1-(5-Oxohexyl)-3,7-Dimethylxanthine, a Phosphodiesterase Inhibitor, Activates MAPK Cascades and Promotes Osteoblast Differentiation by a Mechanism Independent of PKA Activation (Pentoxyfilline Promotes Osteoblast Differentiation)

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We have investigated the effect of 1-(5-oxohexyl)-3,7-dimethylxanthine or pentoxyfilline (PeTx), a nonselective phosphodiesterase inhibitor, on osteoblastic differentiation in vitro by using two mesenchymal cell lines, C3H10T1/2 and C2C12, which are able to acquire the osteoblastic phenotype in the presence of bone morphogenetic protein-2 (BMP-2). PeTx induced the osteoblastic markers, osteocalcin and Osf2/Cbfa1, in C3H10T1/2 and C2C12 cells and enhanced BMP-2-induced expression of osteocalcin, Osf2/Cbfa1, and alkaline phosphatase. This activity was partially attributed to the fact that PeTx is able to enhance BMP-2-induced Smad1 transcriptional activity. Although PeTx clearly stimulates PKA in these cells, neither pretreatment of cells with the PKA inhibitor H89 nor transfection with the specific PKA inhibitor PKI prevented the induction or enhancement of osteoblast markers by PeTx, demonstrating that these effects were independent of PKA activation. On the other hand, PeTx induced the activation of ERK1/2 and p38 kinase pathways independently of the activation of PKA. Selective inhibitors of these MAPK cascades prevented the induction of osteoblastic markers in cells treated with PeTx, suggesting that the activation of these two pathways plays a role in the effect of PeTx on osteoblastic differentiation. (Endocrinology 142: 4673–4682, 2001)

OSTEOPOROSIS AND OTHER diseases of bone loss are a major public health problem. Despite recent successes with drugs that inhibit bone resorption, notably bisphosphonates, there is a clear therapeutic need for bone anabolic molecules (i.e. compounds able to increase bone formation), especially in the case of patients who have already suffered a substantial bone loss (1). PTH and PGE₂ stimulate bone formation in experimental animals and in humans (2, 3). Importantly, the receptors for these two molecules are coupled to a variety of G proteins that activate their specific second messenger signaling cascades: cAMP, calcium, and IP₃ (4–6). Several studies strongly suggest that the anabolic effects of these two molecules are mainly mediated by cAMP, and it has been hypothesized that molecules increasing cAMP could mimic the anabolic effects of PTH and PGE₂ on bone.

Different members of the cyclic nucleotide phosphodiesterase (PDEs) family are enzymes that hydrolyze cAMP and cGMP and therefore play a crucial role in modulating cAMP levels (7). In recent years a number of inhibitors displaying various degrees of selectivity for the different types of PDEs have been developed (8). Interestingly, some PDE inhibitors have been reported to stimulate osteoblastic differentiation and inhibit osteoclastic differentiation in vitro (9, 10). Very recently, Kinoshita et al. (11) demonstrated that the PDE inhibitors 1-(5-oxohexyl)-3,7-dimethylxanthine [known as pentoxyfilline (PeTx)] and rolipram increase bone mass mainly by promoting bone formation in normal mice. Furthermore, PDE inhibitors have been shown to exert therapeutic effects in different experimental osteopenia models (10, 12, 13). Although these effects might be linked to the increase in cAMP levels induced by PDE inhibitors, little is known about the precise mechanisms by which PDE inhibitors stimulate bone cells.

In the present study we have investigated the effect of 1-(5-oxohexyl)-3,7-dimethylxanthine or PeTx, a nonselective PDEs inhibitor, on osteoblast differentiation using two murine pluripotent mesenchymal cell lines, C3H10T1/2 and C2C12, that are able to differentiate in osteoblasts when treated with bone morphogenetic protein-2 (BMP-2). The effect of the PDE inhibitor in the presence or absence of BMP-2 was assessed by measuring the osteoblast-specific markers alkaline phosphatase (ALP), osteocalcin (OC), and Osf2/Cbfa1. In addition, we evaluated the involvement of MAPK, ERK1/2, and p38 signaling pathways in the observed effects.

Materials and Methods
Reagents and antibodies

Myelin basic protein and PeTx were obtained from Sigma (St. Louis, MO); SB203580, PD98059, and H89 were obtained from BIOMOL Re-
search Laboratories, Inc. (Plymouth Meeting, PA). Anti-ERK (K23), anti-p38 (C20), and protein A-Sepharose beads were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). p38/RK/MpK2 assay kit was available through Upstate Biotechnology, Inc. (Lake Placid, NY). [γ-32P]ATP (3000 Ci/mmol) was commercially available from Amer sham Pharmacia Biotech (Arlington Heights, IL). PKA PepTag and the dual luciferase assay system were purchased from Promega Corp. (Madison, WI). Recombinant BMP-2 was purified from CHO cells stably transfected with a plasmid expression vector for human BMP-2.

**Cell culture**

C3H10T1/2, obtained from American Type Culture Collection (Manassas, VA), and MC3T3-E1, provided by Dr. R. Franceschi (University of Michigan, Ann Arbor, MI), cell lines were cultured (5% CO₂ at 37°C) in α-MEM supplemented with 10% heat-inactivated FCS and antibiotics (100 U/ml penicillin-G and 100 μg/ml streptomycin). The mouse myoblast cell line C2C12 was provided by Dr. Gerard Karsenty (Baylor College of Medicine, Houston, TX). C2C12 cells were maintained (5% CO₂ at 37°C) in DMEM containing 15% FBS and antibiotics (100 U/ml penicillin-G and 100 μg/ml streptomycin). For treatment, the cells were plated in 24-well plates at 2 × 10⁵ cells/cm². Twenty-four hours later, the growth medium was replaced with a similar medium containing 2% FBS, then cells were stimulated with the indicated compound for the indicated time period.

**Gene expression analysis by real-time TaqMan PCR**

Murine OC and Osf2/Cbfa1 mRNA expression was determined by RT, followed by real-time TaqMan PCR analysis as previously described (14). RT reactions were carried out using 500 ng total RNA with the following conditions: 42°C for 60 min, 95°C for 5 min, and 4°C for 5 min. RT product was diluted three times in sterile bidistilled water, and 5 μl were used to perform TaqMan PCR. ALP TaqMan PCR was carried out in a 25-μl final volume containing: 1× TaqMan EZ buffer, 5 mm Mn(OAc)₂, 200 μM d[A/dC/dG/deoxy-UTP, 0.625 U AmpliTaq Gold, 300 μM each of murine OC primer (forward and reverse), 40 μM each of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reverse and forward primers, and 200 μM each of GAPDH and OC TaqMan probes. Cycling conditions were 95°C for 15 sec and 60°C for 1 min for 40 cycles. Real-time TaqMan PCR was performed in an ABI PRISM 7700 sequence detector (PE Applied Biosystems, Foster City, CA). Conditions for Osf2 TaqMan PCR were exactly the same as those used for the OC reaction, except that OC primers and probe were replaced by Osf2 ones. All PCR reactions were performed in duplicate, and OC or Osf2 signal was normalized to GAPDH signal in the same reaction.

**Determination of ALP activity**

At the indicated time of treatment cells were lysed at 37°C in lysis buffer containing 1 mM MgCl₂ and 0.2% Nonidet P-40. ALP activity was determined using the ALP Opt kit (Roche, Mannheim, Germany) and was normalized to the protein concentration determined by the bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL).

**Measurement of MAPK and PKA activities**

Cells were stimulated with PeTx for appropriate time intervals and washed twice with ice-cold PBS containing 1 mM Na₂VO₃. One hundred microliters of the following lysis buffer were added to cells: 20 mM 3-(N-morpholino)propanesulfonic acid (pH 7.2), 5 mM EDTA, 1% (vol/vol) Nonidet P-40, 1 mM dithiothreitol, 75 mM β-glycerol phosphate, 1 mM Na₃VO₃, and protease inhibitor mixture (Sigma). Lysis was performed at 4°C for 20 min with continuous shaking. Cell lysates were cleared by centrifugation, then stored at −80°C. The protein concentration in cell lysates was determined by micro-bicinchoninic acid assay (Pierce Chemical Co.).

ERK1/2 and p38 were immunoprecipitated by incubating 250 μg cell lysates with 2 μg anti-ERK1-CT Ab or with 10 μg anti-p38 Ab at 4°C for 4 h with continuous rotation. Then, 30 μl protein A-Sepharose were added, and the incubation was extended for 2 more h. The mixtures were centrifuged (7000 × g for 2 min at room temperature), and protein A-Sepharose beads were washed three times with buffer B [12.25 mM 3-(N-morpholino)propanesulfonic acid (pH 7.2), 0.5 mM EGTA, 1% (vol/vol) Nonidet P-40, 1 mM dithiothreitol, 12.5 mM β-glycerol phosphate, and 7.5 mM MgCl₂] containing 250 mM NaCl. The beads were resuspended in 50 μl buffer B containing 10 mM MgCl₂ and 1 mM MnCl₂ for phosphotransferase assays. For measuring ERK activity, 10 ng myelin basic protein in the presence of 50 μM [γ-32P]ATP were added to ERK1/2 immunoprecipitates. The reaction was conducted at 30°C for 30 min, then terminated by adding SDS sample buffer to a 1:2 final concentration. Samples were analyzed by SDS-PAGE using 12% gels. Gels were fixed in 10% acetic acid and 50% methanol, then embedded in cellophane sheets and dried. Gels were exposed to a phospho-screen and quantitatively assessed by mean of QuantityOne software (Bio-Rad Laboratories, Inc., Hercules, CA).

p38 activation in immunoprecipitates was determined by measuring its phosphotransferase activity toward peptide substrate using the p38/MpK2 detection kit. p38 activity was expressed as [32P]TP counts per min. PKA activity in cell lysates was determined by using the PKA PepTag kit (Promega Corp.) following the manufacturer’s instruction.

**Plasmids and transfections**

pGal4Smad1 and the Gal-dependent luciferase construct, pG5E1b-luc, were provided by Dr. A. Atfi (INSERM, Hôpital Saint Antoine, Paris, France). pRSV-PKI and pRSV-PKImut were provided by Dr. R. A. Mauer (Oregon Health Science University, Portland, OR). The murine OC promoter (mOG2), the luciferase reporter (pmOG2/luc), and mOG2 minimal promoter-luciferase reporter construct (p147/luc) (15) were provided by Dr. G. Karsenty. Cells were transiently transfected with luciferase reporter constructs with or without other expression plasmid (total, 1 μg DNA) using DNA-lipid complex Fugene 6 (Roche) according to the manufacturer’s protocol. To assess transfection efficacy and normalize firefly luciferase signal, 20 ng pRL-TK (Promega Corp.), which encodes a Renilla luciferase gene downstream of a minimal herpes simplex virus-thymidine kinase promoter, was systematically added to the transfection mix. Eighteen hours later, cells were washed and cultivated with fresh medium containing 2% FBS, then stimulated with PeTx for 24 h. Luciferase assays were performed with the Dual Luciferase Assay Kit (Promega Corp.) according to the manufacturer’s instructions. Ten microliters of cell lysate were assayed first for firefly luciferase and then for Renilla luciferase activity. Firefly luciferase activity was normalized to Renilla luciferase activity.

**Statistical analysis**

All experimental data presented were obtained from two independent experiments, each performed in triplicate, and results are expressed as the mean ± s.e. Comparisons between treatments were performed by t test. P < 0.05 was considered statistically significant.

**Results**

PeTx enhances osteoblast differentiation

To determine whether PeTx affects osteoblast differentiation, we investigated the effect of PeTx on the mesenchymal cell line C3H10T1/2 and the pluripotent myoblast cell line C2C12. Treatment with BMP-2 induces in these two cell lines the expression of osteoblast markers, including ALP, OC, and Osf2/Cbfa1 (16). We therefore tested whether PeTx was able to affect the expression of osteoblast markers. C3H10T1/2 and C2C12 cells were treated with increasing concentrations of PeTx in the presence or absence of BMP-2 (100 ng/ml). After 3 d of treatment, ALP induction was determined by measuring its activity in cell lysates. PeTx alone had no effect on ALP activity in C3H10T1/2 cells (Fig. 1A), but enhanced BMP-induced ALP activity in a dose-dependent manner (Fig. 1A). These results were confirmed by the measurement of ALP mRNA using real-time TaqMan
PCR (data not shown). Similar data were obtained with C2C12 cells (data not shown).

Smad proteins are transcriptional comodulators critical for transmitting BMP signals from the cell surface to the nucleus. Phosphorylation and activation of Smad1 have been demonstrated to be necessary for the induction of ALP gene expression and activity by BMP-2 (17, 18). We therefore investigated whether the enhancement of BMP-2-induced ALP by PeTx could be due to a modulation of the transcriptional activity of Smad1. C3H10T1/2 cells were cotransfected with a plasmid containing the Gal4 DNA-binding domain and Smad1 and a Gal4-binding element/luciferase reporter construct. Eighteen hours after transfection, medium was replaced by 2% FCS medium and cotransfected cells were stimulated with BMP-2 (100 ng/ml), PeTx (1.8 mM), or both. After 24-h stimulation, luciferase activity was measured in cell lysates and normalized to Renilla luciferase signal. Data represent the mean ± SD of a representative experiment. *, P < 0.05, cells treated with BMP-2 compared with untreated cells. **, P < 0.05, cells treated with BMP-2/PeTx compared with BMP-2-treated cells.

We then investigated the effect of PeTx on OC mRNA transcription in C3H10T1/2 and C2C12 cells. Interestingly, and in contrast with ALP, PeTx alone induced OC mRNA transcription in addition to enhancing BMP-2-induced OC transcripts (Fig. 2A). The effect of PeTx on OC was further addressed using pmOG2/luc and p147/luc, two reporter plasmids containing, respectively, the wild-type mouse OC promoter and the minimal OC promoter (−147/+13) (15). C3H10T1/2 cells were transiently transfected with p147/luc, then treated with PeTx. As expected, PeTx treatment induced about 3-fold the expression of both versions of the luciferase-OC reporter genes (Fig. 2B).

Osf2/Cbfa1 is the earliest and most specific marker of osteoblast differentiation known. We therefore also tested whether PeTx regulated osf2/Cbfa1 expression. The data presented in Fig. 3A clearly show that PeTx alone induced the expression of this osteoblast-specific marker in both C3H10T1/2 and C2C12 cell lines and enhanced BMP-2-induced Osf2/Cbfa1 expression. These findings were further confirmed using a reporter construct containing a luciferase gene driven by the human Osf2/Cbfa1 promoter, phOsfs2/luc. As described in Fig. 3B, PeTx was able to induce a 3-fold increase in luciferase activity in cells transiently transfected with pOsfs2/luc.

The data obtained for ALP, OC, and Osf2/Cbfa1 expression strongly suggested that PeTx is able to induce osteoblast commitment and enhance the activity of BMP-2.
investigated the mechanism by which PeTx could influence osteoblastic differentiation.

**PeTx activity is independent of PKA activation**

It is well established that PDEs inhibitors increase intracellular cAMP levels, which, in turn, activate PKA. In agreement with that observation, a strong increase in cAMP in C3H10T1/2 and C2C12 cells could be detected after treatment with PeTx (data not shown). In addition and as shown in Fig. 4A, a 15-min treatment with PeTx induced a significant activation of PKA. Similar data were obtained using C2C12 cells (data not shown). To verify the involvement of PKA in the observed PeTx activity, an inhibitor of PKA, H89, was used. Cells were pretreated for 1 h with increasing concentrations of H89 and then stimulated for 15 min with PeTx (1.8 mM) before measuring PKA in cell lysates. A significant inhibition of PeTx-induced PKA activation was already observed at 1 μM H89 (Fig. 4A) and 2–5 μM H89 completely abolished PKA activation without any detectable effect on cell viability (data not shown). C3H10T1/2 cells were preincubated for 1 h with 2 μM H89 before stimulation with PeTx and BMP-2, and ALP activity was determined 3 d after stimulation. H89, at a concentration that blocked PKA activation, had no effect on the enhancement of ALP activity induced by BMP-2/PeTx (Fig. 4B). Furthermore, H89 (at 2 μM) pre-treatment of C3H10T1/2 cells transfected with p147/luc or pOs2/luc constructs had no effect on the luciferase induction by PeTx (Fig. 4C). To further investigate the involvement of PKA in PeTx activity, C3H10T1/2 cells were cotransfected with a plasmid overexpressing PKI (pRSV-PKI), a specific PKA inhibitor, and the p147/luc construct, and then stimulated with PeTx. Control experiments were performed using a mutated form of PKI, pRSV-PKIm. PKI overexpression did not show any inhibitory effect on luciferase induction in response to PeTx.
stimulation (Fig. 4D). Similar results were obtained in C2C12 cells (data not shown). In conclusion, these data clearly demonstrate that PeTx-induced PKA activation is not involved in the enhancement by PeTx of BMP-2-induced ALP production or in the increase in OC and Osf2/Cbfa1 expression.

**PeTx activates ERK1/2 and p38**

MAPKs are important mediators involved in the intracellular network of signal transduction. To date, several MAPKs have been reported to be involved in osteoblastogenesis, including ERK1/2 and p38 (19–21). We therefore investigated whether the effects of PeTx on osteoblasts could be explained by an activation of ERK1/2 and/or p38 in C3H10T1/2 and C2C12 cells. Both cell lines were treated with PeTx for different time intervals, and cell lysates were tested for ERK1/2 and p38 activation by measuring their respective phosphotransferase activities. Activation of ERK1/2 was determined by measuring the phosphorylation of myelin basic protein after immunoprecipitation of lysates with an anti-ERK Ab. ERK1/2 was strongly activated in both C3H10T1/2 and C2C12 cells treated with PeTx (Fig. 5A). In C3H10T1/2 cells, maximum activity was detected after 20-min stimulation, then it rapidly decreased to control levels, while in C2C12 cells, maximum activity was observed at 30 min and remained high up to 60 min (Fig. 5A).

p38 activation was measured after immunoprecipitation with an anti-p38 Ab, using a kinase assay with a p38-specific peptide as substrate. In both C3H10T1/2 and C2C12 cells, a significant increase in p38 activity was observed in lysates from cells treated with PeTx compared with those from untreated cells (Fig. 5B). In both cell lines maximum p38 activation was measured after 10 min of PeTx treatment, then rapidly declined to reach control levels after 30 min.

To determine whether the ability of PeTx to activate MAPK pathways was dependent upon cAMP and PKA activation, cells were pretreated with H89 (2 μM) before challenge with PeTx. H89 has no effect on ERK1/2 and p38 stimulation by PeTx (Fig. 5, C and D). Together these results...
strongly suggest that PeTx is capable of activating the ERK1/2 and p38 MAPK pathways independently of its ability to inhibit PDEs. Thus, PeTx activates both ERK1/2 and p38, suggesting that some of its effects on osteoblast differentiation may be exerted via the MAPK pathway.

**MAPK involvement in PeTx-mediated expression of osteoblast markers**

To investigate the involvement of MAPK in PeTx-mediated activation of osteocalcin and Osf2/Cbfa1, we specifically blocked each of ERK1/2 and p38 pathways and monitored p147/luc or pOs2/luc activation in C3H10T1/2 cells challenged with PeTx.

PD98059 and U0126 are synthetic compounds that specifically inhibit the ERK-activating MAPK kinase, MEK1 (22–24). Both PD98059 (Fig. 6A) and U0126 (data not shown) were able to completely block luciferase activation in C3H10T1/2 cells transiently transfected with p147/luc or pOs2/luc reporter constructs. The culture medium of transfected cells was changed to 2% FCS 18 h after transfection then, cells were stimulated with PeTx (1.8 mM) for an additional 24 h. Luciferase activity was measured in cell lysates and normalized to Renilla luciferase signal. Data represent the mean ± SD of a representative experiment. *, P < 0.001, cells treated with BMP-2 or PeTx compared with untreated control cells. **, P < 0.001, cells treated with BMP-2/PcTx compared with BMP-2-treated cells.
cells transiently transfected with p147/luc, pmOG2/luc, or pOsf2/luc construct and pretreated with SB203580 failed to respond to PeTx stimulation. Comparable results were obtained when experiments were repeated using C2C12 cells (data not shown). We also verified that the inhibitors were used at concentrations that efficiently and specifically inhibited their corresponding targets without affecting cell viability (data not shown).

These results demonstrate that MAPK pathways are involved in PeTx-mediated Osf2 activation and osteocalcin stimulation. Given that these pathways also contribute to the BMP-2 signaling events (21), the enhancement of BMP-2 effects on ALP after PeTx treatment may reflect their additional influence on MAPK pathways.

**Discussion**

Although some PDE inhibitors have been reported to promote bone formation in vivo, the precise mechanisms leading to these effects are currently unknown. Preliminary studies indicate that PeTx and other PDE inhibitors affect osteoblast differentiation at an early stage, based on the fact that immature cell lines, such as C3H10T1/2 or ST2, but not MC3T3-E1 cells, can respond to PeTx by enhancing the BMP-2-induced ALP activity (26) (our unpublished results). In this study we evaluated the effect of pentoxifylline, a nonselective PDE inhibitor, on osteoblast differentiation using two pluripotent mesenchymal cell lines C3H10T1/2 and C2C12, which acquire the osteoblastic phenotype in the presence of BMP-2, and we examined the respective roles of PKA and MAPK in mediating the effects of PeTx on osteoblast differentiation.

In the two cell lines studied, PeTx was able to induce the expression of the osteoblast marker genes osteocalcin and Osf2/Cbfa1 and to significantly enhance BMP-2-induced ALP gene expression and activity. Although treatment with PeTx alone induced Osf2/Cbfa1 and osteocalcin gene expression, ALP gene expression and activity were positively affected by PeTx only in the presence of BMP-2. Osf2/Cbfa1 is recognized as a master gene in osteoblast differentiation, controlling the expression of several genes expressed in osteoblasts, such as OC, α1(I) collagen, bone sialoprotein, and osteopontin, which possess OSE2-like elements in their promoters (27). However, the ALP promoter has not been reported to have any OSE2-like element and is most likely not regulated by Osf2/Cbfa1. This may explain why PeTx alone did not affect ALP expression despite its ability to induce

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**Fig. 5.** PeTx stimulates ERK and p38 pathways in mesenchymal cells. C3H10T1/2 and C2C12 cells were treated with PeTx for different periods (4, 10, 20, 30, and 60 min), and p38 or ERK1/2 were immunoprecipitated from cell lysates using anti-p38 or anti-ERK antibodies as described in Materials and Methods. ERK1/2 (A) or p38 (B) activation was quantified in immunoprecipitates using p38/RK/Mpk2 or p42/44MAPK detection kits, respectively. C and D, Cells were preincubated or not with H89 (2 μM) for 1 h before treatment with PeTx. Then, p38 and ERK1/2 activation was determined in cell lysates after, respectively, 10 or 20 min of treatment. Data represent the mean ± SD of a representative experiment (P < 0.05).
Osf2/Cbfa1. Smad proteins are crucial components of TGFβ-related signal transduction pathways, and Smad1/5/8 are specifically phosphorylated by BMP receptors (28, 29). Importantly, dominant negative Smads have been reported to block the BMP-2-mediated induction of ALP and osteocalcin in different cell models (18, 30–32). Here, using a one-hybrid system, we showed that PeTx is able to enhance BMP-2-induced Smad1 transcriptional activity. This suggests that the synergistic effect of BMP-2 and PeTx on ALP gene expression induction might in part be ascribed to an increase in Smad1 transcriptional activity.

Given that inhibition of PDEs leads to an increase in intracellular cAMP levels, triggering the activation of PKA, this signaling pathway might have been responsible for the effect of PeTx or other PDE inhibitors on osteoblast differentiation. PTH, which is capable of increasing bone mineral density in normal and osteopenic bone in humans and animals (33), activates osteocalcin transcription at least in part via PKA activation (34, 35). Our data strongly suggest, however, that the enhancement of osteoblast differentiation after PeTx treatment is independent of PKA activation. Neither treatment with the specific PKA inhibitor, H89, nor the overexpression of PKI, a PKA antagonist, blocked the effects of PeTx on the osteoblast cell lines. Furthermore, and despite the fact that cAMP inducers have been recently shown to inhibit Osf2/Cbfa1 activity in osteoblastic cells (36), PeTx increased both Osf2/Cbfa1 gene expression and the activity of an osteocalcin minimal promoter containing an Osf2/Cbfa1-binding element, OSE2. Together these data strongly suggest that the effects of PeTx on osteoblast differentiation are independent of cAMP increase and/or PKA activation, thus implying that PeTx activates other signaling pathways that positively affect osteoblast differentiation.

It has been suggested that specific PDE4 inhibitors display a potent bone anabolic activity in vivo (37). A recent study clearly showed that in normal mice both PeTx and rolipram, a PDE4-specific inhibitor, increase cortical and cancellous bone mass (11). In preliminary experiments we investigated
the effects of specific PDE4, PDE5, and PDE3 inhibitors on osteoblast differentiation. Our data suggest that both PDE3 and PDE4 inhibitors enhance osteoblast differentiation, as assessed by ALP activity and OC and Osf2/Cbfa1 expression (data not shown). Comparable results have been reported by Wakabayashi (38) using the stromal cell line ST-2. Interestingly, osteoblastic differentiation of MC3T3-E1 cells was not affected by any of the PDE-selective inhibitors in the absence or presence of BMP-4 (38), suggesting that these compounds only affect immature cells in which osteoblast commitment is promoted.

Several studies have demonstrated that MAPK cascades play an important role in osteoblast differentiation and function. For example, it has been recently reported that ERK is essential for growth, differentiation, and cell function in human osteoblastic cells (39). MAPK cascades play pivotal roles in the stimulation of osteoblast proliferation by both PTHrP and extracellular calcium-sensing receptor agonists (40, 41). Moreover, activation of MAPKs is involved in the regulation of BMP-2-induced osteoblast differentiation in C2C12 cells (21). Finally, the p38 pathway has been reported to regulate ALP activity in response to activation of G1, protein-coupled receptors (42). Exploring the downstream elements capable of regulating osteoblast gene expression, we have demonstrated that PeTx is able to induce the activation of ERK1/2 and p38 MAPK cascades. We demonstrated that activation of MAPK pathways was independent of cAMP and PKA activation, as treatment of cells with the PKA inhibitor, H89, did not affect the activation of ERK1/2 and p38 by PeTx, and we have shown that forskolin also failed to activate these cascades (data not shown). Moreover, the activation of MAPK signaling pathways was clearly involved in the ability of PeTx to stimulate the expression of the osteoblast differentiation markers, osteocalcin and Osf2/Cbfa1. Actually, treatment of C3H10T1/2 or C2C12 cells with specific inhibitors of ERK and p38 pathways abolished both osteocalcin and Osf2/Cbfa1 induction by PeTx. In this context it is important to note that the ERK1/2 cascade has been very recently implicated in the activation and phosphorylation of the osteoblast-specific factor Osf2/Cbfa1 (43), which binds to the osteoblast-specific element-2, a cis-acting sequence present in the promoter of a series of osteoblast-related genes such as OC, α1(1) collagen, bone sialoprotein, and osteopontin (27). PDE inhibitors are able to stimulate bone-like nodule formation in rat bone marrow cultures (37), but the role of MAPK in this effect has not been addressed, because general effects on cell proliferation as well as toxic effects after prolonged treatments by MAPK inhibitors complicate the evaluation of their precise roles during mineralization.

The exact mechanism by which PeTx stimulates MAPK pathways remains to be fully elucidated. At present it is unclear whether PeTx directly stimulates the MAPK pathways or whether these are upstream players. For instance, one cannot exclude the possibility that PeTx may target MAPK pathways via a receptor-mediated signal or affect other molecular targets. Theophyllin and SQ 20006, nonselective PDE inhibitors, were shown to inhibit p70[S6k] activation by a mechanism that is independent of cAMP and cGMP (44). Future studies to better characterize the molecules and pathways involved in PeTx-mediated osteoblast differentiation should contribute to improve our understanding of their anabolic effect on bone.

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