Stimulation of Extracellular Signal-regulated Kinases and Proliferation in Rat Osteoblastic Cells by Parathyroid Hormone Is Protein Kinase C-dependent*

Parathyroid hormone (PTH) is known to have both catabolic and anabolic effects on bone. The dual functionality of PTH may stem from its ability to activate two signal transduction mechanisms: adenylate cyclase and phospholipase C. Here, we demonstrate that continuous treatment of UMR 106-01 and primary osteoblasts with PTH peptides, which selectively activate protein kinase C, results in significant increases in DNA synthesis. Given that ERKs are involved in cellular proliferation, we examined the regulation of ERKs in UMR 106-01 and primary rat osteoblasts following PTH treatment. We demonstrate that treatment of osteoblastic cells with very low concentrations of PTH (10^{-12} to 10^{-11} M) is sufficient for substantial increases in ERK activity. Treatment with PTH-(1–34) (10^{-8} M), PTH-(1–31), or 8-bromo-cAMP failed to stimulate ERKs, whereas treatment with phorbol 12-myristate 13-acetate, serum, or PTH peptides lacking the N-terminal amino acids stimulated activity. Furthermore, the activation of ERKs was prevented by pretreatment of osteoblastic cells with inhibitors of protein kinase C (GF 109203X) and MEK inhibitors (U0126). MAPK activation by PTH is protein kinase C-dependent, but stimulation occurs independently of the EGF receptor and Ras activation.

Parathyroid hormone (PTH) is an essential regulator of calcium homeostasis (1) and has both anabolic and catabolic effects in vivo on bone and in vitro on primary and clonal osteoblastic cells (2, 3). The dual functionality of PTH may stem from its ability to stimulate both adenylate cyclase (4) and phospholipase C (5) as a result of ligand binding to the heterotrimeric G-protein-coupled PTH/PTHrP receptor (6). The first 34 amino acids of PTH are necessary and sufficient for full biological activity of the intact hormone (7). Furthermore, studies with N-terminal peptides of PTH demonstrate a requirement for the first 2 amino acids in the activation of adenylate cyclase (8, 9), that amino acids 29–32 are sufficient for phospholipase C stimulation (10), and that residues 25–34 are the principal receptor-binding region (11). The ability of PTH to activate adenylate cyclase and protein kinase A (PKA) is mediated via the stimulatory GTP-binding protein (G_s). PTH also directs the G_s-mediated activation of phospholipase C and ultimately PKC (5), release of Ca^{2+} from intracellular stores (12), and activation of calcium channels (13, 14). Although these signaling pathways mediate the alterations of gene expression and proliferation in osteoblastic cells, the mechanism(s) involved are still poorly understood.

The MAPK signaling pathway is tightly coupled to the regulation of cell proliferation and viability. The PTH/PTHrP receptor couples to G-protein signaling pathways known to regulate MAPKs in many systems (15). Furthermore, PTH stimulates proliferation in bone (2) and kidney cells (16). This combined evidence suggests that PTH may regulate MAPK in target tissues. Two of the best characterized MAPKs, ERK1/p42mapk and ERK2/p44mapk, are regulated by agonists interacting with growth factor receptor tyrosine kinases and G-protein-coupled receptors (17). ERK1 and ERK2 are activated by dual phosphorylation of threonine and tyrosine by the upstream MAPK kinase MEK1 or MEK2 (18, 19), both of which are activated following phosphorylation by the MAPKKK Raf-1 (20). It is known that activation of the MAPK pathway originates from several distinct classes of cell-surface receptors in a manner dependent on or independent of the small GTP-binding protein Ras (15, 21).

G-protein-coupled receptors have been shown to either activate (22–24) or inhibit (25–27) MAPKs. MAPK activation by G_s-coupled agonists is PKC-dependent, but pertussis toxin-insensitive, and because PKC can directly phosphorylate Raf-1 (28), ERK activation may or may not involve Ras (29–31). G_s-coupled receptors activate or inhibit MAPK in a cell type-specific manner (26, 32, 33). Previously, it was shown that PTH-(1–34) could inhibit MAPK in UMR 106-01 and ROS 17/2.8 cells (27) and inhibits cellular proliferation and DNA synthesis in UMR 106-01 cells (4, 34) via a PKA-dependent pathway. In MC3T3-E1 osteoblasts, PMA was shown to stimulate both proliferation and phosphorylation of ERKs, and these effects were blocked by pretreatment with forskolin (35). Additionally, low concentrations of PTH were shown to activate
ERKs and proliferation in opossum kidney cells (36). Since PKC is known to activate MAPK, it is possible that the ability of PTH to activate PKC and ERKs is a potential mechanism for the anabolic effects of PTH. Here, we demonstrate that activation of ERKs and proliferation in osteoblastic cells occurs following treatment with low concentrations of PTH. This activation is dependent upon PKC, but not PAK, and does not require activation of the small G-protein Ras or phosphorylation of the EGFR. This study supports PTH as a mitogen and demonstrates that the proliferative effects of PTH are dependent upon selective PKC activation, resulting in ERK activation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Synthetic rat PTH (1–34), protein A-acrylic beads, myelin basic protein (MBP), and 8-bromo-cAMP were purchased from Sigma. Isobutylmethylxanthine, ionomycin, PMA, PD 98059, H-89, and GF 109203X were obtained from Calbiochem. Enhanced chemiluminescence (ECL) reagents were obtained from Amersham Pharmacia Biotech. PerkinElmer Life Sciences supplied [γ-32P]ATP and [3H]thymidine. Polynivinilene difluoride membrane (Imnobilon) was purchased from Millipore Corp. (Bed ford, MA). Synthetic PTH peptides (PTH(1–31), (3–34), (13–34), and (28–48)) were purchased from Bachem (King of Prussia, PA). Tissue culture media and reagents were obtained from the Washington University Tissue Culture Center (St. Louis, MO). Fetal bovine serum was a product of JRH Biosciences (St. Louis, MO). horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse antibodies were purchased from Sigma. The polyclonal anti-ERK1 (p44 

**PKC assay**—PKC activity was measured using a Pierce colorimetric PKC assay kit, SpinZyme format (product no. 29542). Cell lysis and PKC assay were performed as described (39). PKC activity was measured in lysates prepared from cytosolic and membrane fractions by incubation with fluorescently labeled MBP substrate. The reaction mixture was applied to a separation unit with an affinity membrane that specifically binds phosphorylated substrate. Quantitation of the phosphorylated product was performed by measuring absorbance at 570 nm, with the amount of peptide obtained being directly proportional to the amount of specific kinase activity present in each sample. Extrapolation of activity was performed from a linear regression generated using known amounts of PKC active enzyme.

**ERK Immune Complex Assay**—Serum-starved osteoblastic cells were treated in the presence and absence of appropriate agents for the indicated time periods at 37°C. The medium was aspirated, and cells were washed twice with cold phosphate-buffered saline and then lysed in ice-cold lysis buffer (20 mM Tris-HCl (pH 8.0), 1% Triton X-100, 2 mM EDTA, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin). Monolayers were scrapped into 1.5-ml Eppendorf tubes and incubated on ice for 30 min. The samples were centrifuged (12,000 rpm, 10 min, 4°C). Protein contents of the supernatants were determined using the Bradford reagent (Bio-Rad) at 595 nm. For each assay, 100 μg of total protein were used; the volume was adjusted to 200 μl; 3 μg of anti-ERK1 and anti-ERK2 antibodies were added; and the samples were rotated for 4 h at 4°C. At this time, 30 μl of protein A-acrylic beads were added, and the samples were rotated from 2 h to overnight at 4°C. The samples were then centrifuged (12,000 rpm, 10 min, 4°C). The immune complex was washed three times with cold lysis buffer, three times with 0.5 M LiCl and 100 mM Tris (pH 7.6), and once with assay buffer (20 mM Tris-HCl (pH 7.2), 1.5 mM EGTA, 0.03% Brij 35, 50 mM β-glycerophosphate, and 1 mM dithiothreitol) and then resuspended in 20 μl of reaction buffer (20 mM Tris-HCl (pH 7.3), 50 μM ATP, 5 μCi of [γ-32P]ATP, and 10 mM MgCl2) containing 10 μg of MBP/assay for 30 min at 30°C. Reactions were terminated via the addition of 7.5 μl of SDS sample buffer (0.063 M Tris (pH 6.8), 1% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.1% bromphenol blue) and boiling for 5 min. Following centrifugation for 10 min at 4°C, 10 μl of each reaction were resolved on a 12.75% SDS-polyacrylamide gel; and after drying, the gel was exposed to x-ray film. Quantitation was performed by scanning the gel with a PhosphorImager (Molecular Dynamics, Inc.)

**Western Blotting of Osteoblastic Cell Lysates**—Cell lysates containing 50 μg of total protein in lysis buffer were boiled for 5 min with 2× SDS sample buffer, centrifuged (12,000 rpm, 3 min), and placed on ice. SDS-polyacrylamide gel electrophoresis was performed with 6 and 12% stacking and resolving gels, respectively. The proteins were transferred electrophoretically to polyvinylidene difluoride membranes for 1 h. After blocking the membrane in 0.1% Tween 20, 138 mM NaCl, 5 mM KCl, and 25 mM Tris-HCl (pH 8.0) containing 5% (w/v) nonfat dry milk, the membrane was probed with anti-ERK1/ERK2 antibodies (diluted 1:1000), followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (diluted 1:10,000). The antigen-antibody complexes were detected by ECL following the manufacturer’s protocol.

**Analysis of EGFR Phosphorylation**—UMR 106-01 cells grown in 100-mm dishes were treated as described. Lysates were prepared as described for the ERK immune complex assay, and equal amounts of protein (0.2 mg) from each sample were incubated overnight at 4°C with 3 μg of polyclonal anti-EGFR or monoclonal anti-phosphotyrosine (PY20) antibody. After 30 min, the samples were centrifuged at 4°C, and the antigen-antibody complexes were collected by centrifugation. Pellets were boiled for 5 min in 2× SDS sample buffer and resolved on 4–15% gradient gels (Bio-Rad). After transfer to polyvinylidene difluoride membranes, samples were immunoblotted with PY20 to assess EGFR phosphorylation content or with anti-EGFR antibody to identify the EGFR.

**Statistical Analysis**—Experimental significance was assessed using one-way analysis of variance with post hoc analysis of multiple comparison using Dunnett’s pairwise method. All other data shown are representative of at least three separate experiments with similar results.

**RESULTS**

**Stimulation of Proliferation in UMR 106-01 Cells by PTH Is PKC-dependent**—Although PTH (10–8 M) activates PKA and is inhibitory to cellular proliferation and DNA synthesis in UMR 106-01 cells (34), lower doses result in significant increases (p < 0.05) in cell number (34). Here, we assessed for changes in DNA synthesis following treatment of UMR 106-01 cells with different doses of PTH. Furthermore, we used N-terminal PTH peptides to determine any involvement of PKC or PKA. Following serum starvation for 24 h, UMR 106-01 cells were treated with PTH (1–34) (10–8 M) alone or with the selective inhibitor PD 98059 (MEK), GF 109203X (PKC), or H-89 (PKA). As shown in Fig. 1A, PTH at this concentration was unable on its own to stimulate proliferation. However, inhibition of the PKA
addition of H-89 resulted in a significant -2-fold increase (p = 0.001) in [3H]thymidine incorporation, whereas GF 109203X and PD 98059 had no effect.

We next examined the ability of N-terminal PTH deletion peptides to stimulate proliferation (Fig. 1B). Following serum starvation, UMR 106-01 cells were incubated with PTH-(1–31) (activates PKA, but not PKC) or with PTH-(3–34) (unable to activate PKA). Since PTH-(3–34) also promotes release of intracellular calcium, UMR 106-01 cells were also treated with PTH-(13–34) and PTH-(28–48), peptides that selectively activate PKC. We observed significant increases in [3H]thymidine incorporation following treatment with PTH-(3–34) (p = 0.003), PTH-(13–34) (p = 0.0007), and PTH-(28–48) (p = 0.01), but not with PTH-(1–31).

Finally, we determined if the significant increases in proliferation we previously observed with low doses of PTH (34) and with PTH-(3–34) were PKC-dependent. Following serum starvation, UMR 106-01 cells were incubated with either PTH-(1–34) (10^{-11} M) or PTH-(3–34) (10^{-8} M) with or without the PKC inhibitor GF 109203X at 5 μM (Fig. 1C). We observed an ~3-fold increase in thymidine incorporation following treatment with 10^{-11} M PTH-(1–34) (p = 0.01) or PTH-(3–34) (p = 0.01), and both were significantly inhibited by the addition of GF 109203X (p = 0.01 and 0.03, respectively).

To correlate enhanced proliferation with an increase in cell number, a complete dose response experiment with PTH-(1–34) followed by cell counting was performed. We observed a significant increase (p = 0.016) in cell number from 3.7 × 10^5/well with controls to 5.2 × 10^5/well following treatment with 10^{-3} M PTH for 72 h.

**PTH Activates PKC at Low Doses**—Since we observed significant increases in proliferation of UMR 106-01 cells at low doses of PTH (10^{-11} M) and these increases appear to be PKC-dependent, we performed a dose response experiment with PTH to determine the minimal concentration required for activation of PKC (Fig. 2). Following 24 h of serum starvation, cells were treated with increasing concentrations of PTH for 2 min, and PKC activity was assayed in both the cytoplasmic and membrane fractions. PKC activity was measured by a colorimetric assay and is defined as picomoles of phosphate incorporated into substrate at a linear regression generated using known amounts of PKC active enzyme. rPTH, rat PTH.

**PTH Activates ERKs in a Dose-dependent Manner**—The ERK subclass (ERK1/p42^{mapk} and ERK2/p44^{mapk}) of MAPKs is activated by major signaling pathways regulating cell proliferation (43–45). It is known that high concentrations of PTH, which
Regulation of ERKs by PTH

result in elevated levels of cAMP and activation of PKA (4, 46), inhibit proliferation (34) and growth factor-stimulated MAPK activity (25, 27) in UMR 106-01 and fibroblast cells. However, since we had demonstrated activation of PKC by N-terminal PTH peptides or low doses of PTH (1–34) that were also able to stimulate proliferation, we investigated the effect of PTH on MAPK activity in UMR 106-01 osteoblastic cells (Fig. 3). To quantify the effect of PTH (1–34) on ERK activity, we performed an in vitro kinase assay with immunoprecipitated ERK1/ERK2 in which MBP was a substrate. ERK activity was measured in osteoblastic cells 15 min after the addition of PTH, which we had found was the time of maximal stimulation (data not shown). Treatment of UMR cells with 10^{-12} and 10^{-11} M PTH resulted in significant increases ($p < 0.05$) in the fold-stimulation of ERK activity (2.9- and 2.8-fold, respectively) above basal levels (Fig. 3). Additionally, UMR cells were treated with 5% FBS as a positive control for ERK activation. Treatment for 15 min with 5% FBS resulted in a 2.8-fold increase in ERK activity above basal levels (Fig. 3). Next, a complete dose response was performed with increasing concentrations of PTH (Fig. 4A). Treatment of UMR cells with 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, or 10^{-8} M PTH or 5% FBS resulted in 3.2-, 2.6-, 2.9-, 1.5-, 1.6-, and 3.3-fold increases above basal activation. The addition of medium containing 5% fetal bovine serum was performed as a positive control for ERK activation. In primary osteoblasts (Fig. 4B), the results were similar, with 3.9-, 5-, 4.7-, 1.2-, 1.4-, and 10-fold increases above basal activation. These results clearly demonstrate that activation of MAPK by PTH is dose-dependent. Treatment of osteoblastic cells with concentrations of PTH in the lower range stimulated ERKs, whereas higher concentrations, which activated PKA maximally, were unable to stimulate ERKs. Interestingly, although treatment with 10^{-12} M PTH was unable to significantly ($p = 0.15$) stimulate PKC above basal levels (Fig. 2), the level of activation was sufficient to stimulate ERK activity. The ERK assays were performed with equal amounts of total protein from whole cell lysates. Therefore, Western blot analysis was performed to examine the relative levels of ERK1/ERK2 protein present for each assay (lower panels).

**Activation of ERKs in Osteoblastic Cells by PTH Is PKC-dependent**—Experiments were performed using the cAMP analog 8-bromo-cAMP (10^{-3} M), the calcium ionophore ionomycin (10^{-5} M), or the phorbol ester PMA (10^{-6} M) to determine which signaling cascade leads to activation of ERKs by PTH (Fig. 5). Following 48 h of serum starvation, cells were treated with the indicated agents, and immune complex kinase assays were performed. Treatment of osteoblastic cells with 5% FBS or PMA stimulated MAPK. Treatment of UMR 106-01 or primary osteoblasts with PMA resulted in 2.5- and 3.0-fold increases in ERK activity, respectively (Fig. 5A and B). Treatment of UMR 106-01 or primary rat osteoblastic cells with 5% FBS resulted in 2.3- and 3.0-fold increases in ERK activity, respectively (Fig. 5C and D).
Regulation of ERKs by PTH

**Fig. 6. Activation of ERKs by PTH is PKC-dependent and requires MEK activation.** UMR 106-01 cells were pretreated with inhibitors of PKA, PKC, and MEK and PTH-treated, and ERK activity was measured. Osteoblastic cells were serum-starved for 48 h and pretreated with H-89 (20 μM, B), GF 109203X (5 μM, C), or PD 98059 (50 μM, D) for 30 min. Treatment with PTH-(1–34) was for 15 min at 10⁻¹¹ M, except in B, where 10⁻⁸ M PTH was used. Treatment with PTH-(13–34) was also for 15 min at 10⁻⁸ M. Treatment with PMA (10⁻⁶ M) and EGF (50 ng/ml) was for 5 min. Western blot analysis was performed to examine the relative levels of ERK1/ERK2 protein present in each assay. C lane, control.

**Fig. 7. Effect of PTH on tyrosine phosphorylation of the EGFR.** Shown are the results from immunoprecipitation of the EGFR following treatment of UMR 106-01 cells with PTH peptides or EGF. Cells were serum-starved for 48 h and then treated for 15 min with the indicated PTH peptides at 10⁻⁸ M, except for PTH-(1–34), which was used at 10⁻¹¹ M. Additionally, osteoblastic cells were treated with EGF (50 ng/ml) for 3 min. A, total protein (200 μg) from each sample was immunoprecipitated (IP) with monoclonal anti-EGFR antibody (aEGFR), and antigen complexes were resolved on 4–15% gradient gels and immunoblotted (IB) with anti-phosphotyrosine antibody (PY20) to assess phosphorylation of the EGFR. B, total protein (200 μg) from each sample was immunoprecipitated with monoclonal anti-phosphotyrosine antibody (PY20), followed by immunoblot analysis with anti-EGFR antibody to identify the EGFR. C, immunoblot analysis of the EGFR was performed to show receptor levels in each lysate. Arrowheads indicate the EGFR. C lane, control.

5, A and B). The addition of 8-bromo-cAMP and ionomycin failed to stimulate MAPK with activities at or below basal levels. Activation of ERKs by the phorbol ester PMA suggested that activation of PKC may be an essential prerequisite to activation of ERKs in both UMR and primary rat osteoblastic cells.

Inhibitors of PKC or PKA were used to further demonstrate that activation of ERKs by PTH is PKC-dependent (Fig. 6). Pretreatment of UMR 106-01 cells with the PKA inhibitor H-89 resulted in a greater than 2-fold increase in basal ERK activity (Fig. 6A) over the control (Fig. 6A). Furthermore, stimulation of ERKs by PTH, PMA, or FBS was not inhibited by pretreatment with H-89 (Fig. 6B). The addition of PTH-(13–34), PMA, or FBS resulted in 2.0-, 3.6-, and 5.0-fold stimulation of ERK activity above basal levels, respectively (Fig. 6B). Furthermore, PTH-(1–34) used at 10⁻⁸ M, a dose that was shown to have no effect when used on its own (Fig. 4), was still able to stimulate ERK activity (~2.0-fold) in the presence of H-89 (Fig. 6B). Treatment with the PKC inhibitor GF 109203X prevented stimulation of ERKs by PTH-(1–34), PTH-(13–34), or PMA (Fig. 6C). However, treatment of cells with 5% FBS resulted in a 5.3-fold increase in ERK activity above basal levels, suggesting that inhibition of PKC does not affect ERK activation by serum growth factors (Fig. 6C).

Finally, because MEK1 and MEK2 activate ERK, we assayed for ERK activity in the presence of the MEK inhibitor PD 98059 (Fig. 6D). Pretreatment with this inhibitor prevented activation of ERKs by PTH, EGF, and 5% FBS, suggesting that activation of ERKs by PKC occurs at or above the level of MEK.

**Effect of PTH on Tyrosine Phosphorylation of the EGFR—**It is known that a number of G-protein-coupled receptor agonists induce phosphorylation of the EGFR preceding MAPK activation (43, 44). Therefore, we examined the ability of PTH to effect tyrosine phosphorylation of the EGFR. UMR cells were serum-starved for 48 h and then treated with the indicated PTH peptides for 15 min or with EGF for 3 min, followed by immunoprecipitation of the EGFR and Western blot analysis for phosphorylation of tyrosines (Fig. 7, A and B). Lysates were immunoprecipitated with anti-EGFR antibody, and immunoblot analysis was performed with anti-phosphotyrosine antibody. Immunoblot analysis was performed with anti-EGFR antibody, and similar results (Fig. 7B). Immunoblot analyses of total protein lysates were performed to ensure that each sample had EGFR protein (Fig. 7C). Activation of ERKs by PTH Is Ras-independent—To determine whether activation of ERKs by PTH requires Ras, UMR 106-01 cells were transiently transfected with the pcDNA3 vector alone as a control or with dominant-negative p21N17Ras prior to treatment with N-terminal peptides or EGF (Fig. 8). Transfection of cells with dominant-negative p21N17Ras reduced ERK activation following treatment with EGF by 72%, reducing the -fold stimulation from 36 to 10 (Fig. 8). However, transfection with dominant-negative p21N17Ras did not prevent ERK activation by the N-terminal PTH peptides (PTH-(1–34) at 10⁻¹¹ M and PTH-(3–34) and PTH-(13–34) at 10⁻⁸ M (Fig. 8). This was not surprising because it is known that activation of the ERK pathway can occur in a manner dependent on or independent of the small GTP-binding protein Ras (15), and activation of the ERK pathway by PKC is typically Ras-independent (29, 30). Therefore, the PKC-dependent stimulation of
ERKs by PTH most likely occurs directly through Raf. As seen in the foregoing experiments, PTH(1–31) and PTH(1–34) at 10^{-8} M did not activate ERKs, nor were they affected by cotransfection of dominant-negative p21N17Ras.

**DISCUSSION**

G-protein-coupled receptors have been shown to either activate (22–24) or inhibit (25–27) ERKs. G_{i}-coupled receptors, such as the α_{5}-adrenergic, m2 muscarinic, and D_{2}-dopaminergic receptors, can trigger MAPK activation via G_{i}-dependent activation of Ras (24, 47–49), similar to the growth factor receptor signaling cascade (50). Presently, there is no strong evidence supporting PTH-mediated signaling by G_{i} in osteoblasts. Furthermore, the addition of pertussis toxin and transient transfection of α-transducin have no effect on ERK activation by PTH (data not shown). G_{i}-coupled receptors, such as the glucagon and β-adrenergic receptors, can activate or inhibit MAPK in a cell type-specific manner (26, 32, 33), and it appears that the activation of PKA interferes with the activation of MAPK at the level of Raf (51). We have previously shown that PTH (10^{-8} M) activates PKA and is inhibitory to cellular proliferation and DNA synthesis in UMR 106-01 cells (4, 34); however, lower doses result in significant increases in cell number (34). Recently, it was shown that PTH(1–34) (10^{-7} M) could inhibit both proliferation and ERKs in UMR 106-01 and ROS 17/2.8 cells via a PKA-dependent pathway (27). Our data support this, as we have shown that when PKA is inhibited, PTH can strongly stimulate proliferation (Fig. 1A) and activate ERKs (Fig. 6B). Furthermore, activation of PKA fails to stimulate proliferation (Fig. 1, A and B) or ERKs (Fig. 5). Nevertheless, in CHO-R15 and PYS-2 cells, PTH(1–34) (10^{-7} M) was shown to stimulate ERKs via a cAMP-dependent pathway independent of phospholipase C and Ras activation (52). Under no circumstances were we able to detect activation of ERKs by PTH when PKA was activated. Furthermore, our data suggest that PKC activation is necessary and sufficient for ERK activation. This discrepancy is most easily explained by the differences between cell types. Therefore, the stimulation or inhibition of ERKs by elevated levels of cAMP following exposure to PTH is cell type-specific; and in actual osteoblastic cells, activation of PKA inhibits ERK activation.

G_{q}-coupled receptors, such as the α_{1H}-adrenergic and m1 muscarinic receptors, can activate ERKs via phospholipase C, resulting in translocation of PKC to the plasma membrane and subsequent activation of Raf (24). ERK1 and ERK2 are activated by dual phosphorylation of threonine and tyrosine by the upstream MAPK kinase MEK1 or MEK2 (19, 53, 54), both of which are activated following phosphorylation by the MAPKK Raf-1 (20, 55). The ability of low doses of PTH to activate PKC (Fig. 2) and to activate ERKs by a PKC-dependent mechanism suggests that the activation of signaling pathways by PTH can be further dissected according to hormone concentration. We used the N-terminal peptides and inhibitors to demonstrate that PKC is necessary and sufficient for activation of the ERK pathway (Figs. 5 and 6). The addition of PKC and MEK inhibitors (Fig. 6) suggests that activation of PKC is required for ERK activation and that the regulation occurs at or above the level of MEK. Finally, we demonstrated that PTH is unable to induce phosphorylation of the EGFR and that dominant-negative Ras is unable to inhibit ERK activation by PTH (Fig. 8). Our results, in conjunction with what is known about activation of ERKs by PKC, suggest that stimulation of this pathway directly follows activation of Raf.

In MC3T3-E1 osteoblasts, PMA was shown to stimulate both proliferation and phosphorylation of ERKs, and these effects were blocked by pretreatment with forskolin (35). Additionally, low concentrations of PTH were shown to activate ERKs and proliferation in opossum kidney cells (36). These results, in addition to the ability of the PTH/PTHrP receptor to activate PKC and ERKs in UMR 106-01 and primary rat osteoblastic cells, indicate a potential mechanism for some of the anabolic effects of PTH. Although our data suggest an important role for PTH and ERK pathways in osteoblast proliferation, the mechanism(s) involved are unknown. Activation of the ERK and SAPK/JNK subclasses of MAPKs has been shown to target and phosphorylate proteins regulating transcription, apoptosis, and cell cycle (56–58). In addition to regulation of ERKs, we have shown that treatment of UMR 106-01 cells with high doses of PTH(1–34) results in inhibition of JNK activity without affecting JNK protein levels and that this inhibition appears to be cAMP-dependent and PKC-independent.\(^2\) Therefore, one function of PTH is to regulate key components of the MAPK signaling cascades that affect proliferation and potentially gene transcription and apoptosis. Furthermore, cell culture studies with osteoblastic cells have demonstrated a wider range of activities for PTH, including regulation of osteoblastic genes (3), DNA synthesis (34, 60–63), and the cell cycle at the G_{1}/S and G_{2}/M transitions. PTH treatment of osteoblastic cells results in increased expression of proto-oncogenes (64) and PKC (65), both associated with enhanced cell proliferation, and regulators of cell cycling (66).

A major question regarding PTH action is how it exerts its paradoxical anabolic and catabolic effects on the osteoblast through one receptor. An obvious answer is that the hormone mediates its effects through two or more receptors. Although a second receptor specific for PTH has been identified, the PTH2 receptor (59), the absence of expression of the PTH2 receptor by osteoblastic cells suggests that this is not the answer. A more likely explanation for the dual functionality of PTH may stem from its ability to activate two signal transduction mechanisms: adenylate cyclase and phospholipase C. Here, we demon‌strate that continuous treatment of UMR and primary osteoblasts with PTH peptides, which selectively activate protein kinase C, results in significant increases in DNA synthesis and ERK activation. Furthermore, PTH causes a concentration-dependent activation of ERKs. This activation is mimicked by

\(^2\) T. A. Doggett, J. T. Swarthout, D. Wilhelm, A. Dieckman, P. Angel, and N. C. Partridge, manuscript in preparation.
PMA, and activation of ERKs by PTH or PMA is inhibited by the PKC inhibitor GF 109203X. The ability of low concentrations of PTH to selectively activate PKC, resulting in activation of ERKs and proliferation in osteoblastic and opossum kidney cells, provides a mechanism explaining the mitogenic effects of PTH, but raises the question of how the PTH/PTHrP receptor can selectively interact with Gq, when physiological concentrations of the hormone are bound. Further experimentation will elucidate this and may provide pharmacological agents for treating osteoporosis.

REFERENCES

1. Strewler, G. J. (2000) N. Engl. J. Med. 342, 177–185
2. Dempster, D. W., Cosman, F., Parfisien, M., Shen, Y., and Lindsay, R. (1993) Endo.
    14, 690–709
3. Partridge, N. C., Bloch, S. R., and Pearman, A. T. (1994) J. Cell. Biochem. 55,
    321–327
4. Partridge, N. C., Kemp, B. E., Veroni, M. C., and Martin, T. J. (1981) J. Cell.
    Biochem. 14, 220–225
5. Civitelli, R., Reid, I. R., Westbroc, S., Avili, L. V., and Hruska, K. A. (1988)
    Am. J. Physiol. 255, E650–E657
6. Abou-Samra, A. B., Juppner, H., Force, T., Freeman, M. W., Kong, X. F.,
    Sciphan, 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
7. Habener, J. F., Rosenblatt from, M., and Potts, J. T. Jr. (1984) J. Cell.
    Biochem. 24, 895–1053
8. Fujimori, A., Cheng, S. L., Avili, L. V., and Civitelli, R. (1992) Endocrinology 130,
    29–36
9. Azaruni, A., Goltzman, D., and Orleowsi, K. (1995) J. Biol. Chem. 270,
    2004–20010
10. Joushishome, H., Whitfield, J. F., Gagnon, L., Maclean, S., Isacs, A.,
    Chakravarty, B., Durkin, J., Neigeberauer, W., Willick, G., and Rixon, R. H.
    (1995) J. Bone Miner. Res. 5, 945–949
11. Gardella, T. J., Wilson, A. K., Keutmann, H. T., Oberstein, R., Potts, J. T., Jr.,
    Kronenberg, H. M., and Nussbaum, S. R. (1993) Endocrinology 132,
    2024–2030
12. Reid, I. R., Civitelli, R., Halstead, L. R., Avili, L. V., and Hruska, K. A. (1987)
    Am. J. Physiol. 255, E45–E51
13. Yamaguchi, T. T., Kleeman, C. F., and Mualem, S. (1987) J. Biol. Chem. 262,
    12107–12113
14. Yamaguchi, T. T., Hahn, T. J., Iida-Klein, A., Kleeman, C. R., and Mualem, S.
    (1987) J. Biol. Chem. 262, 7711–7718
15. Luttrell, L. M., Daaka, Y., and Lefkowitz, R. J. (1999) Curr. Opin. Cell. Biol. 11,
    177–183
16. Garcia-Ocana, A., Gomez-Casero, E., Penaranda, C., and Esbrit, P. (1998) Life.
    Sci. 62, 2267–2273
17. Gutkind, J. S. (1994) J. Biol. Chem. 269, 1839–1842
18. Crews, C. M., Erikson, R. L. (1995) Proc. Natl. Acad. Sci. U. S. A. 89,
    8205–8209
19. Crews, C. M, Alessandrin, A., and Erikson, R. L. (1992) Science 258, 478–480
20. Marshall, M. (1995) Mol. Reprod. Dev. 42, 493–499
21. Luttrell, L., Haben, B. E., van Biesen, T., Luttrell, D. K., Lansing, T. J.,
    and Lefkowitz, R. J. (1995) J. Biol. Chem. 269, 19443–19450
22. Faure, M., Voino-Yasnetzkaya, T. A., and Bourne, H. R. (1994) J. Biol. Chem.
    269, 7851–7854
23. Crespo, P., Xu, N., Simonds, W. F., and Gutkind, J. S. (1994) Nature 369,
    420–4220
24. Hawes, B. E., van Biesen, T., Koch, W. J., Luttrell, L. M., and Lefkowitz, R. J.
    (1995) J. Biol. Chem. 270, 17148–17153
25. Burgering, B. M., Pronk, G. J., van Weeren, P. C., Chardin, P., and Bos, J. L.
    (1995) EMBO J. 12, 4211–4220