Parathyroid hormone (PTH) down-regulates PTH/PTH-related protein receptor gene expression in UMR-106 osteoblast-like cells via a 3′,5′-cyclic adenosine monophosphate-dependent, protein kinase A-independent pathway

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Abstract

Parathyroid hormone (PTH) regulates osteoblast function via a G protein-linked PTH/PTH-related protein (PTHrP) receptor. We have studied the mechanisms of PTH/PTHrP receptor gene repression by PTH in UMR-106 osteoblast-like cells. Inhibition of PTH/PTHrP receptor mRNA expression by rat (r) PTH(1–34) and Insulin-like growth factor-I (IGF-I) at 10−8 M was significant at 1 h and 3 h, and maximal at 2 h and 6 h. A maximal decrease in receptor mRNA abundance by rPTH(1–34) and IGF-I was maintained for 24 h. Inhibition of receptor gene expression by rPTH(1–34) was mimicked in UMR-106 cells by the addition of forskolin (an adenylyl cyclase activator), or 8-(4-chlorophenylthio)-adenine 3′,5′-cyclic monophosphate (8-pCPTcAMP; a cAMP analogue). Although H89, a selective protein kinase A (PKA) inhibitor, completely inhibited PKA activity stimulated by rPTH(1–34) and IGF-I was maintained for 24 h. Inhibition of receptor gene expression by rPTH(1–34) was mimicked in UMR-106 cells by the addition of forskolin (an adenylyl cyclase activator), or 8-(4-chlorophenylthio)-adenine 3′,5′-cyclic monophosphate (8-pCPTcAMP; a cAMP analogue). Although H89, a selective protein kinase A (PKA) inhibitor, completely inhibited PKA activity stimulated by rPTH(1–34), forskolin or 8-pCPTcAMP, suppression of PTH/PTHrP receptor mRNA synthesis induced by these substances in UMR-106 cells was not affected by H89. In primary osteoblast cultures, rPTH(1–34) inhibited synthesis of PTH/PTHrP receptor mRNA irrespective of H89. The down-regulation effect of rPTH(1–34) was also unaltered by PD98059 (an extracellularly regulated kinase 1/2 mitogen-activated protein kinase pathway inhibitor). Pretreatment with cycloheximide, a protein synthesis inhibitor, did not alter the inhibition of PTH/PTHrP receptor mRNA expression by rPTH(1–34), indicating that receptor mRNA suppression does not require new protein synthesis. Transcriptional activation of PTH/PTHrP receptor gene promoter (U3P or U4P)–luciferase constructs was decreased by rPTH(1–34), forskolin and 8-pCPTcAMP irrespective of H89. Thus, PTH transcriptionally down-regulates PTH/PTHrP receptor gene expression in osteoblast-like cells via a cAMP-dependent, PKA-independent pathway.

Introduction

Parathyroid hormone (PTH) maintains calcium ion homeostasis in humans and other animals. PTH-related protein (PTHrP) is a humoral factor produced by various tumors as well as many normal cells and tissues, including keratinocytes, the lactating mammary gland and chondrocytes (Philbrick et al. 1996, Jüppner et al. 1999, Strewler & Nissenson 1999). PTH and PTHrP have both catabolic and anabolic actions in bone. These polypeptides bind to a G protein–coupled receptor, designated the PTH/PTHrP receptor, in target cells such as osteoblasts, chondrocytes and renal tubular cells (Jüppner et al. 1988, Coleman et al. 1994, Vortkamp et al. 1996).

The PTH/PTHrP receptor is a member of a family of seven transmembrane receptors coupled to heterotrimeric G proteins (Jüppner et al. 1991). Binding of PTH or PTHrP to this receptor induces the accumulation of several intracellular second messengers including cAMP, calcium ion and diacylglycerol, activating to protein kinase A (PKA) and protein kinase C (PKC) pathways (Dunlay & Huska 1990, Civitelli et al. 1992, Fukayama et al. 1994). Activation of these second messenger systems alters gene transcription in osteoblasts. For example, PTH not only stimulates the expression of many mRNAs such as those encoding collagenses and receptor activator of nuclear factor-κB ligand, but also suppresses the transcription of several genes, including alkaline phosphatase, type I

Mechanisms involved in the desensitization of cellular responsiveness to PTH have been described in osteoblastic cells, mainly representing decreases in receptor expression on the cell surface (down-regulation). For the PTH/PTHrP receptor, down-regulation results from lower rates of receptor biosynthesis (Gonzalez & Martin 1996, Jongen et al. 1996, Kawane et al. 2001) or accelerates clearance of functional cell surface receptors via their internalization and subsequent proteolysis (Huang et al. 1995, Ferrari et al. 1999, Tawfeek et al. 2001, 2002). Prolonged exposure to PTH and other substances such as insulin-like growth factor-I (IGF-I) and 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) lowers the amounts of PTH/PTHrP receptor mRNA in osteoblastic cells (Gonzalez & Martin 1996, Jongen et al. 1995, 1996, Kawane & Horiiichi 1999, Kawane et al. 2001). Down-regulation of the cAMP response to PTH, and also PTH/PTHrP receptor down-regulation has been observed in primary cultures of osteoblasts (Jongen et al. 1995, 1996) and in various osteoblastic cell lines (Mitchell & Goltzman 1990, Fukayama et al. 1994, Gonzalez & Martin 1996). In these investigations, desensitization involved elevation of intracellular cAMP concentration accompanied by down-regulation of the receptor. PTH has been found to decrease the abundance of PTH/PTHrP receptor mRNA in osteoblasts (Fukayama et al. 1994, Gonzalez & Martin 1996, Jongen et al. 1996, Kawane et al. 2001); prolonged activation of the PKA signaling pathway caused down-regulation of receptor expression (Fukayama et al. 1994). However, Jongen et al. (1996) suggested that other signaling pathways were also involved since bovine PTH(1–34), an analogue that does not stimulate the cAMP/PKA pathway but rather increases PKC activity, downregulated receptor mRNA abundance in osteoblasts. However, the potency of bovine PTH(1–34) in inducing down-regulation was much weaker than that of the full agonist, bovine PTH(1–34). Although diverse mechanisms of homologous down-regulation of PTH/PTHrP receptor mRNA are present in osteoblasts, receptor mRNA down-regulation is strongly dependent upon cAMP (Mitchell & Goltzman 1990, Fukayama et al. 1994, Jongen et al. 1996).

We have previously demonstrated that PTH suppresses PTH/PTHrP receptor gene transcription, identifying the receptor gene sequence most important for repression of PTH (Kawane et al. 2001). The present study was undertaken to understand the events, following PTH binding to the PTH/PTHrP receptor, that bring about the suppression of receptor gene expression in UMR–106 rat osteoblast-like cells. We have demonstrated that PTH transcriptionally inhibited PTH/PTHrP receptor gene expression via a cAMP-mediated pathway that was independent of PKA.

Materials and Methods

Cell cultures

Rat osteoblast-like osteosarcoma UMR–106 cells (ATCC; CRL 1661) were grown by routine methods in monolayer culture at 37 °C in an atmosphere of 5% CO2/95% air. Dulbecco’s modified Eagle’s medium (DMEM; ICN Pharmaceuticals, Costa Mesa, CA, USA) supplemented with 5% fetal bovine serum (FBS; Filtron, Brooklyn, Australia) was used. Cell cultures were passed once per week. At 70% confluence, the culture medium was replaced for 24 h with serum-free medium containing 0.1% bovine serum albumin (BSA). Cells were then exposed to a test substance such as 100 nM rat (r) PTH(1–34) (Peninsula Laboratories, Belmont, CA, USA), human PTH(3–34) (Peptide Institute, Osaka, Japan), 100 nM human IGF-I (Austral Biologicals, San Roman, CA, USA), 100 µM 8-(4-chlorophenylthio)-adenine 3’,5’-cyclic monophosphate (8-pCPTcAMP; Alexis Biochemicals, San Diego, CA, USA), 10 nM forskolin (Sigma Chemical Co., St Louis, MO, USA) and phorbol-12-myristate-13-acetate (TPA; Sigma) in DMEM with 0.1% BSA for the duration indicated. In studies of inhibitors, serum-deprived cells were treated with 10 µM H89 (Seikagaku Corp., Tokyo, Japan), a PKA inhibitor, 100 µM PD98059 (Alexis Biochemicals), an extracellularly regulated kinase 1/2 (ERK1/2) mitogen-activated protein (MAP) kinase pathway inhibitor or 35 µM cycloheximide (Sigma), a protein synthesis inhibitor, for 1 h preceding incubation for various time-periods with 10−7 M rPTH(1–34) (Kawane & Horiiichi 1999, Kawane et al. 2001).

Measurement of PKA activity

UMR–106 cells, plated at a density of 4 × 104 cells/cm2 in 96-well tissue culture clusters, were grown in DMEM containing 5% FBS for 24 h. Cells then were cultured in serum-free DMEM for 18 h and treated with test agents. H89 (10 µM) was pretreated for 1 h before the addition of test agents. After stimulation by test agents such as 100 nM rPTH(1–34), 10 nM forskolin or 100 µM 8-pCPTcAMP for the period indicated, the medium was aspirated and the cells were incubated with 40 µl assay mixture consisting of a salt solution (20 mM HEPES (pH 7.2), 137 mM NaCl, 5.4 mM KCl, 0.3 mM Na2HPO4, 0.4 mM KH2PO4, 10 mM MgCl2, and 20 mM Na2VO4), supplemented with 25 mM sodium β-glycerophosphate, 1 mg/ml digitonin, 5 mM EGTA, 10 µM ATP, [γ-32P]ATP (148 TBq/mmol; ICN Pharmaceuticals) and 5 µg kemptide (Sigma) as substrate, with or without 50 ng protein kinase inhibitor.
(Sigma) as the inhibitory peptide. After a 10-min incubation at 30 °C, the reaction was stopped by placing the reaction mixture on ice and adding 10 µl 25% (w/v) trichloroacetic acid. The soluble reaction mixture was spotted onto phosphocellulose filters (Whatman P-81; Clifton, NJ, USA), which were subsequently washed three times with 75 mM H₃PO₄. Radioactivity trapped on the filters was determined by liquid scintillation counting. The difference in incorporated radioactivity values was defined as PKA activity (Verheijen & Defize 1995).

Determination of PTH/PTHrP receptor mRNA

To assess the effect of rPTH(1–34) on the abundance of PTH/PTHrP receptor mRNA, Northern blot analysis was performed as previously described (Kawane & Horiuchi 1999). Briefly, total RNA was extracted from cells using guanidine thiocyanate. Total RNA was fractionated electrophoretically on a 1·2% agarose gel containing formaldehyde and transferred onto nylon membranes. Membranes were hybridized with the PTH/PTHrP receptor cDNA labeled with [α-32P]dCTP. Hybridization was carried out for 2 days at 42 °C, followed by washing with 0·1–2 × SSPE containing 0·1% SDS at 65 °C. For standardization, blots were rehybridized with a cyclophilin cDNA probe. Signal intensity on membranes was quantitated by a Molecular Imager FX (BioRad, Hercules, CA, USA) equipped with Quantity One 4·1·1 image analysis software (BioRad).

Transient transfections and reporter assays

Promoter constructs used in transfaction assays were assembled as previously described (Kawane et al. 2001). Briefly, promoter constructs used in transfaction assays were derived from rPRP12BB (an approximately 7 kb clone of PTH/PTHrP receptor promoter containing exons U1, U2 and U3) and rPRP3BN (an approximately 1·3 kb clone of the receptor promoter containing exon U4) (refer to Fig. 7, left panel). The XhoI/BamH1 and BglII/BamH1 fragments from rPRB122BB were subcloned into the XhoI/BglII site and the BglII site of the pGL3 basic vector respectively. Transient transfections were performed with UMR-106 cells grown to 70% confluence in six-well plates. Cells were cotransfected with 0·5 µg luciferase-reporter plasmid and 0·5 µg β-galactosidase expression vector (pEFBOS-LacZ) as an internal control (Takahata et al. 1998), using GenePorter transfection reagent (Gene Therapy System, San Diego, CA, USA), for 3 h under serum-free conditions. Transfected cells were cultured in the medium containing 10% (final concentration) FBS for 3 h and thereafter the medium was changed into serum-free DMEM containing 0·1% BSA. Total RNA was extracted and subjected to Northern blot analysis for mRNAs encoding the PTH/PTHrP receptor (PTH1R) and cyclophilin (Cyclo.).

Statistical analysis

The data are presented as the means ± s.e.m. Statistical analysis was performed using analysis of variance followed by determination of Fisher’s protected least significant difference (StatView 4·02; Abacus Concepts, Berkeley, CA, USA). A P value less than 0·05 was considered to indicate statistical significance.

Results

Suppression of PTH/PTHrP receptor mRNA by rPTH(1–34) and IGF-I

We examined the effects of PTH on PTH/PTHrP receptor mRNA abundance in UMR-106 osteoblast-like cells. Northern blot analysis detected PTH/PTHrP receptor transcripts of approximately 2·5 kb. Their amounts decreased in a time-dependent manner after treatment at 10−7 M (Fig. 1). They were decreased as early as 1 h after initiation of treatment with rPTH(1–34). Inhibition was already maximal at 2 h and was maintained maximally for up to 24 h. A ligand inducing heterologous down-regulation of the PTH/PTHrP receptor, IGF-I (10−7 M), also reduced receptor mRNA abundance.
Down-regulation of receptor mRNA induced by IGF-I was significant at 3 h and maximal for 6–24 h (Fig. 2).

Involvement of cAMP on PTH/PTHrP receptor mRNA expression

To determine the mechanisms of PTH/PTHrP receptor mRNA suppression by rPTH(1–34), cells were incubated with rPTH(1–34), forskolin or 8-pCPTcAMP (Fig. 3). Homologous down-regulation of PTH/PTHrP receptor mRNA by rPTH(1–34) was mimicked by 8-pCPTcAMP ($10^{-4}$M), a cAMP analogue and forskolin ($10^{-8}$M), an adenylyl cyclase activator. In the presence of H89, an inhibitor of PKA, down-regulation of PTH/PTHrP receptor mRNA expression elicited by rPTH(1–34), forskolin or 8-pCPTcAMP was completely intact (Fig. 3a). When we measured PKA activities in UMR-106 cells, a clear increase was found to be induced by rPTH(1–34), forskolin or 8-pCPTcAMP; H89 abrogated PKA activities of the cells treated with vehicle, rPTH(1–34), forskolin or 8-pCPTcAMP (Fig. 3b). H89 inhibited PKA activities for up to 48 h in UMR-106 cells treated with vehicle, $10^{-7}$M rPTH(1–34), $10^{-8}$M forskolin or $10^{-8}$M 8-pCPTcAMP (data not shown).

Figure 4 depicts the effect of H89 on intracellular cAMP-mediated down-regulation of PTH/PTHrP receptor mRNA in primary cultures of osteoblasts. The reduction of PTH/PTHrP receptor mRNA induced by rPTH(1–34) persisted despite treatment with the PKA inhibitor, H89. Homologous down-regulation of PTH/PTHrP receptor mRNA expression was also induced by...
PKC activators (Jongen et al. 1996) and we further showed that down-regulation of PTH/PTHrP receptor mRNA expression induced by IGF-I was mediated via the ERK1/2 MAP kinase pathway (Kawane & Horiuchi 1999). Next, to determine whether MAP kinase was responsible for PTH-induced inhibition of PTH/PTHrP receptor mRNA synthesis, we added a highly selective inhibitor of the ERK1/2 MAP kinase pathway, PD98059. This inhibitor did not block the decrease in receptor mRNA induced by rPTH(1–34) in UMR-106 cells, but addition of the inhibitor completely abolished the down-regulation of receptor mRNA expression induced by PKC pathway activators such as human PTH(3–34) and TPA (Fig. 5).

**Transcriptional regulation of the PTH/PTHrP receptor gene by rPTH(1–34)**

To determine whether suppression of PTH/PTHrP receptor mRNA synthesis required protein synthesis in UMR-106 cells, the reduction of receptor gene expression by rPTH(1–34) was assessed in the presence of a protein synthesis inhibitor, cycloheximide. Cycloheximide (10 μg/ml) did not block PTH-induced inhibition of PTH/PTHrP receptor mRNA expression, demonstrating that de novo protein synthesis was not required to achieve inhibition (Fig. 6).

We next examined whether rPTH(1–34) suppressed the transcriptional activities of a PTH/PTHrP receptor gene. For this purpose, we constructed a restriction/genomic map of the rat PTH/PTHrP receptor gene including a 7 kb upstream flanking region with exons U1, U2 and U3, and a 1.3 kb downstream region with exon U4 (Fig. 7). The upstream constructs contained two promoters, U1P and U3P, and the downstream constructs included the U4 promoter for the receptor gene. When we assessed which promoter participated in repression of the gene by rPTH(1–34), the hormonal peptide markedly decreased the promoter activity of the construct (p1<sup>6812</sup> +103), which contained U1P and U3P (Fig. 7). Since the construct (p1<sup>3365</sup> +103), containing only the U3 promoter, showed inhibition of the promoter activity upon treatment with rPTH(1–34). Having identified U4P for the rat PTH/PTHrP receptor gene, we tested promoter activity in a construct (+104/+1334) containing it in the absence of other promoters. Here, promoter activity was highly inhibited by rPTH(1–34). Thus, U3P and U4P were involved in rPTH(1–34)-induced suppression of the PTH/PTHrP receptor gene (Fig. 7).

Finally, we tested the effect of cAMP and PKA on the receptor gene promoter construct (p1<sup>3365</sup> +103)
containing U3P (Fig. 8, left) and on the construct (+104/+1334) containing U4P (Fig. 8, right). Rat PTH(1–34), forskolin and 8-pCPTcAMP (the cAMP analogue) markedly suppressed U3 and U4 promoter activities, while treatment with H89 (the PKA inhibitor) did not affect suppression of the promoter activities induced by rPTH(1–34), forskolin or 8-pCPTcAMP. These results indicate that inhibition of the receptor gene promoter by PTH is mediated by intracellular cAMP followed by a PKA-independent pathway (Fig. 8).

Discussion

Desensitization of cells to PTH has been demonstrated in culture systems using bone-derived cells as well as in cells derived from other tissues (Mitchell & Goltzman 1990, Fukayama et al. 1994, Okano et al. 1994, Gonzalez & Martin 1996, Jongen et al. 1996, Guo et al. 1997). Inhibition of PTH/PTHrP receptor gene expression by PTH in osteoblastic cells is of a transcriptional nature (Fukayama et al. 1994, Gonzalez & Martin 1996). Recently we identified the PTH-suppressive region of PTH/PTHrP receptor gene in osteoblasts (Kawane et al. 2001). In the present study, we have demonstrated that the mechanism underlying PTH/PTHrP receptor gene repression by PTH in UMR–106 osteoblast-like cells is cAMP dependent but PKA independent.

PTH/PTHrP receptor expression is controlled not only by homologous down-regulation with pretreatment of PTH or PTHrP but also by unrelated agonists such as IGF-I (Goad & Tashjian 1993, Kawane & Horiuchi 1999), prostaglandin E1 (Civitelli et al. 1992), calcitonin (Guo et al. 1997) and 1,25(OH)2D3 (Gonzalez & Martin 1996, Xie et al. 1996), which act at different receptors. We have previously shown that heterologous desensitization to PTH in UMR–106 cells, as induced by IGF-I, results from reduced expression of the PTH/PTHrP receptor gene (Kawane & Horiuchi 1999). The present study compared the time-course of suppression of PTH/PTHrP receptor mRNA induced by PTH, the prototype agonist at the receptor, with that induced heterologously by IGF-I. More rapid down-regulation of receptor mRNA was achieved by rPTH(1–34) than by IGF-I. Furthermore, cycloheximide, a protein synthesis inhibitor, completely blocked the decrease in receptor mRNA expression induced by IGF-I (Kawane & Horiuchi 1999), but did not affect suppression of receptor mRNA by rPTH(1–34). These observations documented that de novo protein synthesis is not required for PTH-induced repression of receptor gene transcription in osteoblastic cells, but is necessary for IGF-I-induced repression (Kawane & Horiuchi 1999, Kawane et al. 2001). Our previous report indicated that IGF-I suppressed gene expression for PTH/PTHrP receptor in osteoblastic cells via the ERK1/2 MAP kinase pathway (Kawane et al. 2001), while PTH-induced down-regulation of receptor mRNA is mediated mainly by a different mechanism, specifically cAMP produced by adenylyl cyclase linked with the PTH/PTHrP receptor (Mitchell & Goltzman 1990, Jongen et al. 1996, Jüppner et al. 1999).

Much information concerning homologous down-regulation of the PTH/PTHrP receptor in osteoblastic cells has been obtained by receptor binding studies and by determination of PTH-induced accumulation of cAMP (Mitchell & Goltzman 1990, Fukayama et al. 1994, Gonzalez & Martin 1996, Jongen et al. 1996, Guo et al. 1997). We have demonstrated that homologous, PTH-induced down-regulation of PTH/PTHrP receptor mRNA can be observed in the UMR–106 osteosarcoma cell line. Primary cultures of osteoblasts may more accurately reflect conditions in vivo than experiments on a
clonal osteosarcoma cell line, so we also assessed the effect of PTH on the receptor mRNA in these osteoblasts. Treatment of primary osteoblast cultures with rPTH(1–34) rapidly suppressed expression of PTH/PTHrP receptor mRNA, indicating that this suppression is common to osteoblastic cells.

We examined the mechanisms underlying homologous down-regulation of receptor gene expression. PTH/PTHrP receptor can activate several second messenger pathways in the process of cAMP-mediated signaling (Abou-Samra et al. 1992). In fact, rapid down-regulation of PTH/PTHrP receptor mRNA was induced by treatment with a PKC activator such as the active phorbol ester TPA (Fukayama et al. 1994). Human PTH(3–34), an analogue that does not stimulate PKA (Fujimori et al. 1992), was able to down-regulate receptor gene

![Figure 7](image1.png)

**Figure 7** PTH-regulated promoter activities of U1P, U3P and U4P. Left: Schematic representation of deletion mutants of the 5’-flanking region nucleotides (from −7.0 kb to 1.3 kb) of the rat PTH/PTHrP receptor gene. Numbers in the name of each deletion mutant indicate their 5’- and 3’-ends. Restriction enzyme sites are indicated as Bam H1 (B), AatI (A), XhoI (X), EcoRV (EV) and NaeI (N). Right: Luciferase activity of the 5’-flanking region of the rat PTH/PTHrP receptor gene transfected into UMR-106 cells with or without 10⁻⁷M rPTH(1–34) (PTH). Luciferase activity is shown as units relative to the promoterless plasmid pGL-3 basic vector. Data from triplicate determinations are expressed as the means ± S.E.M.

![Figure 8](image2.png)

**Figure 8** Effect of a PKA inhibitor (H89) on transcriptional activity of the PTH/PTHrP receptor gene in UMR-106 cells. Cells were transfected with 1 µg PTH/PTHrP receptor reporter construct (−3365/+103) containing the promoter U3 promoter (left) or 1 µg of a construct (+104/+1334) containing U4 promoter (right), and cultured in the presence of vehicle, 10⁻⁷M rPTH(1–34), 10⁻⁸M forskolin or 10⁻⁴M 8-pCPT-cAMP, each with (solid bars) or without (open bars) H89 at 10⁻⁴M. Luciferase activity is shown as units relative to the promoterless plasmid pGL-3 basic vector. β-gal, β-galactosidase. Data from triplicate determinations are expressed as the means ± S.E.M.
expression, although less potently than a full agonist, PTH(1–34) (Jongen et al. 1996). We also observed that treatment of UMR-106 cells with human PTH(3–34) or TPA moderately down-regulated PTH/PTHrP receptor gene expression. Thus, although activation of PKC could decrease PTH/PTHrP receptor mRNA expression in osteoblast-like cells, PKC activation was not highly involved in homologous down-regulation of PTH/PTHrP receptor mRNA. PKC transduces the signal to the ERK1/2 MAP kinase pathway, but an inhibitor of the MAP kinase pathway, PD98059, influenced PKC-mediated down-regulation of gene expression. These findings indicate that suppression dependent upon PKC activity is mediated via the ERK1/2 MAP kinase pathway, which is a heterologous down-regulation pathway with IGF-I as the initial stimulus (Fig. 9).

In the present study, we found that homologous down-regulation of PTH/PTHrP receptor mRNA was strongly dependent on intracellular concentrations of cAMP and was readily mimicked by a cAMP analogue to which the cell membrane is permeable (8-pCPTcAMP) as well as an activator of adenylyl cyclase (forskolin). Although PTH-induced cAMP accumulation correlated well with down-regulation of PTH/PTHrP receptor mRNA expression in UMR-106 cells, PKA activity, which is stimulated by cAMP, is not involved in down-regulation of the receptor gene. Even in the presence of H89, a highly selective PKA inhibitor, the increase in intracellular cAMP concentration induced by rPTH(1–34), forskolin, or 8-pCPTcAMP was able to decrease PTH/PTHrP receptor mRNA abundance. These findings indicated that PTH reduces expression of PTH/PTHrP receptor mRNA through a pathway mediated by cAMP but independent of PKA (Fig. 9). In a previous report, homologous down-regulation of PTH/PTHrP receptor mRNA in SaOS-2 osteoblast-like cells was shown to depend upon cAMP-dependent PKA (Fukayama et al. 1994). We cannot yet explain the apparently different pattern of regulation of PTH/PTHrP receptor mRNA in these systems. Different results may reflect different states of differentiation of the osteoblastic cell lines or culture conditions among them.

We have sought for an involvement of cAMP-dependent signal transduction pathways in addition to the one mediated by PKA. A second enzyme target of cAMP, designated cAMP-guanidine nucleotide exchange factors (cAMP-GEFs or Epacs) transduces the cAMP signal into

**Figure 9** Proposed signaling pathways mediating down-regulation of PTH/PTHrP receptor mRNA expression in osteoblast-like cells. A major pathway of PTH-induced suppression of the PTH/PTHrP receptor gene expression was shown to be cAMP dependent and PKA independent.
the Rap1/B-Raf pathway in a PKA- and Ras-independent manner (de Rooij et al., 1998, Richards 2001). According to a recent report (Fujita et al., 2002), PTH stimulates bone cell proliferation by signaling via Epacs independently of PKA. Although Epacs activate MAP kinases in the target cells of thyrotropin, melanocyte-stimulating hormone and follicle-stimulating hormone (Richards 2001), as well as in bone cells (Fujita et al., 2002), our experiments using PD98059, an ERK1/2 MAP kinase pathway inhibitor, indicated that cAMP-dependent down-regulation of PTH/PTHrP receptor mRNA in osteoblast-like cells does not involve the ERK1/2 MAP kinase pathway. Further studies are needed to identify the pathway mediated by cAMP on down-regulation of PTH/PTHrP receptor gene expression.

We previously showed that the minimal region for basal promoter activity of the rat PTH/PTHrP receptor gene was located within the U3 promoter, while PTH clearly suppressed U3 promoter activity as shown by luciferase in transiently transfected UMR-106 cells (Kawane et al., 2001). The present study demonstrated the presence of a U4 promoter adjacent to exon 5 in rats, and this promoter activity is also inhibited by rPTH(1–34) in UMR-106 osteoblast-like cells. We have demonstrated that suppression of U3 and U4 promoters is mediated via intracellular cAMP, by the findings that forskolin and 8-cPTcAMP mimicked the effect of rPTH(1–34) in cells transfected with plasmids containing U3 or U4 promoters. Further, since addition of H89 did not affect the repression of rat promoter activity induced by rPTH(1–34), forskolin or 8-cPTcAMP, the inhibitory effect is independent of PKA. Other pathways must be involved.

In conclusion, we have found that rPTH(1–34) transcriptionally down-regulated gene expression of PTH/PTHrP receptor via a cAMP-dependent, PKA-independent pathway in UMR-106 osteoblast-like cells.

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