Induction of osteoblast differentiation indexes by PTHrP in MG-63 cells involves multiple signaling pathways

LUISA CARPIO, JULIENNE GLADU, DAVID GOLTZMAN, AND SHAFAAT A. RABBANI
Department of Medicine, McGill University Health Center, Montreal, Quebec H3A 1A1, Canada

Received 29 January 2001; accepted in final form 26 April 2001

Carpio, Luisa, Julienne Gladu, David Goltzman, and Shafaat A. Rabbani. Induction of osteoblast differentiation indexes by PTHrP in MG-63 cells involves multiple signaling pathways. Am J Physiol Endocrinol Metab 281: E489–E499, 2001.—Parathyroid hormone (PTH)-related peptide (PTHrP) can modulate the proliferation and differentiation of a number of cell types including osteoblasts. PTHrP can activate a G protein-coupled PTH/PTHrP receptor, which can interface with several second-messenger systems. In the current study, we have examined the signaling pathways involved in PTHrP-induced type I collagen and alkaline phosphatase expression in the human osteoblast-derived osteosarcoma cells, MG-63. By use of Northern blotting and histochemical analysis, maximum induction of these two markers of osteoblast differentiation occurred after 8 h of treatment with 100 nM PTHrP-(1–34). Chemical inhibitors of adenylate cyclase (H-89) or of protein kinase C (chelerythrine chloride) each diminished PTHrP-mediated type I collagen and alkaline phosphatase stimulation in a dose-dependent manner. These effects of PTHrP could also be blocked by inhibiting the Ras-mitogen-activated protein kinase (MAPK) pathway with a Ras farnesylation inhibitor, B1086, or with a MAPK inhibitor, PD-98059. Transient transfection of MG-63 cells with a mutant form of Go, which can sequester βγ-subunits, showed significant downregulation of PTHrP-stimulated type I collagen expression, as did inhibition of phosphatidylinositol 3-kinase (PI 3-kinase) by wortmannin. Consequently, the βγ-PI 3-kinase pathway may be involved in PTHrP stimulation of Ras. Collectively, these results demonstrate that, acting via its G protein-coupled receptor, PTHrP can induce indexes of osteoblast differentiation by utilizing multiple, perhaps parallel, signaling pathways.

parathyroid hormone-related peptide; osteoblast; cell differentiation; hypercalcaemia

THE SEARCH for the responsible pathogenetic factor in the development of hypercalcaemia of malignancy culminated in the discovery of parathyroid hormone (PTH)-related peptide (PTHrP) a little more than a decade ago (6, 26, 32). Since then, PTHrP has been observed to be expressed by a wide variety of normal adult and fetal tissues (12, 30). Due to its wide tissue distribution and the degree to which it is conserved across evolution, it was proposed that PTHrP may well have a significant developmental role. It was subsequently discovered that PTHrP plays a role in the proliferation and differentiation of a variety of cell types, including chondrocytes, osteoblasts (18), and keratinocytes (16, 17). Despite the evidence that PTHrP plays an important role in cellular turnover and maturation, little is currently known of the molecular mechanisms involved in PTHrP-mediated cellular differentiation. Due to the NH2-terminal sequence homology between PTH and PTHrP, these two peptides can interact with a common receptor, PTH/PTHrP receptor (1). The presence of this receptor in bone is well established, and high receptor levels are seen in osteoblasts that are actively differentiating (24), suggesting a role for the receptor in osteoblast development. Active mineralization of bone matrix involves the production of type I collagen and alkaline phosphatase, which, among others, are established markers of osteoblast differentiation (4).

The common PTH/PTHrP receptor is a member of the family of seven transmembrane receptors that are coupled to heterotrimeric G proteins. PTH and PTHrP are known to activate several second-messenger pathways that are linked by distinct mediators to the PTH/ PTHrP receptor (27). For example, ligand binding is known to stimulate both intracellular cAMP and inositol trisphosphate through Goq and Gqα, respectively (1, 15). G protein-coupled receptors are also known to activate mitogen-activated protein kinase (MAPK) activity in a manner that is dependent on the profile of the involved G protein, the receptor to which it is coupled, and the cell type in which they are found (33). Previous studies have reported differential activation of protein kinase A (PKA) or PKC pathways, and this may be cell type specific and vary according to the differentiation stage and exposure time to the ligand. Activation of MAPK has been shown to be essential in the differentiation of several cell types, and it has been shown that PKA and/or PKC activation can influence MAPK activity. G protein activation will also involve the release of a βγ-dimer subunit (5, 21), and this subunit may regulate the phosphorylation of the protein Shc, which can then lead to the formation of a protein complex involving Shc-Grb2-Sos and the subsequent activation of the protooncogene Ras (11). Ras activation is known to result in activation of Raf and

Address for reprint requests and other correspondence: S. A. Rab- bani, Calcium Research Laboratory, Royal Victoria Hospital, 687 Pine Ave. West, Rm. H4.72, Montreal, QC H3A 1A1 Canada

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

http://www.ajpendo.org 0193-1849/01 $5.00 Copyright © 2001 the American Physiological Society
then in activation of the enzymes MAPK kinase (MEK) and MAPK (22). Receptors coupled to trimeric G proteins may therefore activate one or more of these possible pathways. Although it has been definitively established that PTH/PTHrP receptor signaling may occur via $\gamma_\alpha$, and/or $\gamma_\alpha_{\beta}$, the question of $\beta_\gamma$-mediated signaling by this receptor is not yet so clearly proven.

The present study was undertaken to further examine the possible role of multiple signal transduction pathways in the stimulation of osteoblast differentia-

![Fig. 1. Effect of parathyroid hormone (PTH)-related peptide (PTHrP) on MG-63 cell differentiation. Human osteoblast-like osteosarcoma MG-63 cells were grown in 10% serum to 80% confluence and then incubated in serum-free conditions. Cells were then treated with vehicle or with 100 nM PTHrP-(1–34) for timed intervals. A: total cellular RNA was extracted from MG-63 cells. Fifteen micrograms of total cellular RNA for each time point (1–16 h) were electrophoresed onto a 1.1% agarose-formaldehyde gel. Filters were probed with type I collagen and 18S cDNA to determine the ratio of I collagen to 18S mRNA expression. B: alkaline phosphatase activity in control and PTHrP-treated MG-63 cells was detected by a histochemical reaction. Cells were fixed in citrate-buffered acetone. Slides were then immersed in alkaline-dye solution containing diazonium salts and incubated for 30 min. Cells were stained with Mayer's hematoxylin solution for 10 min to detect the insoluble pigments formed as a result of alkaline phosphate activity. Slides were then evaluated microscopically. C: alkaline phosphatase activity was quantified using the NIH Image-based Scion Image analysis program. Quantification is presented as staining density per field. Results represent means ± SE of 3 different experiments. *Significant differences from control ($P < 0.05$).]
tion by PTHrP. Through the use of chemical inhibitors of signal transduction and transient transfection of a \(\beta\)-sequestering mutant form of \(\alpha_\text{Ga_s}\), and employing a human osteoblast-like osteosarcoma model, MG-63 cells, we show the importance of activation of PKA, PKC, and Ras by PTHrP in inducing osteoblast differentiation. Our results also demonstrate the involvement of MAPK, which may be a point of convergence of these activated signaling pathways in this system. These results, therefore, emphasize the involvement of multiple pathways in PTHrP-induced indexes of osteoblast differentiation.

**MATERIALS AND METHODS**

*Cell culture.* MG-63 osteosarcoma cells were maintained in vitro in MEM [with Earle’s Salts (ES)] supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin-streptomycin-sulfoxide (GIBCO-BRL). For transient transfection assays, cells were plated at 1 \(\times\) 10\(^6\) cells/60-mm dish 24 h before transfection and growth in 5% CO\(_2\) in MEM-ES. Cells were incubated with 10 \(\mu\)g/ml lipofectin (GIBCO-BRL) and cultured overnight in serum-free MEM-ES culture medium with 0.1, 1, or 10 \(\mu\)g of plasmid DNA. After overnight incubation with lipofectin, fresh culture medium containing 10% FBS was added. PTHrP treatment assays were performed within 48 h after transfection.

PDG-98059 (Biomol), wortmannin (Sigma Canada), B108 (Essal Research Institute, Anover MA), H-89 (Biomol), and chelerythrine chloride (Biomol) were dissolved in dimethyl sulfoxide and stored at appropriate stock concentrations and were diluted to the desired concentrations immediately before use.

The plasmid encoding the \(\alpha_\text{Ga} \) triple mutant was obtained from the laboratory of Dr. H. R. Bourne (University of California, San Francisco, CA) and has been previously described (13).

*Northern blot analysis.* Total cellular RNA was extracted by TRIzol extraction from control and experimental cells after treatment with vehicle alone, PTHrP-(1–34) alone, or graded concentrations of the chemical inhibitors. Ten micrograms of total cellular RNA were electrophoresed on a 1.1% agarose-formaldehyde gel, transferred to a nylon membrane (Nytran; S&S, Keene, NH) by capillary blotting, and then incubated with lipofectin, fresh culture medium containing 10% FBS was added. PTHrP treatment assays were performed within 48 h after transfection.

Fig. 2. Effect of protein kinase C (PKC) inhibitor chelerythrine chloride (Ch Cl) on type I collagen gene expression in MG-63 cells. Top: MG-63 cells were grown in 10% serum to 80% confluence and then incubated overnight in serum-free conditions (Ctl). Cells were then pretreated with vehicle or Ch Cl for 1 h. Cells were then treated with 100 nM PTHrP-(1–34) for 8 h. Ctl represents the results of treatment with vehicle only for both the pretreatment and treatment periods. Levels of type I collagen and ratios of type I collagen to 18S mRNA were determined by Northern blot analysis, as described in MATERIALS AND METHODS. Bottom: results depict the ratios of type I collagen to 18S mRNA and are expressed as % change relative to Ctl, which was assigned a value of 100%. Bars represent means ± SE of 6 different experiments. *Significant differences in the ratios from Ctl cells (\(P < 0.05\)); **significant differences in the ratios from PTHrP-only-treated cells (\(P < 0.05\)).
0.05. The mean, SE, and $P$ value measurements were performed using Excel software (Microsoft, Port Redmond, WA).

RESULTS

Effect of PTHrP on indexes of osteoblast differentiation. MG-63 osteoblast-like osteosarcoma cells, which exhibit characteristics of early, immature osteoblasts, were incubated in the absence or presence of 100 nM PTHrP-(1–34) for 0, 1, 3, 6, 8, 12, and 16 h. As shown in Fig. 1A, treatment with PTHrP-(1–34) increased type I collagen mRNA transcript levels within 6 h, and peak levels were reached after 8 h of treatment. Type I collagen levels were augmented more than twofold at this time point compared with control, untreated cells. Type I collagen levels decreased to basal by $\sim$16 h. A second marker of osteoblast differentiation, alkaline phosphatase, was detected by histochemical reaction.

After treatment of cells with 100 nM PTHrP-(1–34) for 6, 12, and 24 h, cells were fixed, and alkaline phosphatase activity was detected by a histochemical reaction. As seen in Fig. 1, B and C, PTHrP induced alkaline phosphatase activity, with highest levels occurring at 24 h. Although staining density increased slightly in untreated cells, the overall increase in staining in treated cells was considerably greater, reaching levels as high as sevenfold after 12 h of treatment. These results indicate that PTHrP can induce indexes of differentiation in MG-63 osteoblastic cells.

Effects of inhibiting PKC on indexes of osteoblast differentiation. To determine the involvement of PKC in PTHrP-mediated MG-63 cell differentiation, cells were pretreated with chelerythrine chloride (3, 7), a specific inhibitor of PKC, followed by coincubation with 100 nM PTHrP-(1–34) for 8 h, the time of maximal
induction of type I collagen transcript levels by PTHrP treatment. As seen in Fig. 2, inhibition of PKC by chelerythrine chloride treatment resulted in a decrease in PTHrP-stimulated type I collagen levels in a dose-dependent manner. Cell morphology (Fig. 3), viability as determined by trypan blue dye exclusion (>95% viable), and basal levels of type I collagen mRNA were unaffected by treatment of MG-63 cells with chelerythrine chloride alone. These results were paralleled by a reduction in alkaline phosphatase levels. Pretreatment with 5.0 μM chelerythrine chloride followed by coincubation with 100 nM PTHrP-(1–34) for 24 h resulted in alkaline phosphatase levels that were comparable to levels in untreated cells cultured for the same time period (Fig. 3). The ability of this inhibitor to curtail the effects of PTHrP on MG-63 cell differentiation suggests that PKA activation is involved in this phenomenon.

Effects of inhibiting PKA on indexes of osteoblast differentiation. In a number of cell types, PTHrP is known to be a strong activator of adenylate cyclase via its stimulation of Gαs. The cAMP generated then leads to activation of PKA. To determine whether activation of PKA is involved in PTHrP-induced MG-63 cell differentiation, cells were pretreated with the PKA inhibitor H-89. After incubation in serum-free medium for ~16 h, cells were pretreated with H-89 at 15 and 30 μM concentrations for 1 h, followed by coincubation with 100 nM PTHrP-(1–34). Inhibition of PKA by H-89 treatment resulted in a dose-dependent decrease in PTHrP-stimulated type I collagen mRNA levels (Fig. 4). The specificity of this response was confirmed by the treatment of MG-63 cells with H-89 alone, which showed little to no effect on basal type I collagen mRNA levels. Cell viability by trypan blue dye exclusion (>94% viable) and morphology (Fig. 3) were unaffected by H-89 at the doses indicated. These results were paralleled by a reduction in alkaline phosphatase levels. Pretreatment with 30 μM H-89 for 1 h, followed by coincubation with 100 nM PTHrP-(1–34) for 24 h, resulted in levels of alkaline phosphatase that were comparable to those in untreated cells (Fig. 3). The ability of this inhibitor to curtail the effects of PTHrP on MG-63 cell differentiation suggests that PKA activation is involved in this phenomenon.

Effects of a Gαs mutant on indexes of osteoblast differentiation. To confirm results obtained regarding PTHrP signaling via Gαs in PTHrP-mediated osteoblast differentiation, MG-63 cells were transiently transfected with a Gαs triple mutant. The Gαs mutant used in our studies is designed to stabilize a receptor-Gαs-βγ-complex, effectively blocking signaling from both Gαs and βγ. After transient transfection, MG-63 cells were treated with 100 nM PTHrP-(1–34) for 8 h. As seen in Fig. 5, in cells transfected with the Gαs mutant, PTHrP stimulation of type I collagen mRNA levels was significantly reduced, confirming the involvement of Gαs in PTHrP-stimulated osteoblast differentiation and suggesting the possible involvement of βγ-subunits.

![Image](https://example.com/image.png)

**Fig. 4.** Effect of the PKA inhibitor H-89 on type I collagen gene expression in MG-63 cells. Top: MG-63 cells were grown in 10% serum to 80% confluence and incubated overnight in serum-free conditions. Cells were then pretreated with vehicle or H-89 for 1 h and then incubated with 100 nM PTHrP-(1–34) for 8 h. Ctl represents results of treatment with vehicle only for both the pretreatment and treatment periods. Levels of type I collagen and ratios of type collagen to 18S mRNA were determined by Northern blot analysis, as described in MATERIALS AND METHODS. Bottom: results depict the ratios of type I collagen to 18S mRNA and are expressed as %change relative to Ctl, which was assigned a value of 100%. Bars represent means ± SE of 3 different experiments. *Significant differences in the ratios from Ctl cells (P < 0.05); **significant differences in the ratios from PTHrP-only-treated cells (P < 0.05).

Effects of inhibiting PI 3-kinase on indexes of osteoblast differentiation. To explore signal transduction components related to the βγ-complex that might be implicated in PTHrP-induced osteoblast differentiation, we examined the involvement of PI 3-kinase by using the chemical inhibitor wortmannin. In preliminary experiments, wortmannin was confirmed to have no effects on cellular viability by trypan blue dye exclusion (>97%) or morphology (Fig. 3) at the doses indicated. Cells were pretreated with wortmannin for 3 h, followed by coincubation with 100 nM PTHrP-(1–34). Type I collagen mRNA levels were minimally affected in cells treated with wortmannin alone. The ability of wortmannin to cause a dose-dependent decrease in PTHrP-stimulated type I collagen levels (Fig. 6) suggests that PI 3-kinase is a component of the PTHrP-induced signaling involved in MG-63 cell differentiation. This was confirmed by the ability of wortmannin to abrogate the PTHrP-mediated increase in alkaline phosphatase activity after 24 h of coincubation (Fig. 3). Alkaline phosphatase activity in the wortmannin-treated cells is comparable to basal, untreated cells (Fig. 4). Taken together, these results suggest the involvement of PI 3-kinase in PTHrP-mediated osteoblast differentiation.

*AJP-Endocrinol Metab • VOL 281 • SEPTEMBER 2001 • www.ajpendo.org*
Effects of Ras inhibition on indexes of osteoblast differentiation. Ras proteins are a major point of convergence of numerous signal transduction pathways. Ras is required to be anchored to the plasma membrane to function and must undergo the posttranslational addition of a farnesyl group, which facilitates Ras insertion into the plasma membrane (28, 36). We therefore examined the effects of B1086, which is an inhibitor of the enzyme farnesyltransferase and therefore inhibits Ras activity. In preliminary experiments, B1086 was confirmed to have no effects of cellular viability as assessed by trypan blue dye exclusion (>97%) or morphology (Fig. 3) at the doses indicated. Overnight pretreatment of MG-63 cells with B1086 was followed by 8 h of coincubation with 100 nM PTHrP-(1–34). Total RNA was collected and subjected to Northern blot analysis. Type I collagen mRNA levels were significantly decreased in B1086-treated cells (Fig. 7), whereas type I collagen mRNA levels were little affected in cells treated with B1086 alone. Histochernical examination of alkaline phosphatase activity after identical treatment conditions also revealed a significant decrease in this parameter in B1086-treated cells (Fig. 3).

Effects of MAPK inhibition on indexes of osteoblast differentiation. The MAPK family of serine/threonine kinases includes extracellular signal-regulated kinases (ERKs). Activation of the ERK group of MAP kinases may occur via Ras and may involve stimulation of the enzyme MEK, or MAPK kinase. MEK activates ERK MAPK directly and thus serves as a point of control in that selective inhibition by the chemical inhibitor of
MEK, PD-98059 (2, 23), results in inhibition of MAPK. To determine the involvement of MAPK in the PTHrP-mediated induction of type I collagen expression, MG-63 cells were incubated overnight in serum-free conditions. Cells were pretreated with vehicle or PD-98059 (5 μM, 10 μM) for 16 h. The cells were then treated with 100 nM PTHrP for 8 h. Ctl represents results of treatment with vehicle only for both the pretreatment and treatment periods. Levels of type I collagen and ratios of type I collagen to 18S mRNA were determined by Northern blot analysis as described in MATERIALS AND METHODS. Bottom: results depict the ratios of type I collagen to 18S mRNA and are expressed as % change relative to Ctl, which was assigned a value of 100%. Bars represent means ± SE of 3 different experiments. *Significant differences in the ratios from Ctl cells (P < 0.05); **significant differences in the ratios from PTHrP-only-treated cells (P < 0.05).

Fig. 7. Effect of the Ras inhibitor B1086 on type I collagen gene expression MG-63 cells. Top: MG-63 cells were grown in 10% serum to 80% confluence and then incubated overnight in serum-free conditions. Cells were pretreated with vehicle or Ras inhibitor B1086 (5 μM, 10 μM) for 16 h. The cells were then treated with 100 nM PTHrP for 8 h. Ctl represents results of treatment with vehicle only for both the pretreatment and treatment periods. Levels of type I collagen and ratios of type I collagen to 18S mRNA were determined by Northern blot analysis as described in MATERIALS AND METHODS. Bottom: results depict the ratios of type I collagen to 18S mRNA and are expressed as % change relative to Ctl, which was assigned a value of 100%. Bars represent means ± SE of 3 different experiments. *Significant differences in the ratios from Ctl cells (P < 0.05); **significant differences in the ratios from PTHrP-only-treated cells (P < 0.05).

We next investigated effects of the various inhibitors of upstream signaling on the ability of PTHrP to induce peak MAPK activity after 15 min of treatment (Fig. 10). All five inhibitors inhibited the ability of PTHrP to induce MAPK activity, thereby confirming the role of MAPK as a downstream target of these signaling pathways.

DISCUSSION

Previous studies in our laboratory and others have demonstrated the capacity of PTH and PTHrP to affect the differentiation of a number of cell types, including osteoblasts. The current evidence indicates that PTHrP can act as an autocrine or paracrine growth and/or differentiation factor in a number of tissues (40–42). The existence of both PTHrP and the PTH/PTHrP receptor in bone and the ability of a variety of levels of MBP phosphorylation by MAPK were observed at 15 min, with activity remaining significantly elevated at 20 and 25 min. By 30 min, levels of MBP phosphorylation had returned to basal, untreated levels.

Fig. 8. Effect of the MAPK kinase (MEK) inhibitor PD-98059 on type I collagen (α1 collagen) gene expression in MG-63 cells. Top: MG-63 cells were grown in 10% serum to 80% confluence and then incubated overnight in serum-free conditions. Cells were pretreated with vehicle or PD-98059 (50 μM, 100 μM) for 16 h, followed by PTHrP treatment for 8 h. Ctl represents results of treatment with vehicle only for both the pretreatment and treatment periods. Levels of type I collagen and ratios of type I collagen to 18S mRNA were determined by Northern blot analysis, as described in MATERIALS AND METHODS. Bottom: results depict the ratios of type I collagen to 18S mRNA and are expressed as % change relative to Ctl, which was assigned a value of 100%. Bars represent means ± SE of 3 different experiments. *Significant differences in the ratios from Ctl cells (P < 0.05); **significant differences in the ratios from PTHrP-only-treated cells (P < 0.05).

E495

AJP-Endocrinol Metab • VOL 281 • SEPTEMBER 2001 • www.ajpendo.org

by 10.220.33.1 on April 19, 2017 http://ajpendo.physiology.org/ Downloaded from
bone-derived cells to produce PTHrP both in culture and in vivo strongly suggest a local role in bone. Evidence for such a role was also provided by the effects of targeted disruption of the PTHrP gene in mice (19). Thus mice heterozygous for the null mutation displayed haploinsufficiency and decreased bone volume as the mice aged. Such observations, confirmed by targeted overexpression of the same gene (38), suggest that PTHrP may modulate the maturation and differentiation of osteoblasts.

Treatment of MG-63 cells with 100 nM PTHrP-(1–34) was seen to induce increased expression of the osteoblast differentiation markers type I collagen and alkaline phosphatase. Although production of type I collagen is not exclusive to the differentiating osteoblast but is also produced by fibroblastic cells, type I collagen is considered a useful osteoblast differentiation marker when expressed in an established sequence with other bone markers such as alkaline phosphatase (4). Both catabolic and anabolic effects of PTHrP and PTH may be observed in bone via the PTH/PTHrP receptor (8). Ishizuya et al. (14) have shown that these discrepancies appear to be a function of exposure time, such that intermittent exposure of cells to PTHrP-(1–34) of ~6 h will cause osteoblast differentiation. Treatment of MG-63 osteoblastic cells with PTHrP-(1–34) were consistent with these observations, in that levels of type I collagen mRNA were seen to rise at ~6 h, peak at 8 h, and begin to decline with extended exposure time. These results suggest that there may exist an inhibitory effect of prolonged exposure of osteoblasts to PTHrP. Subsequent experiments were therefore performed with 8-h incubation periods in keeping with these observations. The ability of the MG-63 cells to withstand prolonged exposure to each inhibitor was first verified by trypan blue dye exclusion viability experiments as well as by close observation for any adverse changes in cell morphology. It seems possible that receptor desensitization due to internalization did occur during this time interval, but this was not examined, and signaling events leading to eventual differentiation would appear to have been adequately initiated during the incubation times that were employed.

It is known that PTH/PTHrP receptor activation can lead to activation of multiple G proteins, namely Goq and Gαs, with subsequent activation of phospholipase C (PLC) and adenylyl cyclase (25), respectively. Stimulation of PLC will result in subsequent production of inositol 1,4,5-trisphosphate and diacylglycerol (1, 2, 39), leading to mobilization of calcium and PKC, respectively. Although there are varying reports regarding the involvement of PLC in osteoblast differentiation, we have seen that inhibition of PKC by treatment of MG-63 cells with PTHrP both in culture and in vivo strongly suggest a local role in bone. Evidence for such a role was also provided by the effects of targeted disruption of the PTHrP gene in mice (19). Thus mice heterozygous for the null mutation displayed haploinsufficiency and decreased bone volume as the mice aged. Such observations, confirmed by targeted overexpression of the same gene (38), suggest that PTHrP may modulate the maturation and differentiation of osteoblasts.

Treatment of MG-63 cells with 100 nM PTHrP-(1–34) was seen to induce increased expression of the osteoblast differentiation markers type I collagen and alkaline phosphatase. Although production of type I collagen is not exclusive to the differentiating osteoblast but is also produced by fibroblastic cells, type I collagen is considered a useful osteoblast differentiation marker when expressed in an established sequence with other bone markers such as alkaline phosphatase (4). Both catabolic and anabolic effects of PTHrP and PTH may be observed in bone via the PTH/PTHrP receptor (8). Ishizuya et al. (14) have shown that these discrepancies appear to be a function of exposure time, such that intermittent exposure of cells to PTHrP-(1–34) of ~6 h will cause osteoblast differentiation. Treatment of MG-63 osteoblastic cells with PTHrP-(1–34) were consistent with these observations, in that levels of type I collagen mRNA were seen to rise at ~6 h, peak at 8 h, and begin to decline with extended exposure time. These results suggest that there may exist an inhibitory effect of prolonged exposure of osteoblasts to PTHrP. Subsequent experiments were therefore performed with 8-h incubation periods in keeping with these observations. The ability of the MG-63 cells to withstand prolonged exposure to each inhibitor was first verified by trypan blue dye exclusion viability experiments as well as by close observation for any adverse changes in cell morphology. It seems possible that receptor desensitization due to internalization did occur during this time interval, but this was not examined, and signaling events leading to eventual differentiation would appear to have been adequately initiated during the incubation times that were employed.

It is known that PTH/PTHrP receptor activation can lead to activation of multiple G proteins, namely Goq and Gαs, with subsequent activation of phospholipase C (PLC) and adenylyl cyclase (25), respectively. Stimulation of PLC will result in subsequent production of inositol 1,4,5-trisphosphate and diacylglycerol (1, 2, 39), leading to mobilization of calcium and PKC, respectively. Although there are varying reports regarding the involvement of PLC in osteoblast differentiation, we have seen that inhibition of PKC by treatment
with chelerythrine chloride can block induction of type I collagen and alkaline phosphatase. Stimulation of the PTH/PTHrP receptor also results in activation of adenylate cyclase followed by a rapid increase in intracellular cAMP and subsequent activation of PKA. We show that the inhibition of adenylate cyclase, by treatment with H-89, results in an abrogation of the PTHrP-(1–34)-stimulated increase in type I collagen mRNA expression and also interrupts the production of the later marker of MG-63 cell differentiation, alkaline phosphatase.

Although activation of PKA and PKC is known to occur in minutes, the ability of these short-term signals to influence such a late-developing cellular phenomenon as differentiation is well documented in the literature. Previous studies by Tsai et al. (35) show that 100 nM PTHrP induces cAMP in UMR106 osteosarcoma cells in minutes, with subsequent occurrence of differentiation at later time points. In several studies investigating the effects of PTH on osteoblast activity, the PKC and PKA signaling pathways appear to be activated simultaneously and seem to cooperate in the anabolic effects of the shared receptor in bone cells. Cross talk between these two signal transduction systems may therefore occur in osteoblasts after PTH stimulation. Further studies to elucidate this possibility were also undertaken by combined inhibitor treatment of MG-63 cells, but these show additive, and not synergistic, effects (data not shown).

Although it has been well established that the PTH/PTHrP receptor can signal through both Goα and Goq (1), the question of PTHrP signal transduction via βγ-subunit-dependent pathways remains to be definitively answered. The ability of the transient transfection of a Goα mutant (29), which sequesters βγ-subunits, to abrogate the effects of PTHrP treatment on osteoblast differentiation markers may implicate βγ-signaling. However, because the dominant negative Goα (8) mutant utilized also inhibits signaling via the endogenous Goα subunit, the results obtained from our experiment may have simply confirmed results obtained by inhibition of adenylate cyclase by H-89. Nevertheless, data obtained by chemical inhibition of PI 3-kinase are consistent with a role for the βγ-subunits in mediating some of the effects of PTHrP. Further studies in which βγ-subunits are selectively inhibited will be required, but such studies are currently technically challenging.

PI 3-kinase is a heterodimeric cytosolic protein composed of an 85-kDa regulatory subunit and a 110-kDa catalytic subunit (9). PI 3-kinase is stimulated by both G protein-coupled receptors and receptor tyrosine kinases. Stephens et al. (31) first discovered that PI 3-kinase is specifically activated by purified βγ-subunits. The ability of the chemical inhibitor of PI 3-kinase, wortmannin, to cause a significant reduction in the expression of both osteoblast differentiation markers in our studies suggests that PI 3-kinase is involved in PTHrP-mediated MG-63 cell differentiation. Because PI 3-kinase inhibition is seen to decrease βγ-dependent MAPK activation, it is commonly presumed that βγ-subunits activate PI 3-kinase and thus initiate a cascade of events that leads to the phosphorylation of the protein Shc. Whether Rac or other GTP-exchange proteins are involved in PI 3-kinase-mediated pathways was not tested. Nevertheless, the interaction of βγ-subunits with SH2-containing proteins can be mediated by PI 3-kinase, such that Shc complexes to the SH2-containing adaptor protein Grb2. Grb2 can then stably associate with the Ras guanine nucleotide ex-
change factor Sos. Ras is anchored to the plasma membrane, where Sos can stimulate the exchange of GTP, thereby leading to Ras activation. We have found that chemical inhibition of Ras by treatment with B1086, a farnesyltransferase inhibitor, also diminished the effect of PTHrP-(1–34) on induction of both type I collagen levels and alkaline phosphatase levels.

One of the best-characterized downstream targets of Ras is the family of MAPKs (20). MAPKs are a family of serine/threonine kinases involved in the transduction of cellular signals to the nucleus. These proteins regulate a vast range of cellular processes including proliferation, differentiation, transformation, inflammation, apoptosis, and cytoskeletal rearrangement. Previous data also support the pivotal role of MAPKs in the induction of phenotypic indexes of osteoblast differentiation (34, 43). Upstream of MAPK, the activation of Ras will lead to the sequential activation of Raf1, a serine/threonine kinase, and MEK, whose substrates are the ERK1 and ERK2 of the MAPK family. It has also been shown that activation of MAPK can occur independently of Ras activation, presumably via direct activation of Raf or MEK by PKA (37) or PKC. Treatment of MG-63 cells with the MAPK inhibitor PD-98059 interrupted PTHrP stimulation of indexes of osteoblast differentiation, thereby suggesting that MAPK is involved in this process. The ability of PTHrP to induce MAPK activity in MG-63 osteoblastic cells complements the results of the studies on the effect of PD-98059 on PTHrP induction of markers of differentiation. Furthermore, the capacity of multiple inhibitors of upstream signaling to diminish MAPK activity suggests that these multiple signaling pathways converge to some extent at MAPK. Whether other members of the MAPK family, such as p38K or c-Jun NH2-terminal protein kinase, are also involved in PTHrP signaling remains to be determined.

It is probable, then, that PTH/PTHrP activation in response to PTHrP initiates parallel signaling via Goα leading to PKA activation, via Goq leading to activation of PKC, and via Gβγ leading to activation of PI3-kinase and Ras (Fig. 11). Convergence of these signals may then occur via activation of MAPK. Further study is now required to extend these observations to other osteoblastic cell models and to determine whether other kinases, such as the stress-activated kinases, play a role in PTHrP-mediated osteoblast differentiation. The elucidation of the signaling pathways involved in the effects of PTHrP on enhancing differentiation of osteoblastic cells clearly has important implications with regard to our understanding of the actions of this hormone in normal physiology and particularly with respect to its anabolic actions in bone.

REFERENCES


23. Mayer AM, Brenic S, and Glaser KB. Pharmacological target- ing of signaling pathways in protein kinase C-stimulated superoxide generation in neutrophil-like HL-60 cells: effect of phosphol ester, arachidonic acid and inhibitors of kinase(s), phosphatase(s) and phospholipase A2. J Pharmacol Exp Ther 279: 633–644, 1996.