAMP produced per 15 min per well.

Protein Kinase C Activity—PKC activity was assayed according to previously described procedures (32, 33). Briefly, cells were washed twice with serum-free media and stimulated with different concentrations of PTH or PTHRP synthetic fragments or PMA for a period of 2 min. At the end of the stimulation period, the cells were washed twice with ice-cold PBS and then gently scraped in a buffer (800 μl) containing 2 mM Tris-Cl (pH 7.5), 250 mM sucrose, 2 mM EDTA, 5 mM EGTA, 1 mM dithiothreitol, 50 mM phenylmethylsulfonyl fluoride, and 2.5 μg/ml of leupeptin. These cells were then sonicated twice on ice for a period of 10 s each using a Branson Sonifier (model 450) set at low intensity. This was followed by a 60-min ultracentrifugation at 100,000 $\times$ *g*. The supernatant containing the cytosolic fraction was removed. The pellet containing the membrane fraction was resuspended in the same buffer (800 μl) containing 10% Triton X-100 and sonicated twice on ice for 10 s at medium intensity. The sonicate was shaken at 4 °C for 1 h and then subjected to ultracentrifugation at 100,000 $\times$ *g* for 60 min. The supernatant containing the solubilized membrane fraction was then collected. The cytosol and solubilized membrane fractions were then each applied to a DEAE-cellulose column that was washed with buffer containing 2 mM Tris-Cl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 50 mM phenylmethylsulfonyl fluoride, and 2.5 μg/ml leupeptin. The PKC enzyme was eluted from the column with buffers containing 130 and 150 mM NaCl. The PKC activity of the eluate was assayed by incorporation of [γ-^{32}P]ATP into a 7 amino acid synthetic peptide (FKKSFKL-NH_2). The assay mixture contained 10 mM Tris-Cl (pH 7.4), 5 mM magnesium acetate, 0.5 μg/ml of leupeptin, 100 μM ATP, 80 μCi/ml of [γ-^{32}P]ATP, 0.7 mM $CaCl_2$, 1 μg/ml of phosphatidylserine, 10 μM diolein, and 9 mM of the 7-amino acid peptide. The incubation was started by the addition of 50 μl of either the cytosolic or solubilized membrane fraction to 200 μl of the above mixture at 30 °C and ended after a period of 5 min by the addition of 125 μl of concentrated acetic acid. A 100-μl aliquot of this mixture was transferred to a 3MM Whatman filter paper washed three times with 25 ml of 75 mM phosphoric acid for a period of 15 min, dried by methanol, transferred into scintillation vials, and counted in 10 ml of scintillation fluid using a β-counter. All counts were then corrected for the amount of protein present in 50 μl of the cytosolic or the solubilized membrane fraction. Results were calculated as the amount of PKC present in the membrane relative to the amount of PKC present in the cytosol, and control ratios were taken as basal activity and normalized to a value of one.

Northern Blot Analysis—Total RNA was extracted from cell cultures according to the method of Chomczynski and Sacchi (34). The RNA preparation was denatured in 37% formaldehyde and separated by

agarose gel (1.5%) electrophoresis, transferred onto nitrocellulose membrane filters, and baked at 80 °C for 2 h. The RNA blots were then hybridized with cDNAs encoding either rat NHE-1 (*Pst*I fragment, nucleotides +478 to +1850; number +1 begins at the ATG translation start codon), NHE-2 (*Bam*HI-*Sac*II fragment, nucleotides +269 to +1314), NHE-3 (*Pst*I fragment, nucleotides +1153 to +2434), or NHE-4 (*Kpn*I-*Dra*I fragment, nucleotides +1427 to +2535). Probes were labeled with [α-^{32}P]dCTP using an Amersham multiprime DNA labeling kit. The filters were prehybridized, hybridized, and washed as described previously (2). The filters were then exposed to autoradiographic film with an intensifying screen for 8 days at −80 °C.

$^{22}Na^+$ Influx Measurements—Amiloride-inhibitable $^{22}Na^+$ Influx was used as a measure of Na^+/H^+ exchanger activity as described previously (7). Briefly, OK cells were grown to confluence in 24-well plates. Cells were incubated in serum-free media overnight. The cells were then washed twice with a Na^+-saline solution containing 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 20 mM HEPES-Tris (pH 7.4) followed by a 15-min period of preincubation at 37 °C in the same Na^+-saline solution supplemented with appropriate concentrations of PTH or PTHRP fragments, forskolin, 1,9-dideoxyforskolin, PMA, or 4α-PMA. The cells were then washed twice with a solution containing 130 mM choline chloride, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM glucose, and 20 mM HEPES-Tris (pH 7.4). $^{22}Na^+$ influx assays were initiated by incubating the cells with the respective stimulatory agents prepared in choline chloride solution containing 1 mM ouabain and 1 μCi of ^{22}NaCl (carrier free)/ml, and in the absence or presence of 1 mM amiloride for the indicated periods of time at room temperature. The nominal absence of K^+ in the influx buffer and the presence of ouabain was used to prevent the transport of $^{22}Na^+$ catalyzed by the Na-K-2Cl cotransporter and Na,K-ATPase, respectively. The incubation was terminated by adding 1 ml of ice-cold NaCl stop solution containing 130 mM NaCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 20 mM HEPES-Tris (pH 7.4). The solution was quickly aspirated and then rapidly repeated an additional three times.

To extract the radiolabel, the cells were solubilized in 250 μl of 0.5 N NaOH and the wells washed with 250 μl of 0.5 N HCl. Both the solubilized cell extracts and the wash solutions were added to scintillation vials, and the radioactivity was quantitated in a β-counter. Amiloride-sensitive Na^+/H^+ exchanger activity was defined as the difference between the rates of $^{22}Na^+$ influx in the absence and presence of 1 mM amiloride.

RESULTS

Influence of PTH and PTHRP Fragments on Signal Transduction in OK Cells—Earlier studies (35–37) have shown that PTH and PTHRP require the first two N-terminal amino acids and amino acids 25–34 to stimulate adenylate cyclase activity. In contrast, amino acids 3–34 and even smaller regions (amino acids 28–34) appear sufficient to activate translocation of PKC to the plasma membrane (32, 38, 39). Therefore, to investigate the linkage of the PTH receptor to these two signaling pathways in the OK cell line, experiments were conducted using a series of synthetic fragments of human PTH and PTHRP that should allow one to distinguish between activation of the adenylate cyclase-cAMP-PKA and/or phospholipase C-DAG-PKC pathways. Prior studies had demonstrated that PTH-(1–34) and PTHRP-(1–34) were equipotent ($K_{0.5} \sim 10^{-9}$ M) in stimulating adenylate cyclase activity in OK cells (37). Therefore, to obtain maximal stimulation, a single hormone concentration of 100 nM was selected to evaluate the effects of the different PTH and PTHRP analogues. As illustrated in Fig. 1, 100 nM PTH-(1–34) and PTHRP-(1–34) stimulated adenylate cyclase activity 6–7-fold in OK cells, whereas no increase in enzyme activity was detected with fragments PTH-(3–34), PTH-(28–42), PTH-(28–48), and PTHRP-(1–16), all lacking either the first 2 amino acids or 25–34. The effect of these same fragments on PKC activity was then determined (Fig. 2). In contrast to the effect on the adenylate cyclase system, hormone-stimulated translocation of PKC activity from cytosol to membrane was observed with all the fragments in a concentration-dependent manner ($K_{0.5} \sim 10^{-11}$ M) except PTHRP-(1–16) which was inert in the PKC assay as it was in the adenylate cyclase assay. Thus, unlike renal MCT cells (26), the PTH receptor in OK cells is

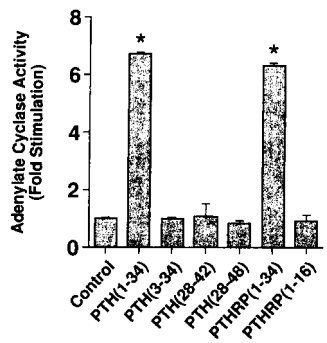

FIG. 1. **Effect of PTH and PTHRP peptide fragments on adenylate cyclase activity in OK cells.** OK cells were grown to confluence in 24-well plates and treated with various PTH and PTHRP fragments (each at 100 nM). Experiments were performed as described under "Experimental Procedures," and results are the mean ± S.E. of up to six determinations from three separate experiments. Values are reported as the -fold stimulation of adenylate cyclase activity. Control adenylate cyclase activity was 190 ± 20 cpm [^{3}H]cAMP/15 min. Significance from control values was determined by Student's *t* test and is indicated by an *asterisk* ($p < 0.002$).

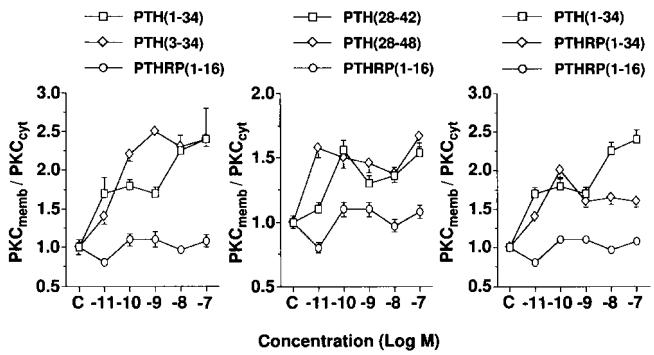

FIG. 2. **Concentration dependence of PTH and PTHRP peptide fragments on PKC activity of OK cells.** Confluent cultures of OK cells were treated with increasing concentrations of PTH and PTHRP peptide fragments and then assayed for PKC activity which was defined as the translocation of PKC from cytosol to membranes. Experiments were performed as described under "Experimental Procedures." Results are the mean ± S.E. of up to nine determinations from three separate experiments and are presented as an index of the ratio of PKC activity present in the membrane per PKC activity present in the cytosol (PKC_{memb}/PKC_{cyt}). The ratio of PKC_{memb}/PKC_{cyt} in the absence of PTH or PTHRP (0.057 ± 0.004) served as the control (*C*) and was normalized to a value of 1 for comparative purposes. Statistical analyses (analysis of variance) were performed on the data and all peptide fragments, with the exception of PTHRP-(1–16), were found to significantly stimulate PKC activity ($p < 0.02$).

directly linked to both the PKA and PKC pathways. Interestingly, PTH-(1–34) and PTHRP-(1–34) were more effective in activating PKC at picomolar concentrations compared with their stimulation of adenylate cyclase (37). This most likely reflects the differential sensitivities of the two signaling pathways to fractional occupancy of a single receptor.

Identification of the Na^+/H^+ Exchanger Isoform in OK Cells—Recent pharmacological and immunological data (40) support the notion that the apical Na^+/H^+ exchanger in OK cells is NHE-3 or NHE-3-like. However, the expression of other isoforms has not been thoroughly examined. To verify the nature of the Na^+/H^+ exchanger isoform(s) present in OK cells, Northern blot analyses were performed on mRNA extracted from OK cells and from fibroblastic cells independently transfected with isoform-specific cDNA fragments encoding rat NHE-1, -2, -3, or -4 under low hybridization stringency conditions to enhance cross-species detection. As shown in Fig. 3, although cDNAs encoding each rat NHE isoform hybridized with mRNA from the corresponding fibroblastic cell trans-

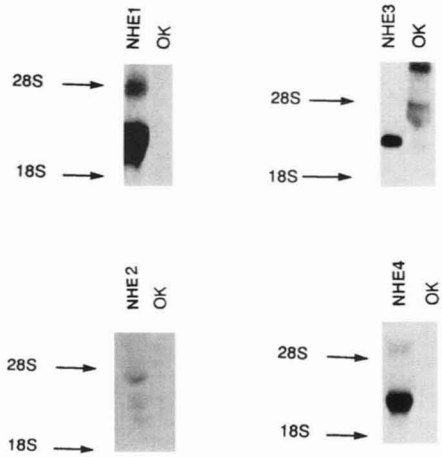

FIG. 3. **Determination of Na^+/H^+ exchanger isoform expression in OK cells.** Total cellular RNA was extracted from the following cell lines: oppossum renal OK cells; Chinese hamster ovary AP-1 cell transformants that stably express either rat NHE-1, NHE-2, or NHE-3; and Chinese hamster lung fibroblasts that stably express rat NHE-4. Total cellular RNA (20 μg) was analyzed by Northern blot hybridization (see "Experimental Procedures" for further details). ^{32}P-Labeled isoform-specific cDNA fragments were used as probes. The positions of the 28 and 18 S rRNAs were used as markers and are shown on the *left* of the figure panels.

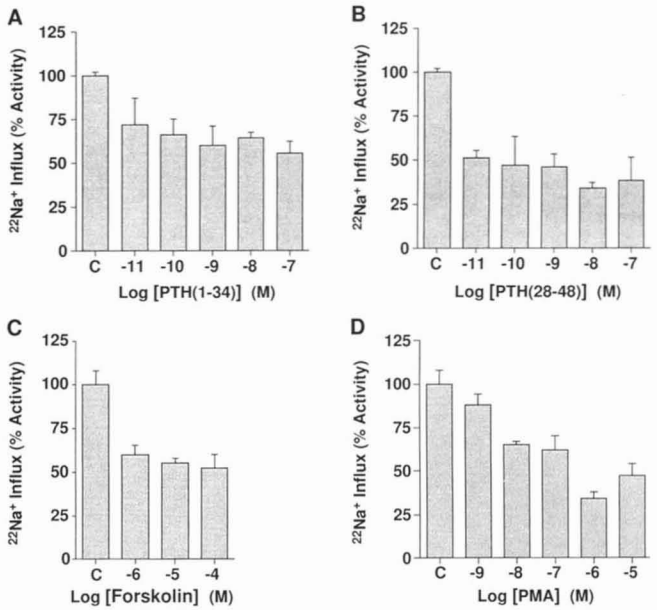

FIG. 4. **Concentration response profiles for PTH-(1–34), PTH-(28–48), forskolin, and PMA on Na^+/H^+ exchanger activity in OK cells.** OK cells were grown to confluence in 24-well plates. Prior to $^{22}Na^+$ influx measurements, the cells were preincubated with increasing concentrations of PTH-(1–34) (*A*), PTH-(28–48) (*B*), forskolin (*C*), and PMA (*D*) for 15 min in isotonic NaCl solution. The cells were rapidly washed with Na^+-free isotonic choline chloride solution and then incubated in assay medium containing 1 μCi of $^{22}NaCl$ (carrier free)/ml and the various agents for a 12-min period. Control (*C*) cells were treated with diluent alone. Na^+/H^+ exchanger activity was determined as amiloride-inhibitable ^{22}Na influx as described under "Experimental Procedures." Each value is the mean ± S.E. of up to six determinations from three experiments.

formants, only the cDNA encoding NHE-3 hybridized with mRNA species from OK cells. One major mRNA band was detected at ~9.0 kilobases (the size was estimated using a commercially prepared RNA ladder) and a minor diffuse band (~5 kilobases) at the 28 S rRNA marker. At present, it is unclear if the bands represent alternative-splicing of a single NHE-3 gene transcript or represent two distinct NHE-3-like gene products. Alternatively, the smaller band may represent nonspecific hybridization of the NHE-3 cDNA probe to the highly abundant 28 S rRNA, which occasionally occurs with some cDNA probes under low stringency conditions. Washing the Northern blots at moderate to high stringency resulted in the loss of all hybridization signals; probably as a consequence of species nucleotide differences between the rat cDNA probe and the opossum mRNAs. However, based on the similar biochemical and pharmacological properties of the rat NHE-3 isoform expressed in AP-1 cells (7) and those described for the apical Na^+/H^+ exchanger in OK cells (40), it is likely that one or both of the mRNA species detected in OK cells corresponds to NHE-3. Molecular cloning of the OK Na^+/H^+ exchanger will be required to make a definitive conclusion.

Influence of PTH and PTHRP Fragments on NHE-3 Activity—The data presented above demonstrated that synthetic peptide fragments of PTH and PTHRP were capable of activating either adenylate cyclase and PKC (*i.e.* PTH-(1–34) and PTHRP-(1–34)) or only PKC (*i.e.* PTH-(3–34), PTH-(28–42), and PTH-(28–48)). Therefore, these fragments provide a useful means of determining whether stimulation of one or both pathways are associated with PTH or PTHRP regulation of NHE-3. To this end, OK cells were pretreated for 15 min in Na^+-saline medium containing increasing concentrations of the individual peptide hormone fragments and then, in their continuing presence, assayed for NHE-3 activity that was defined as initial rates of amiloride-inhibitable $^{22}Na^+$ influx. PTH-(1–34) inhibited $^{22}Na^+$ influx into OK cells in a concentration-dependent manner, approaching maximal inhibition (40%) at ~10^{-10} M (Fig. 4*A*). Likewise, PTH-(28–48), which activated only PKC, reduced NHE-3 activity with similar efficacy (*i.e.* ~50–60%) (Fig. 4*B*). It is noteworthy that the concentration inhibition profile of NHE-3 activity by both analogues mirrored their concentration-dependent activation of PKC activity. Similarly, PTHRP-(1–34), PTH-(3–34), and PTH-(28–42), each at a single concentration of 100 nM, significantly inhibited NHE-3 activity to levels of 52 ± 3%, 52 ± 1%, and 51 ± 6%, respectively, relative to control (100 ± 7%) ($p < 0.02$). As expected, the biologically inert analogue PTHRP-(1–16) had no significant effect (93 ± 6%) on NHE-3 activity.

The above data supported the notion that PTH-(1–34) and PTHRP-(1–34) may be mediating their effects on NHE-3 activity via a PKC, and possibly PKA, pathway. In this regard, earlier studies (25) have demonstrated that pharmacological activation of these two protein kinases can inhibit the apical Na^+/H^+ exchanger in OK cells, as assessed by measuring changes in pH_i using a pH-sensitive fluorescent dye. To corroborate these observations using radioisotope techniques, the effects of forskolin, a direct agonist of adenylate cyclase, and PMA, a potent activator of PKC, were examined. Treatment of OK cells with increasing concentrations of either forskolin (Fig. 4*C*) or PMA (Fig. 4*D*) maximally suppressed NHE-3-mediated $^{22}Na^+$ influx by ~50–65%. In contrast, the corresponding biologically inert analogues, 1,9-dideoxyforskolin (10 μM) and 4α-PMA (1 μM) lacked any inhibitory effect and supported the notion that the effects of these compounds were biologically relevant (data not shown). To assess whether pharmacological activation of PKA and PKC could act in a synergistic manner, the combined effects of 10 μM forskolin and 1 μM PMA were investigated. Treatment of OK cells with both compounds gave similar results (64 ± 4% inhibition) to those obtained separately. Thus, these results are in accordance with previous data (25), suggesting that activation of either of these two regulatory cascades, at least at this signaling juncture, can ultimately lead to inhibition of NHE-3.

The conclusions reached using cells at resting pH_i were also

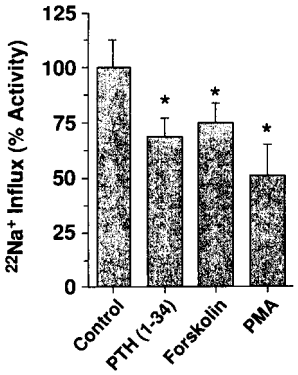

FIG. 5. **Influence of PTH-(1–34), forskolin, and PMA on Na^+/H^+ exchanger activity in OK cells under acidic pH_i conditions.** Confluent OK cells were incubated with 25 mM NH_4Cl in isotonic saline buffer for 30 min to acidify the cells to ~pH_i 6.6 (pH_i was assessed by microfluorometry using the dye BCECF as described previously (8)). During the last 15 min of the NH_4^+ prepulse, cells were treated with PTH-(1–34) (100 nM), forskolin (10 μM), and PMA (1 μM). The cells were then rapidly washed with Na^+-free isotonic choline chloride solution and then incubated in assay medium containing 1 μCi of $^{22}NaCl$ (carrier free)/ml and the different agents for a 5-min period. Control cells were treated with diluent alone. Na^+/H^+ exchanger activity was determined as amiloride-inhibitable ^{22}Na influx. Each value is the mean ± S.E. of four determinations from two experiments. Significance from control measurements was calculated using the Student's t test and is indicated by an *asterisk* ($p < 0.05$).

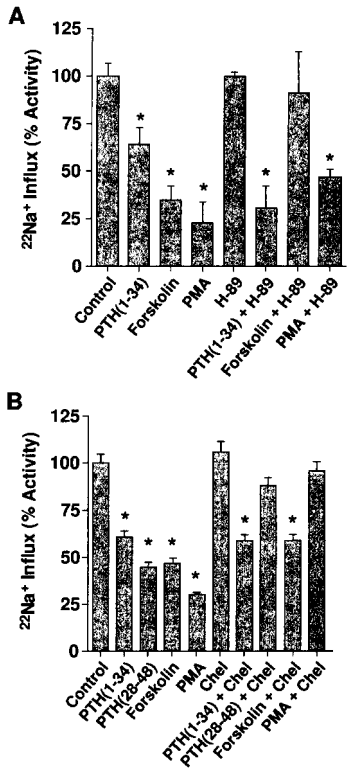

FIG. 6. **Effect of PKA and PKC inhibitors on PTH analogue-, forskolin-, and PMA-mediated inhibition of Na^+/H^+ exchanger activity in OK cells.** Confluent OK cells were preincubated in the absence or presence of 100 μM H-89, a selective inhibitor of PKA (*A*) or 1 μM chelerythrine chloride (*Chel*), a selective inhibitor of PKC (*B*), for 1 h prior to treatment with PTH analogues (100 nM), forskolin (10 μM), and PMA (1 μM). Results are shown as the difference between initial rates of $^{22}Na^+$ influx in the absence and presence of 1 mM amiloride and are expressed as a percentage of the control value. Each value is the mean ± S.E. of six to eight determinations from two experiments. Significance from control measurements in the absence of any agent other than diluent was calculated using the Student's t test and is indicated by an *asterisk* ($p < 0.02$).

confirmed and extended by complementary studies measuring exchanger activity in cells that were acid-loaded (~pH_i 6.6) by an NH_4^+ prepulse (Fig. 5). NHE-3 activity was significantly reduced by ~25–50% ($p < 0.05$) in cells pretreated with PTH-(1–34), forskolin, and PMA. Although the percentage inhibition was lower compared with resting pH_i conditions, this represented only an apparent decrease in the percentage since the absolute rates of amiloride-inhibitable ^{22}Na influx were greatly increased in both control and treated cells under acid-loaded conditions. These data also exclude the remote possibility that the inhibitory effects mediated by these agents were due to a generalized reduction in cellular metabolic activity and H^+ concentration.

To confirm the signaling circuitry involving PTH → PKA/PKC → NHE-3 in greater detail, highly selective antagonists of PKA (*i.e.* H-89) (41) and PKC (*i.e.* chelerythrine chloride) (42) were used. OK cells were preincubated for 1 h with either 100 μM H-89 or 1 μM chelerythrine chloride followed by coincubation in the absence or presence of PTH analogues (100 nM), forskolin (10 μM), and PMA (1 μM). As illustrated in Fig. 6*A*, H-89 abolished the effects of forskolin but had no influence on PTH-(1–34) or PMA. This indicated that PTH-(1–34) and PMA could inhibit NHE-3 activity independently of PKA. This also confirmed that the effects of both compounds were not mediated by subsequent PKC activation of adenylate cyclase type II; such a signaling route has been observed in some cell types (27, 28). In comparison, chelerythrine chloride (Fig. 6*B*) completely abrogated the negative regulation mediated by PTH-(28–48) and PMA; agents that act exclusively through PKC. However, it was ineffective in preventing the inhibition of NHE-3 by PTH-(1–34), as was H-89, and was unable to negate the actions of forskolin. The inability of chelerythrine chloride to prevent the inhibitory effects of forskolin verified that activation of the PKA pathway does not lead to enhanced PKC activity which could subsequently inhibit NHE-3. Furthermore, these data suggested that PTH-(1–34) was capable of acting independently through either PKA or PKC or possibly a novel third pathway involving $G\alpha_{13}$ (30). To test this postulation, the combined influence of H-89 and chelerythrine chloride was assessed. However, for reasons which remain unclear, the combination of these two protein kinase antagonists caused the cells to detach from the culture wells during the multiple washing steps of the $^{22}Na^+$ influx assay which did not occur when the compounds were used separately. As an alternative protocol to circumvent this problem, OK cells were incubated overnight (~20 h) in the presence of PMA (400 nM) to down-regulate PKC activity (43) followed by a 1-h pretreatment with H-89. Under these conditions, the cells remained adherent throughout the assay procedures. Using this approach, inhibition of both PKA and PKC prevented the suppressive effects of PTH-(1–34) on NHE-3; its activity being 92 ± 7% ($p > 0.01$) of that obtained for control cells (100 ± 5%). This provided direct evidence that the inhibitory actions of PTH-(1–34) on NHE-3 involved both PKA and PKC. Furthermore, this indicated that the PTH/PTHRP receptor did not transduce its signal to NHE-3 by a potential third pathway that was independent of adenylate cyclase and phospholipase C.

DISCUSSION

PTH inhibits apical Na^+/H^+ exchanger activity in renal proximal tubule OK cells (19, 20). The results from this study provide direct evidence that activation of the PTH/PTHRP receptor in OK cells stimulates both PKA and PKC, each of which can independently lead to inhibition of the Na^+/H^+ exchanger NHE-3 isoform. Kinetically, this alteration has been demonstrated to occur by a reduction in the V_{max} and affinity for

protons of the Na^+/H^+ exchanger (44).

Previous studies had implicated the involvement of PKA and/or PKC pathways in the PTH-mediated inhibition of Na^+/H^+ exchanger activity in both renal OK (20, 25) and MCT (26) cells by the use of pharmacological agents. Our own findings in OK cells confirmed that both forskolin, an activator of the adenylate cyclase → PKA pathway, and PMA, a direct PKC agonist, were able to similarly inhibit NHE-3 activity. These respective effects were only prevented with the corresponding specific inhibitors of PKA (H-89) and PKC (chelerythrine chloride), with no evidence for cross-talk between the two pathways. This provided direct evidence for the involvement of PKA and PKC and is consistent with their capacity to independently inhibit NHE-3 activity. Similar conclusions were drawn using a series of synthetic peptide fragments of PTH and PTHRP that differentially activated the adenylate cyclase-cAMP-PKA and phospholipase C-DAG-PKC pathways or only the latter signaling route. Most notably, PTH analogues such as PTH-(1–34) triggered both signaling pathways which, at least theoretically, could independently influence NHE-3 activity. Antagonizing the actions of either PKA with H-89 or PKC with chelerythrine chloride failed to inhibit the suppressive effect of PTH-(1–34) on NHE-3 activity. This suggested a role for both protein kinases in the action of this fragment and supported the thesis that activation of either pathway is sufficient for similar effects on NHE-3 activity. This was confirmed when simultaneous inhibition of PKA and PKC prevented the actions of PTH-(1–34). Consequently, each pathway appears independently capable of altering exchanger activity, a conclusion consistent with previous findings in OK cells (25).

Notwithstanding the above conclusion, it is noted that there are significant concentration-dependent differences between PTH-(1–34) and PTHRP-(1–34) activation of adenylate cyclase ($K_{0.5} \sim 10^{-9}$ M) (37) and that reported herein for PKC ($K_{0.5} \sim 10^{-11}$ M). Since plasma circulating concentrations of PTH are in the picomolar range, it is likely that PTH-mediated inhibition of renal proximal tubule NHE-3 activity is transduced primarily by the PKC pathway. However, the PKA pathway may play a more significant role in modulating renal NHE-3 activity in certain tumorigenic malignancies associated with elevated paracrine secretions of PTHRP that cause humoral hypercalcemia and hypophosphatemia (23). However, the relative importance of one or the other pathway to PTH/PTHRP regulation of renal apical NHE-3 activity is unclear.

In addition to the regulation of renal NHE-3 cited above, other more recent studies have provided supporting evidence that NHE-3 can be regulated by serine/threonine protein kinases in heterologous cell expression systems. Rabbit NHE-3 stably expressed in fibroblastic cells (PS120) is inhibited following acute cell stimulation by phorbol esters, although elevating intracellular cAMP had no effect (45). In contrast, preliminary data in our laboratory have shown that rat NHE-3 stably expressed in Chinese hamster ovary AP-1 cells is inhibited by both phorbol esters and agonists that increase cAMP accumulation.[2] Thus, regulation of NHE-3 exhibits species and/or cell-specific differences.

By Northern analysis, the only NHE isoform expressed in cultured OK cells was NHE-3. Interestingly, the size of the major NHE-3 transcript (~9 kilobases) was considerably larger than that observed in other mammalian species which ranged from 5.4 to 5.6 kilobases (2, 46). The reason for this discrepancy is unknown but may reflect species variations in the size of the 5′ and/or 3′ mRNA untranslated regions. Although we did not detect other NHE isoforms, we cannot exclude the possibility that small quantities were present but below the detection threshold of this technique, particularly since our cDNA probes were from a different species. Nonetheless, the NHE-3 isoform clearly appears to be the most abundant isoform of this gene family in OK cells. Moreover, NHE-3 immunoreactivity has previously been localized along the microvillar membrane of the brush border of rabbit proximal tubule epithelia (15) and OK cells (40). Inasmuch as OK cells are believed to express a proximal tubule cell phenotype, our finding of NHE-3 expression in these cells is consistent with those previous observations.

In common with the other isoforms of the Na^+/H^+ exchanger, NHE-3 is believed to contain 10–12 putative transmembrane segments followed by a hydrophilic cytoplasmic domain. The membrane-spanning region of the exchanger is required for transport, whereas the cytoplasmic domain appears to function in a regulatory capacity (47). Our studies suggest that PKA and PKC may both exert important regulatory activities at either the same phosphorylation site or at discrete phosphorylation sites which, nevertheless, similarly influence exchanger activity. In this regard, the cytoplasmic domain contains consensus sequences for PKA (R-R/K-*X*-S*/T*) as well as for PKC ((R/K)$_{1-3}$, X_{2-0})-S*/T*-(X_{2-0}, R/K$_{1-3}$)), although there is overlap in consensus sequence determinants among protein kinases (48). However, the molecular signaling events that occur between protein kinase activation and NHE-3 inhibition are unclear. At present, it is unknown whether these protein kinases mediate their effects by direct phosphorylation of NHE-3 or indirectly via phosphorylation-dependent ancillary proteins. With regard to the latter, there is some *in vitro* evidence that PKA-mediated inhibition of the rabbit renal apical Na^+/H^+ exchanger requires the involvement of a regulatory protein that is separate from the kinase and transporter (49, 50). Cell-specific expression of these factors could account for the differential responsiveness of NHE-3 to individual protein kinases. Further studies are required to identify the precise molecular mechanisms involved.

[2] R. Kandasamy, R. Harris, F. H. Yu, A. Boucher, J. W. Hanrahan, and J. Orlowski, manuscript in preparation.

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