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Parathyroid Hormone and Parathyroid Hormone-related Peptide Activate the Na^+/H^+ Exchanger NHE-1 Isoform in Osteoblastic Cells (UMR-106) via a cAMP-dependent Pathway*

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Arezou Azarani‡§¶, John Orlowski‡‖, and David Goltzman‡§**

*From the Departments of ‡Physiology and **Medicine, McGill University, and the §Calcium Research Laboratory, Royal Victoria Hospital, Montreal, Quebec H3A 1A1, Canada*

Parathyroid hormone (PTH) and parathyroid hormone-related peptide (PTHRP) regulate Na^+/H^+ exchanger activity in osteoblastic cells, although the signaling components involved are not precisely defined. Since these peptide hormones can stimulate production of diverse second messengers (*i.e.* cAMP and diacylglycerol) that activate protein kinase A (PKA) and protein kinase C (PKC) in target cells, it is conceivable that either one or both of these pathways can participate in modulating exchanger activity. To discriminate among these possibilities, a series of synthetic PTH and PTHRP fragments were used that stimulate adenylate cyclase and/or PKC. In the osteoblastic cell line UMR-106, human PTH(1–34) and PTHRP(1–34) augmented adenylate cyclase activity, whereas PTH(3–34), PTH(28–42), and PTH(28–48) had no effect. Nevertheless, all these peptide fragments were found to enhance PKC translocation from the cytosol to the membrane in a dose-dependent (10^{-11} to 10^{-7} M) manner. PTHRP(1–16), a biologically inert fragment, was incapable of influencing either the PKA or PKC pathway. PTH(1–34) and PTHRP(1–34), but not PTH(3–34), PTH(28–42), PTH(28–48), or PTHRP(1–16), elevated Na^+/H^+ exchanger activity, implicating cAMP as the transducing signal. In accordance with this observation, forskolin (10 μM), which directly stimulates adenylate cyclase, also activated Na^+/H^+ exchanger activity. The involvement of PKA was verified when the highly specific PKA inhibitor, H-89, completely abolished the stimulatory effect of PTH(1–34) and forskolin on Na^+/H^+ exchange. In addition, Northern blot analysis revealed the presence of only the NHE-1 isoform of the Na^+/H^+ exchanger in UMR-106 cells. In summary, these results indicated that PTH and PTHRP activate the Na^+/H^+ exchanger NHE-1 isoform in osteoblastic UMR-106 cells exclusively via a cAMP-dependent pathway.

Parathyroid hormone (PTH)[1] and PTH-related peptide (PTHRP) are important regulators of normal and pathological bone remodeling (1). In UMR-106 cells, a well characterized osteoblastic cell line (2, 3), both PTH and PTHRP can interact with a common G protein-coupled receptor that is functionally associated with both the adenylate cyclase-cAMP-protein kinase A (PKA) and phospholipase C-diacylglycerol-protein kinase C (PKC) pathways (4, 5). Thus, the ability of this receptor to couple to multiple effector systems is believed to account for the pleiotropic actions of these peptides in bone as well as other tissues. Nonetheless, the precise linkage of the PTH/PTHRP-receptor to these distinct signaling pathways and various downstream molecular targets remains poorly defined.

A recently identified target of PTH/PTHRP action in osteoblasts is the plasma membrane Na^+/H^+ exchanger, which plays a central role in modulating intracellular pH (pH_i) (6–8). In osteoblastic cells, regulation of pH_i is a critical component of hormone-stimulated bone remodeling where local fluctuations occur in the osseous microenvironment (9). Numerous studies have shown that Na^+/H^+ exchanger activity is acutely regulated by a wide variety of stimuli that activate diverse signal transduction systems (*i.e.* PKA, PKC, Ca^{2+}/calmodulin-dependent protein kinase II, tyrosine kinases), although in many cases the precise mechanisms have yet to be fully elucidated (reviewed in Ref. 10). This extensive functional and regulatory diversity appears to be generated by tissue-specific expression of multiple isoforms of the Na/H exchanger as well as by variations in the signaling repertoire of individual cell types (reviewed in Ref. 11). To date, four members (NHE-1 to NHE-4) of this gene family have been identified and characterized by cDNA cloning (11–14) and functional expression studies (15–18). Overall, they share ~40–60% amino acid identity and contain one or more potential sites for phosphorylation by different serine/threonine protein kinases in their putative cytoplasmic carboxyl-terminal region. More recently, a putative fifth (19) and possibly sixth (20) isoform have been located by chromosomal mapping in humans.

In osteoblastic cell lines, there are conflicting reports regarding the regulation of the Na^+/H^+ exchanger and pH_i by PTH/PTHRP and second messengers. Elevation of intracellular cAMP levels by PTH, PTHRP, forskolin, or cAMP analogues has been reported to inhibit (6, 7, 21) or stimulate (22) Na^+/H^+ exchanger activity in UMR-106 cells. Aside from methodological considerations, the reason for this difference is unclear. In addition, phorbol 12-myristate 13-acetate (PMA treatment) also leads to stimulation of Na^+/H^+ exchanger activity, presumably through activation of PKC (22). Thus, at least two distinct signaling pathways appear to regulate this ion transporter in bone cells. In contrast to UMR-106 cells, PTH as well as epinephrine, a β-adrenergic agonist that also activates a G protein-linked receptor coupled to adenylate cyclase, were found to activate the Na^+/H^+ exchanger independent of

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‖ Recipient of a Chercheur-Boursier (Scholarship) from the Fond de la Recherche en Santé du Québec. To whom correspondence should be addressed: Dept. of Physiology, McGill University, McIntyre Medical Sciences Bldg., 3655 Drummond St., Montréal, Québec H3G 1Y6, Canada. Tel.: 514-398-8335; Fax: 514-398-7452.

[1] The abbreviations used are: PTH, parathyroid hormone; PTHRP, parathyroid hormone-related peptide; PKA, protein kinase A; PKC dithiothreitol; αMEM, α minimum essential medium.

changes in cAMP accumulation in rat osteosarcoma ROS 17/2.8 cells (23). Further studies revealed that β-adrenergic receptor-mediated activation of the Na^+/H^+ exchanger involved a G protein ($G\alpha_{13}$) that was not linked to either adenylate cyclase or phospholipase C (24, 25), thereby implicating the involvement of a novel third pathway. Whether this also applies to the PTH receptor is unknown. Nonetheless, it is conceivable that the PTH/PTHRP signal to modulate Na^+/H^+ exchanger activity in osteoblasts is transduced by more than two signaling pathways. In addition, the regulatory diversity mentioned above may also be reflected by cellular expression of one or more isoforms of the Na^+/H^+ exchanger that are differentially responsive to distinct signaling pathways. The identity of specific Na^+/H^+ exchanger isoforms in osteoblastic cells has yet to be determined.

The purpose of this study was two-fold. First, using UMR-106 cells as a model system, we wished to examine in greater detail the reported linkage of PTH/PTHRP to the PKA and PKC signaling pathways, and possibly to another pathway involving $G\alpha_{13}$, in regulating Na^+/H^+ exchanger activity. 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 inhibitors of the PKA and PKC pathways. Second, we wished to identify which isoform(s) of the Na^+/H^+ exchanger is(are) present in these cells. Our results demonstrated that only the NHE-1 isoform is expressed in UMR-106 cells and that PTH and PTHRP selectively activate NHE-1 via a pathway involving PKA.

EXPERIMENTAL PROCEDURES

Materials—Phosphatidyl L-serine, diolein, dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), 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 Scientific and BDH.

Tissue Culture—Rat osteoblastic osteosarcoma cells (UMR-106) 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 (13, 15). 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) (18). 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 (3). 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) to each 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. The [^{3}H]cAMP was isolated by chromatography on Dowex and Alumina columns and quantitated in a β-counter. Adenylate cyclase activity was expressed as [^{3}H]cAMP produced/15 min/well.

Protein Kinase C Activity—PKC activity was assayed according to previously described procedures (26, 27). 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 DTT, 50 mM PMSF, and 2.5 μg/ml 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 × *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 × *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, which was washed with buffer containing 2 mM Tris-Cl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 50 mM PMSF, 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 seven-amino acid synthetic peptide (FKKSFKL-NH_2) and quantitated 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 1.

Northern Blotting—Total RNA was extracted from cell cultures according to the method of Chomczynski and Sacchi (28). 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 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 (12). 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 (15). Briefly, UMR-106 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 the various stimulatory agents. 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 agents prepared in choline chloride solution containing 1 mM ouabain and 1 μCi/ml $^{22}NaCl$ (carrier-free), and in the absence or presence of 1 mM amiloride for 12 min (unless indicated otherwise) at room temperature. The incubation was terminated by adding 1 ml of an 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.

In experiments where exchanger activity was to be determined from the rate of $^{22}Na^+$ influx at constant $H^+{}_i$ concentration, pH_i was clamped by incubating the cells in medium of varying K^+ concentration containing the K^+/H^+ exchange ionophore nigericin (29). Because at equilibrium $[K^+{}_i]/[K^+{}_o] = [H^+{}_i]/[H^+{}_o]$, the desired pH_i was calculated from the imposed $[K^+]$ gradient and the extracellular pH (pH_o = 7.4), assuming an intracellular $[K^+]$ of 140 mM. Briefly, the cell monolayer were washed twice with Na^+-saline solution and preincubated for 15 min in KCl solution. In this study, the pH_i was set at 6.6; the pH_i clamping solution contained 142 mM *N*-methyl-D-glucamine methanesulfonate, 14 mM KCl, 8 mM potassium glutamate, 2 mM NaCl, 1 mM $MgCl_2$, 10 μM nigericin, and 10 mM HEPES. Then, the solution was removed and measurements were made in KCl solution supplemented with 1 mM ouabain and 1 μCi/ml $^{22}NaCl$ in the absence or presence of 1 mM amiloride.

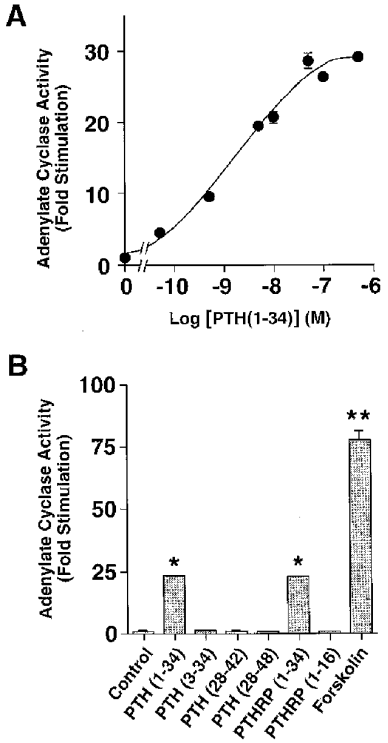

FIG. 1. **Effect of PTH and PTHRP peptide fragments on adenylate cyclase activity in UMR-106 cells.** UMR-106 cells were grown to confluence in 24-well plates. *A*, cells were treated with increasing concentrations of PTH(1–34) and then assayed for adenylate cyclase activity. *B*, UMR-106 cells were treated with various PTH and PTHRP fragments (each at 100 nM) and forskolin (10 μM). Experiments were performed as described under "Experimental Procedures," and results are the mean ± S.E. of up to 6 determinations from three separate experiments. Values are reported as the -fold stimulation of adenylate cyclase activity. Control adenylate cyclase activity was 256 ± 21 cpm [^{3}H]cAMP/15 min. Significance from control values was determined by Student's *t* test and is indicated by asterisks ($^*p < 0.01$; $^{**}p < 0.001$).

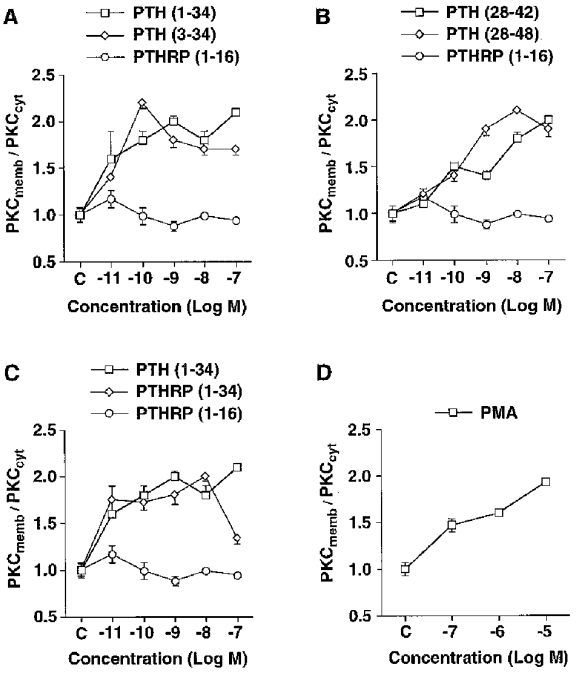

FIG. 2. **Concentration dependence of PTH and PTHRP peptide fragments and PMA on PKC activity of UMR-106 cells.** Experiments were performed as described under "Experimental Procedures." Results are the mean ± S.E. of up to nine determinations from three separate experiments and are shown as an index 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 any agent (0.170 ± 0.003) served as the control (*C*) and was normalized to a value of 1 for comparative purposes.

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 UMR-106 Cells—Earlier studies (30–33) have shown that PTH and PTHRP require the first two NH_2-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 PKC translocation to the plasma membrane (26, 34, 35). To verify the linkage of the PTH receptor to these signaling pathways in our UMR-106 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 PKA and PKC pathways. As an initial experiment, the influence of PTH(1–34) concentration on adenylate cyclase activity was assessed. As illustrated in Fig. 1*A*, PTH(1–34) increased adenylate cyclase activity 29-fold in a concentration-dependent manner, achieving half-maximal stimulation at $\sim 10^{-9}$ M. Based on this result, a single hormone concentration of 100 nM was used to evaluate the effect of the other synthetic PTH and PTHRP analogues. As shown in Fig. 1*B*, both PTH(1–34) and PTHRP(1–34) stimulated adenylate cyclase activity ~25-fold, while 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 amino acids 1–2 or 25–34. As expected, forskolin caused a large increase (75-fold) in adenylate cyclase activity. The effect of these same fragments on PKC activity was then determined (Fig. 2, *A–C*). In contrast to the effects on the adenylate cyclase system, hormone-stimulated PKC activity was observed with all the fragments in a concentration-dependent manner except PTHRP(1–16), which was inert in the PKC assay as it was in the adenylate cyclase assay. Interestingly, PTH(1–34) was more effective in activating PKC than adenylate cyclase, with half-maximal stimulation being achieved at $\sim 10^{-11}$ M. This most likely reflects the differential sensitivities of the two signaling pathways to fractional occupancy of a single receptor. The phorbol ester PMA also similarly enhanced PKC activity in a concentration-dependent manner (Fig. 2*D*). Thus, in these cells, the PTH receptor is linked to both the adenylate cyclase-cAMP-PKA and phospholipase C-diacylglycerol-PKC pathways.

Identification of the Na^+/H^+ Exchanger Isoform in UMR-106 Cells—To determine which isoform of the Na^+/H^+ exchanger is present in UMR-106 cells, Northern blot analyses were performed. The rat NHE-1 cDNA probe clearly hybridized with mRNA extracted from UMR-106 cells as well as with mRNA from control AP-1 cell transformants expressing rat NHE-1 (Fig. 3, *top left panel*). The UMR-106 transcript was of the expected size (~4.8 kilobases) (12), whereas the transfected NHE-1 transcript was ~3.6 kilobases, reflecting the size of the truncated cDNA used for stable expression. In contrast, while transcripts encoding rat NHE-2 and NHE-3 were detected in AP-1 cells transfected with the respective cDNAs, no hybridization signals were observed with UMR-106 mRNA (Fig. 3, *bottom left* and *top right panels*). Similarly, NHE-4 mRNA was not detected in UMR-106 cells, although it was readily detected

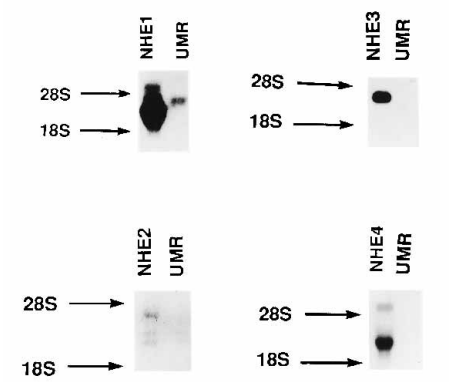

FIG. 3. **Determination of Na^+/H^+ exchanger isoform expression in UMR-106 cells.** Total cellular RNA was extracted from the following cell lines: rat UMR-106 cells; AP-1 cell transformants that stably expressed either rat NHE-1, NHE-2, or NHE-3; and PS120 cells that stably expressed rat NHE-4. Total 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.

in PS120 cells transfected with rat NHE-4 (Fig. 3, *bottom right panel*). Thus, UMR-106 cells appear to express only the NHE-1 isoform.

Influence of PTH and PTHRP Fragments on NHE-1 Activity—As demonstrated above, synthetic peptide fragments of PTH and PTHRP were capable of activating either adenylate cyclase and PKC or just PKC. Hence, these fragments provide a useful means of determining whether stimulation of one or both pathways are associated with PTH/PTHRP regulation of NHE-1. To this end, preliminary studies were conducted to define appropriate assay conditions for measuring Na^+/H^+ exchanger activity, defined as initial rates of amiloride-inhibitable $^{22}Na^+$ influx, as a function of uptake time and hormone concentration. Following pretreatment of UMR-106 cells with 100 nM PTH(1–34) for 15 min in Na^+-saline medium, amiloride-inhibitable $^{22}Na^+$ influx was found to increase linearly in the continual presence of the PTH analogue for a 12-min period (Fig. 4*A*). In view of this observation, subsequent measurements were performed using this time interval. PTH(1–34) also stimulated NHE-1 activity in a concentration-dependent manner, reaching levels of ~250–300% at 100 nM PTH(1–34); the maximum concentration tested (Fig. 4*B*). Thereafter, a concentration of 100 nM was adopted for the other peptide hormone fragments. As illustrated in Fig. 5, both PTH(1–34) and PTHRP(1–34) stimulated amiloride-inhibitable ^{22}Na influx to a similar extent (~250%). However, PTH(28–42), which activated only PKC, had no effect on NHE-1 activity. An identical lack of potency was also observed for PTH(3–34) and PTH(28–48) (data not shown) as well as the biologically inert fragment PTHRP(1–16) (Fig. 5).

The above data supported the notion that PTH(1–34) and PTHRP(1–34) may be selectively mediating their effects on NHE-1 activity via a cAMP-dependent pathway. This possibility was tested by increasing intracellular levels of cAMP with forskolin, which can directly activate adenylate cyclase. As shown in Fig. 5, 10 μM forskolin also increased NHE-1 activity to ~340% of control values, whereas a biologically inert forskolin analogue, 1,9-dideoxyforskolin (10 μM), had no effect. The absence of stimulation by 1,9-dideoxyforskolin indicated that forskolin-induced activation of NHE-1 activity was biologically relevant and not a nonspecific effect of the compound. To test this hypothesis further, the effect of a highly selective inhibitor of PKA, H-89 (36), was investigated. This cell permeant compound has been shown to effectively inhibit PKA while having

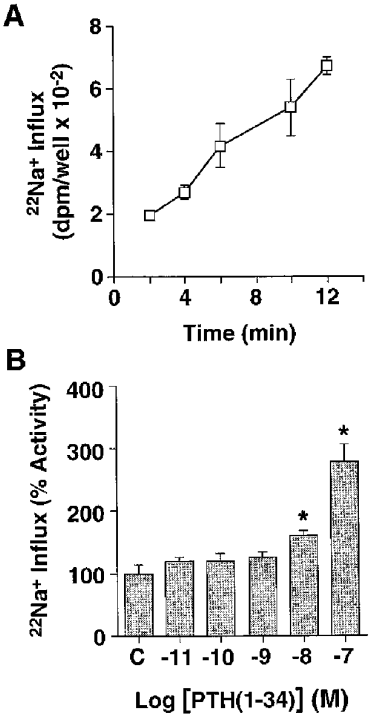

FIG. 4. **Effect of PTH(1–34) as a function of uptake time and concentration on Na^+/H^+ exchanger activity in UMR-106 cells.** UMR-106 cells were grown to confluence in 24-well plates. *A*, prior to $^{22}Na^+$ influx measurements, the cells were preincubated in PTH(1–34) (100 nM) for 15 min in isotonic NaCl solution. The cells were rapidly washed with Na^+-free isotonic choline chloride solution and then incubated in assay medium containing 1 μCi/ml ^{22}NaCl (carrier-free). $^{22}Na^+$ influx was measured at increasing time intervals in the continuing presence of PTH(1–34). Low levels of background $^{22}Na^+$ influx that were not inhibitable by 1 mM amiloride were subtracted from the total influx. Na^+/H^+ exchanger activity was expressed as initial rates of amiloride-inhibitable $^{22}Na^+$ influx. Each value is the mean ± S.E. of 4–6 determinations. *B*, prior to $^{22}Na^+$ influx measurements, the cells were preincubated with increasing concentrations of PTH(1–34) (10^{-11} to 10^{-7} M) for 15 min in isotonic NaCl solution. The cells were then assayed for Na^+/H^+ exchanger activity at the different concentrations of PTH(1–34) using a 12-min uptake period as described above. Each value is the mean ± S.E. of up to 6 determinations from three experiments. Significance from control (*C*) measurements in the absence of PTH(1–34) is indicated by an *asterisk* (*, $p < 0.02$).

no detectable effect on the activities of PKC, protein kinase G, and Ca^{2+}/calmodulin-dependent protein kinase II when applied to whole cells at high micromolar concentrations (36). Therefore, UMR-106 cells were preincubated with 100 μM H-89, followed by coincubation in the absence or presence of 10 μM forskolin and 100 nM PTH(1–34). As illustrated in Fig. 5, H-89 abrogated the effects of these agents and confirmed the involvement of PKA. Furthermore, the complete abolition of PTH(1–34) activation of NHE-1 by H-89 also indicated that the PTH receptor did not transduce its signal to the exchanger by a potential third pathway that was independent of adenylate cyclase and phospholipase C.

Forskolin also accelerated the NHE-1 activity to 129 ± 4.8% ($p < 0.05$) of control values (100 ± 6.5%) under acid-loaded, clamped pH_i conditions (pH_i = 6.6) using the ionophore K^+-nigericin. A similar effect was also observed for PTH(1–34) (127 ± 3.9%; $p < 0.05$). Although the percentage stimulation was lower compared to unclamped conditions, this represented only an apparent decrease in the percentage since the absolute rates of amiloride-inhibitable ^{22}Na influx are greatly increased in both control and forskolin-treated cells under acid-loaded conditions. Thus, these compounds were able to accelerate NHE-1 activity even when the H^+ substrate concentration was held

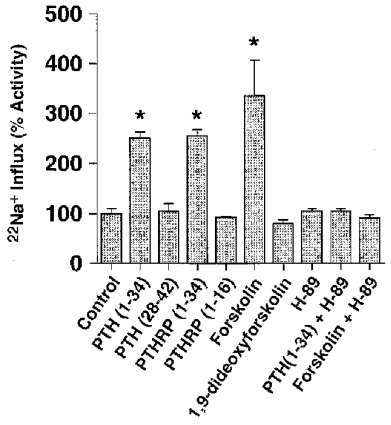

FIG. 5. **Influence of PTH and PTHRP fragments and forskolin on Na^+/H^+ exchanger activity in UMR-106 cells.** Prior to measurements of Na^+/H^+ exchanger activity, confluent UMR-106 cells were pretreated for 15 min with peptide fragments of PTH and PTHRP (each at 100 nM), forskolin (10 μM), or 1,9-dideoxyforskolin (10 μM), or pretreated for 1 h with the PKA antagonist H-89 (100 μM) to which PTH(1–34) or forskolin were added during the last 15 min. 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 percent change in amiloride-inhibitable $^{22}Na^+$ influx. Each value is the mean ± S.E. of 6 determinations from three 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* (*, $p < 0.02$).

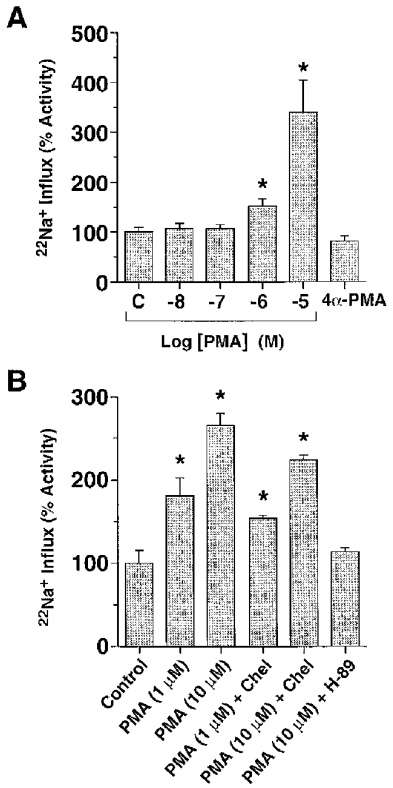

FIG. 6. **Effect of phorbol ester on Na^+/H^+ exchanger activity in UMR-106 cells.** *A*, prior to amiloride-inhibitable $^{22}Na^+$ influx measurements, UMR-106 cells were preincubated with increasing concentrations of PMA (10^{-8} to 10^{-5} M) or 4α-PMA (10 μM) for 15 min in isotonic NaCl solution. The cells were then assayed for Na^+/H^+ exchanger activity in their continuing presence. *B*, UMR-106 cells were preincubated for 1 h in the absence or presence of 1 μM chelerythrine chloride, a PKC antagonist, or 100 μM H-89, a PKA antagonist. PMA was added at the indicated concentrations during the last 15 min of the preincubation period, followed by measurements of amiloride-inhibitable $^{22}Na^+$ influx. Each value is the mean ± S.E. of 6 determinations from two to three experiments. Significance from control measurements in the absence of any agent other than diluent was calculated using Student's *t* test and is indicated by an *asterisk* (*, $p < 0.02$).

constant. Mechanistically, this suggested that the effect of both agents was not a consequence of increased metabolic production of acid.

Because other studies (8, 22) had indicated that Na^+/H^+ exchanger activity could be stimulated by PKC in osteoblastic cells, it was somewhat surprising that PTH fragments (*i.e.* PTH(3–34), PTH(28–42), and PTH(28–48)) capable of increasing PKC activity were without effect. Since the earlier studies had evaluated the influence of PKC on Na^+/H^+ exchanger activity by treatment of cells with phorbol esters, this class of agents was also examined. Consistent with the results obtained with PTH(3–34), PTH(28–42), and PTH(28–48), nanomolar concentrations (100 nM) of PMA that significantly activated PKC (see Fig. 2) had no effect on NHE-1 activity (Fig. 6*A*). In contrast, 1 and 10 μM PMA significantly increased NHE-1 activity. Interestingly, the increase in PKC activity induced with 1 μM PMA was not significantly different from that achieved with 0.1 μM PMA. The biologically inactive PMA analogue, 4α-PMA (10 μM), had no effect (Fig. 6*A*) and supported the view that PMA was acting in a specific and biologically relevant manner. Thus, only high concentrations of PMA (>0.1 μM) stimulated NHE-1 and suggested that a novel mechanism other than activation of PKC may be involved.

To investigate the validity of this supposition, the effect of chelerythrine chloride, a potent and selective inhibitor of PKC (37), was investigated. This cell permeant compound, used at a concentration of 1 μM, effectively inhibits the catalytic domain of PKC but does not affect the binding of diacylglycerol or phorbol esters to the regulatory domain (37). As shown in Fig. 6*B*, 1 μM chelerythrine chloride had only a marginal suppressive effect (~25–35%) on 1 and 10 μM PMA-mediated stimulation of NHE-1. In contrast, 100 μM H-89 completely prevented elevation of NHE-1 activity by 10 μM PMA. These data supported the possibility that PMA may be modulating NHE-1 activity by a pathway involving PKA rather than PKC. Indeed, PMA has been reported to modulate adenylate cyclase type II activity in some cell systems via a mechanism that is partially (~50%) independent of PKC (38, 39). To explore this possibility, UMR-106 cells were treated with increasing concentrations of PMA and found to elevate adenylate cyclase activity, but only by a modest 2.5-fold at 10 μM PMA (Fig. 7). This level of stimulation does not appear to be sufficient to activate the PKA $\rightarrow$ NHE-1 pathway, since a minimum 20-fold increase in $cAMP_i$ by 10 nM PTH(1–34) was required to significantly stimulate NHE-1 activity (see Figs. 1*A* and 4*B*). However, at present, one cannot exclude the possible existence of small compartmentalized accumulations of $cAMP_i$ induced by high concentrations of PMA that are sufficient to activate localized pools of specific PKA subtypes (discussed below).

DISCUSSION

This study has identified that the Na^+/H^+ exchanger isoform present in UMR-106 cells is NHE-1, which is the most broadly distributed isoform in mammalian tissues. Activation of the PTH/PTHRP receptor in these cells stimulates both the adenylate cyclase and phospholipase C signaling pathways and causes an increase in NHE-1 activity. However, only PTH analogues (*i.e.* PTH(1–34) and PTHRP(1–34)) capable of activating adenylate cyclase and PKA were able to stimulate NHE-1 activity, whereas those that retained the ability to enhance only PKC activity (*i.e.* PTH(3–34), PTH(28–42), and PTH(28–48)) were ineffective.

Prior studies examining the regulation of Na^+/H^+ exchanger activity in UMR-106 cells are conflicting, with some reports

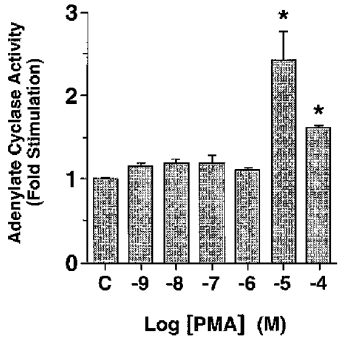

FIG. 7. **Effect of PMA concentration on adenylate cyclase activity of UMR-106 cells.** Experiments were performed as described under "Experimental Procedures." Results are the mean ± S.E. of 6 determinations from three separate experiments. Control (*C*) adenylate cyclase activity was 146 ± 11 cpm [^{3}H]cAMP/15 min and was normalized to a value of 100%. Significance from control measurements in the absence of PMA is indicated by an *asterisk* (*, $p < 0.05$).

showing that elevation of $cAMP_i$ levels by various agents was inhibitory (6, 7, 21) or stimulatory (22). Our results are in complete agreement with the latter study by Gupta and coworkers (22). These investigators, using the pH-sensitive fluorescent dye 2′,7′-bis-(2-carboxyethyl)-5,6-carboxyfluorescein, demonstrated that forskolin could stimulate the rate of ethylisopropylamiloride-inhibitable H^+ efflux, which is an alternative means of assessing Na^+/H^+ exchanger activity, in UMR-106 cells at resting pH_i and following an intracellular acid load. These microflurometric analyses of pH_i complemented our measurements of forskolin- and PTH(1–34)-induced increases in amiloride-inhibitable ^{22}Na influx under similar conditions. Since these compounds accelerated NHE-1 activity even under an imposed acid load, it is unlikely that their effects can be solely accounted for by a mechanism involving cAMP enhancement of metabolic H^+ production which can occur in some cell types (40). Moreover, the kinetic analyses of Gupta *et al.* (22) showed that forskolin increased both the magnitude and H^+ affinity of the Na^+/H^+ exchanger activity in UMR-106 cells. In contrast, other studies (6, 7, 21) have observed cAMP-mediated inhibition of Na^+/H^+ exchanger activity in UMR-106 cells. The reason for the discrepancy is unclear but may reflect different experimental conditions. Both Gupta *et al.* (22) and our own studies were performed using adherent cell cultures, whereas the other studies determined Na^+/H^+ exchanger activity in suspensions of UMR-106 cells after enzymatic digestion of the cultures. Cell attachment has been implicated in the regulation of pH_i through the action of integrins (41) and cytoskeletal components (42). In view of the fact that osteoblastic and particularly pre-osteoblastic cells *in vivo* (1) may communicate via cellular processes, the maintenance of the integrity of cell-cell communication may be important for accurately assessing hormone-regulated Na^+/H^+ exchanger activity.

Additional evidence supporting a role for cAMP in the regulation of NHE-1 is rather sparse. Human (40) and rabbit (17) NHE-1 expressed in PS120 fibroblastic cells did not respond to cAMP analogues. In contrast, primary rat hepatocytes (43) and murine macrophages (44) showed significant cAMP-induced stimulation of Na^+/H^+ exchanger activity. Subsequent investigations indicated that these tissues expressed only NHE-1 mRNA (12, 13).[2] Furthermore, preliminary data has shown that rat NHE-1 stably expressed in Chinese hamster ovary AP-1 cells is also activated by agonists that increase cAMP accumulation.[3] In addition to mammalian NHE-1, the trout red cell also expresses a Na^+/H^+ exchanger, called βNHE, that is cAMP-activable and has a primary structure with highest identity to that of mammalian NHE-1 (40). Thus, the diverse regulation of NHE-1 by increasing $cAMP_i$ appears to be partly influenced by the signaling repertoire of individual cell types and perhaps by species variation. Further studies are in progress to define the precise molecular mechanism by which NHE-1 is regulated by agents that activate PKA.

Stimulation of Na^+/H^+ exchange by phorbol esters has also been reported in rat UMR-106 (22) and human osteoblastic SaOS-2 (8) cells, presumably via activation of PKC. Our examination of the effects of PMA in UMR-106 cells produced mixed results that were concentration-dependent. At low concentrations of PMA (*i.e.* 100 nM), PKC activity was significantly increased, but no effect on NHE-1 activity was observed. This was consistent with data obtained using PTH analogues that exclusively activated PKC but did not alter NHE-1 activity. However, higher pharmacological concentrations (≥1 μM) of PMA caused significant stimulation of NHE-1 and appeared to corroborate results from the previous study of UMR-106 cells (22) where a similar concentration of PMA was used. To resolve this seemingly contradictory result, our analyses were extended by assessing the effects of highly specific antagonists of PKA and PKC. Surprisingly, the effects of PMA were abolished by the PKA antagonist H-89 while being relatively unaffected by the PKC inhibitor chelerythrine chloride. This suggested that high concentrations of PMA could activate PKA via a nonclassical mechanism. At present, only limited circumstantial evidence exists for PKC-independent effects mediated by PMA. For example, in human embryonic kidney (HEK-293) cells, PMA has been reported to modulate adenylate cyclase type II activity in the range of 2–9-fold via a pathway that is only partially prevented (~50%) by inhibition of PKC (38, 39), thereby implicating the involvement of additional mechanisms that are PKC-independent. This may also partly explain other observations that acute treatment of UMR-106 (45), ROS 17/2.8 (46), or fetal rat osteoblasts (47) with phorbol esters significantly enhanced PTH-mediated stimulation of adenylate cyclase activity. Regardless of the precise mechanism, but assuming that PMA activated PKA by elevating $cAMP_i$, it remained perplexing that 10 μM PMA could substantially elevate NHE-1 activity by raising $cAMP_i$ levels only 2.5-fold, whereas a 25-fold increase in $cAMP_i$ levels was required to achieve a similar degree of stimulation by 100 nM PTH(1–34) or PTHRP(1–34). Although speculative, one possible explanation to account for this difference is that PTH(1–34) and PMA may mediate their effects on NHE-1 activity in UMR-106 cells by differential activation of distinct adenylate cyclase (48) and PKA (49) isoforms that reside in unique subcellular regions (for review, see Ref. 50). Indeed, compartmentalized increases in $cAMP_i$ have been observed in living cells by microfluorescent techniques (51). Furthermore, individual hormones have been found to preferentially activate distinct PKA isoforms in a tissue-specific manner. For example, in UMR-106 cells, PTH and prostaglandin E2 (PGE_2) were found to preferentially activate type I PKA, whereas in normal rat clavarial osteoblasts, PTH activated type I and II PKA to similar extents while PGE_2 stimulated type II PKA almost exclusively (52). Regionalized membrane localization of NHE-1 has also been observed in adherent fibroblasts (42). Compartmentation of small changes in $cAMP_i$ below the detection sensitivity of our assay may also explain why 1 μM PMA activated NHE-1 without causing an

[2] Demaurex, N., Orlowski, J., Brisseau, G., Woodside, M., and Grinstein, S. (1995) *J. Gen Physiol.* **106,** 85–111.

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

observable change in adenylate cyclase activity. Thus, the magnitude and spatial localization of increases in $cAMP_i$ resulting from different stimuli may be important in the signaling activation of NHE-1. Further experimentation will be required to resolve this phenomenon, but is beyond the scope of the current study.

Notwithstanding the unique regulation of NHE-1 by PMA in UMR-106 cells, a number of other studies have provided convincing data that NHE-1 is regulated by PKC. Stable expression of human (53) and rabbit (17) NHE-1 in fibroblastic cells (PS120) has shown that this isoform is rapidly activated following acute cell stimulation by nanomolar concentrations of phorbol esters as well as growth factors and other mitogens. Mechanistically, this response is associated with increased phosphorylation of a common set of tryptic peptide fragments in the cytoplasmic tail-region of the exchanger (53–55). Moreover, this isoform contains several putative PKC consensus sequences (56) in its carboxyl-terminal region. Interestingly, this region does not contain a classical consensus site (RR*X*S) for PKA. However, since there is overlap in consensus sequence determinants among protein kinases (56), one cannot exclude the potential for PKA phosphorylation of NHE-1. At present, however, it is unclear if phosphorylation of NHE-1 *in vivo* is mediated directly or indirectly by these protein kinases and, indeed, whether phosphorylation *per se* is responsible for the altered activity. It is conceivable that the true targets of protein kinases are cell-specific ancillary factors that subsequently interact with the Na^+/H^+ exchanger and regulate its activity. Cell-specific expression of these factors could account for the differential responsiveness of NHE-1 to individual protein kinases.

REFERENCES

1. Rouleau, M. F., Mitchell, J., and Goltzman, D. (1990) *J. Bone Miner. Res.* **5,** 1043–1053
2. Partridge, N. C., Alcorn, D., Michelangeli, V. P., Ryan, G., and Martin, T. J. (1983) *Cancer Res.* **43,** 4308–4314
3. Bernier, S. M., Rouleau, M. F., and Goltzman, D. (1991) *Endocrinology* **128,** 2752–2760
4. Abou-Samra, A. B., Jüppner, H., Force, T., Freeman, M. W., Kong, X. F., Schipani, E., Urena, P., Richards, J., Bonventre, J. V., Potts, J. T., Jr., Kronenberg, H. M., and Segre, G. V. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 2732–2736
5. Pausova, Z., Bourdon, J., Clayton, D., Mattei, M.-G., Seldin, M. F., Janicic, N., Rivière, M., Szpirer, J., Levan, G., Szpirer, C., Goltzman, D., and Hendy, G. N. (1994) *Genomics* **20,** 20–26
6. Reid, I. R., Civitelli, R., Avioli, L. V., and Hruska, K. A. (1988) *Am. J. Physiol.* **255,** E9–E15
7. Sugimoto, T., Kano, J., Fukase, M., and Fujita, T. (1992) *J. Cell. Physiol.* **152,** 28–34
8. Graham, C. S., and Tashjian, A. H., Jr. (1992) *Biochem. J.* **288,** 137–143
9. Redhead, C. R., and Baker, P. F. (1988) *Calcif. Tissue Int.* **42,** 237–242
10. Grinstein, S., Rotin, D., and Mason, M. J. (1989) *Biochim. Biophys. Acta* **988,** 73–97
11. Tse, C.-M., Levine, S., Yun, C., Brant, S., Counillon, L. T., Pouysségur, J., and Donowitz, M. (1993) *J. Membr. Biol.* **135,** 93–108
12. Orlowski, J., Kandasamy, R. A., and Shull, G. E. (1992) *J. Biol. Chem.* **267,** 9331–9339
13. Wang, Z., Orlowski, J., and Shull, G. E. (1993) *J. Biol. Chem.* **268,** 11925–11928
14. Sardet, C., Franchi, A., and Pouysségur, J. (1989) *Cell* **56,** 271–280
15. Orlowski, J. (1993) *J. Biol. Chem.* **268,** 16369–16377
16. Yu, F. H., Shull, G. E., and Orlowski, J. (1993) *J. Biol. Chem.* **268,** 25536–25541
17. Levine, S. A., Montrose, M. H., Tse, C. M., and Donowitz, M. (1993) *J. Biol. Chem.* **268,** 25527–25535
18. Bookstein, C., Musch, M. W., DePaoli, A., Xie, Y., Villereal, M., Rao, M. C., and Chang, E. B. (1994) *J. Biol. Chem.* **269,** 29704–29709
19. Klanke, C., Su, Y. R., Callen, D. F., Wang, Z., Meneton, P., Baird, N., Kandasamy, R. A., Orlowski, J., Otterud, B. E., Leppert, M., Shull, G. E., and Menon, A. (1995) *Genomics* **25,** 615–622
20. Szpirer, C., Szpirer, J., Rivière, M., Levan, G., and Orlowski, J. (1994) *Mamm. Genome* **5,** 153–159
21. Green, J., and Kleeman, C. R. (1992) *Am. J. Physiol.* **262,** C111–C121
22. Gupta, A., Schwiening, C. J., and Boron, W. F. (1994) *Am. J. Physiol.* **266,** C1083–C1092
23. Ganz, M. B., Pachter, J. A., and Barber, D. L. (1990) *J. Biol. Chem.* **265,** 8989–8992
24. Voyno-Yasenetskaya, T., Conklin, B. R., Gilbert, R. L., Hooley, R., Bourne, H. R., and Barber, D. L. (1994) *J. Biol. Chem.* **269,** 4721–4724
25. Dhanasekaran, N., Vara Prasad, M. V. V. S., Wadsworth, S. J., Dermott, J. M., and Van Rossum, G. (1994) *J. Biol. Chem.* **269,** 11802–11806
26. Jouishomme, H., Whitfield, J. F., Chakravarthy, B., Durkin, J. P., Gagnon, L., Isaacs, R. J., Maclean, S., Neugebauer, W., Willick, G., and Rixon, R. H. (1992) *Endocrinology* **130,** 53–60
27. Heasley, L. E., and Johnson, G. L. (1989) *J. Biol. Chem.* **264,** 8646–8652
28. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159
29. Thomas, J. A., Buchsbaum, R. N., Zimniak, A., and Racker, E. (1979) *Biochemistry* **18,** 2210–2218
30. Goltzman, D., Peytremann, A., Callahan, E., Tregear, G. W., and Potts, J. T., Jr. (1975) *J. Biol. Chem.* **250,** 3199–3203
31. Rabbani, S. A., Kaiser, S. M., Henderson, J. E., Bernier, S. M., Mouland, A. J., Roy, D. R., Zahab, D. M., Sung, W. L., Goltzman, D., and Hendy, G. N. (1990) *Biochemistry* **29,** 10080–10089
32. Rabbani, S. A., Mitchell, J., Roy, D. R., Hendy, G. N., and Goltzman, D. (1988) *Endocrinology* **123,** 2709–2716
33. Rosenblatt, M. (1986) *N. Engl. J. Med.* **315,** 1004–1013
34. Fujimori, A., Cheng, S. L., Avioli, L. V., and Civitelli, R. (1992) *Endocrinology* **130,** 29–36
35. Jouishomme, H., Whitfield, J. F., Gagnon, L., Maclean, S., Isaacs, R., Chakravarthy, B., Durkin, J., Neugebauer, W., Willick, G., and Rixon, R. H. (1994) *J. Bone Miner. Res.* **9,** 943–949
36. Chijiwa, T., Mishima, A., Hagiwara, M., Sano, M., Hayashi, K., Inoue, T., Naito, K., Toshioka, T., and Hidaka, H. (1990) *J. Biol. Chem.* **265,** 5267–5272
37. Herbert, J. M., Augereau, J. M., Gleye, J., and Maffrand, J. P. (1990) *Biochem. Biophys. Res. Commun.* **172,** 993–999
38. Yoshimura, M., and Cooper, D. M. F. (1993) *J. Biol. Chem.* **268,** 4604–4607
39. Lustig, K. D., Conklin, B. R., Herzmark, P., Taussig, R., and Bourne, H. R. (1993) *J. Biol. Chem.* **268,** 13900–13905
40. Borgese, F., Sardet, C., Cappadoro, M., Pouysségur, J., and Motais, R. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 6765–6769
41. Schwartz, M. A., Ingber, D. E., Lawrence, M., Springer, T. A., and Lechene, C. (1991) *Exp. Cell Res.* **195,** 533–535
42. Grinstein, S., Woodside, M., Waddell, T. K., Downey, G. P., Orlowski, J., Pouysségur, J., Wong, D. C. P., and Foskett, J. K. (1993) *EMBO J.* **12,** 5209–5218
43. Moule, S. K., and McGivan, J. D. (1990) *Eur. J. Biochem.* **187,** 677–682
44. Kong, S. K., Choy, Y. M., Fung, K. P., and Lee, C. Y. (1989) *Biochem. Biophys. Res. Commun.* **165,** 131–137
45. Kitten, A. M., Hymer, T. K., and Katz, M. S. (1994) *Am. J. Physiol.* **266,** E897–E904
46. Rao, L. G., and Murray, T. M. (1989) *Calcif. Tissue Int.* **45,** 354–359
47. Bos, M. P., van Leeuwen, J. P., and Herrmann-Erlee, M. P. (1991) *J. Cell. Physiol.* **147,** 87–92
48. Tang, W.-J., and Gilman, A. G. (1992) *Cell* **70,** 869–872
49. Taylor, S. S., Buechler, J. A., and Yonemoto, W. (1990) *Annu. Rev. Biochem.* **59,** 971–1005
50. Coghlan, V. M., Bergeson, S. E., Langeberg, L., Nilaver, G., and Scott, J. D. (1993) *Mol. Cell. Biochem.* **127/128,** 309–319
51. Adams, S. R., Harootunian, A. T., Buechler, Y. J., Taylor, S. S., and Tsien, R. Y. (1991) *Nature* **349,** 694–697
52. Livesey, S. A., Kemp, B. E., Re, C. A., Partridge, N. C., and Martin, T. J. (1982) *J. Biol. Chem.* **257,** 14983–14987
53. Sardet, C., Fafournoux, P., and Pouysségur, J. (1991) *J. Biol. Chem.* **266,** 19166–19171
54. Wakabayashi, S., Bertrand, B., Shigekawa, M., Fafournoux, P., and Pouysségur, J. (1994) *J. Biol. Chem.* **269,** 5583–5588
55. Winkel, G. K., Sardet, C., Pouysségur, J., and Ives, H. E. (1993) *J. Biol. Chem.* **268,** 3396–3400
56. Kennelly, P. J., and Krebs, E. G. (1991) *J. Biol. Chem.* **266,** 15555–15558