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Phosphodiesterase 4 inhibitor regulates the TRANCE/OPG ratio via COX-2 expression in a manner similar to PTH in osteoblasts

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Abstract

Phosphodiesterase 4 (PDE4) inhibitors stimulate osteoclast formation by increasing the TRANCE/OPG mRNA ratio via cAMP-mediated pathways in a manner similar to parathyroid hormone (PTH) in osteoblasts. We investigated the role of cyclooxygenase-2 (COX-2) in osteoclast formation induced by the PDE4 inhibitor rolipram. Rolipram induced COX-2 expression in mRNA and protein levels, followed by increased prostaglandin E_2 production in osteoblasts. PKA, ERK, and p38 MAPK pathways regulate COX-2 mRNA expression induced by rolipram, in which PKA is a central regulator of the ERK and p38 MAPK pathways. A COX-2 inhibitor reversed the up-regulation of the TRANCE/OPG mRNA ratio induced by rolipram in osteoblasts, resulting in decreased osteoclast formation. These data suggest that COX-2 mediates rolipram induced osteoclast formation by regulating the TRANCE/OPG mRNA ratio in osteoblasts. Furthermore, the effects of the PDE4 inhibitor on osteoblasts were very similar to those of PTH, indicating that the PDE4 inhibitor largely shares the biological actions of PTH in osteoblasts.

Keywords: Phosphodiesterase 4; Parathyroid hormone; COX-2; TRANCE; OPG; Osteoblast; Osteoclast

Osteoclasts are multinucleated giant cells responsible for bone resorption [1]. Osteoblasts, as well as stromal cells, are essential for osteoclastogenesis through cell–cell interactions with osteoclast precursors of monocyte/macrophage lineage [2,3]. In co-cultures of mouse bone marrow cells and calvarial osteoblasts, osteoclasts are formed in response to several factors such as 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], parathyroid hormone (PTH), interleukin (IL)-6 plus soluble IL-6 receptor, and prostaglandin E_2 (PGE_2) [2,3]. These factors induce the expression of TNF-related activation-induced cytokine (TRANCE, also known as RANKL, ODF, or OPGL) in osteoblasts, which triggers osteoclast differentiation [4,5]. Osteoblasts also produce osteoprotegerin (OPG), a decoy receptor for TRANCE, to inhibit osteoclast formation [6].

Cyclooxygenase (COX) is the rate-limiting enzyme in the conversion of arachidonic acid released from membranes to prostanoids [7]. Two COX enzymes, COX-1 and COX-2, are encoded by separate genes. In most tissues, COX-1 is expressed constitutively, whereas COX-2 is induced transiently. Recent *in vitro* studies suggest that COX-2 is regulated by multiple local and systemic factors important to bone metabolism [7], and is involved in osteoclast differentiation [8]. Factors that promote osteoclast differentiation and may act through COX-2 pathways include interleukins (IL-1, IL-6, and IL-17), basic fibroblast growth factor (bFGF), and $1,25(OH)_2D_3$. These factors stimulate COX-2 gene expression and PGE_2 production in bone marrow cells and osteoblasts in osteoblast/spleen co-culture systems [8]. Some of these factors often regulate osteoclast differentiation by acting via the induction of TRANCE expression in osteoblasts and stromal cells. The induction of TRANCE can be blocked by

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inhibitors of COX-2, demonstrating that PGE_2 production is involved in TRANCE expression in osteoblasts [9,10].

Phosphodiesterases (PDEs) are enzymes that degrade and inactivate cAMP/cGMP [11]. The PDE family consists of 11 isozymes, PDE1–11, with some of these PDE isozymes classified further into subtypes. Those involved in the degradation of cAMP are PDE1, 2, 3, 4, 7, 8, 10, and 11 [11]. We previously reported that 3-isobutyl-1-methylxanthine (IBMX), a nonspecific PDE inhibitor, and rolipram, a selective PDE4 inhibitor, increased osteoclast formation as a result of TRANCE expression via cAMP-mediated pathways [12]. Interestingly, this pathway also induces TRANCE expression in osteoblasts following stimulation with parathyroid hormone (PTH) [13].

Since the precise action of PDE4 inhibitors in osteoclast formation has not yet been fully elucidated, we investigated the mechanism of PDE4 inhibitor action in osteoclast formation in comparison with PTH. We found that COX-2 mediates the effect of both rolipram and PTH on TRANCE/OPG-regulated osteoclast formation. We further examined the signaling pathways involved in COX-2 mRNA expression induced by rolipram or PTH, and determined how they crosstalk in osteoblasts.

Materials and methods

Reagents. PTH (human, 1–34) was purchased from Anygen Co., Ltd. (Gwang-ju, Korea). Antibodies against ERK, phospho-ERK, p38, and phospho-p38 were purchased from Cell Signaling Technology (Beverly, MA, USA). All other reagents were from Sigma–Aldrich (St. Louis, MO).

Cells and culture system. Primary calvarial osteoblasts were obtained from the calvariae of neonatal ICR mice (Samtako, Inc., Korea) as described before [12]. Bone marrow cells were obtained from the long bones of 4- to 6-week-old ICR male mice. To examine osteoclast formation, mouse bone marrow cells (1×10^5 cells) were co-cultured with calvarial osteoblasts (5×10^3 cells) in the presence or absence of rolipram (10 μM) and/or NS398 (5 μM) in 96-well culture plates (CORNING, MA, USA). After 6 days of culture, cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP), a marker enzyme of osteoclasts, as described previously [12].

Northern blot analysis. Calvarial osteoblasts were cultured with agents for the indicated periods and then subjected to total RNA extraction using Trizol reagent (Invitrogen, CA, USA). Total RNA (20 μg) was electrophoresed on 1.2% agarose-formaldehyde gels, transferred to nylon membrane filters (Hybond N+, Amersham Biosciences, Bukinghamshire, UK), and hybridized with ^{32}P-labeled cDNA probes. After the final wash, the membranes were exposed to X-ray film (BioMax, Kodak, Rochester, NY) at −70 °C.

Luciferase assay. Luciferase reporter pGL3 plasmid including 5′-flanking region (from −1398 to +123 bp) of the human COX-2 gene was kindly provided by Dr. S. Prescott (University of Utah, Salt Lake). For luciferase assay, calvarial osteoblasts were transfected with 1.5 μg pGL3-basic or pGL3-COX-2 promoter luciferase reporter plasmid and 0.5 μg pCMV-β-galactosidase plasmid. Cells were then incubated with PTH (100 nM) or rolipram (10 μM) for 8–12 h. Cells were harvested and lysed in a reporter lysis buffer, and the cleared lysates were transferred to each tube, and luciferase assay reagent (Promega, WI, USA) was added. The light intensity of the reaction was determined using a luminometer (Turner Biosystems Inc., Sunnyvale, CA) and the luciferase activity was normalized to β-galactosidase activity.

Immunoblot analysis. Total cell lysates were isolated, separated by SDS–PAGE, and transferred onto Immobilon-P membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% nonfat-milk in TBS-T, and then immunostained with anti-phospho p38 (1:1000), anti-phospho ERK (1:1000), anti-p38 (1:1000) or with anti-ERK antibody (1:1000) followed by secondary horseradish peroxidase-conjugated antibody (1:5000). The membranes were developed using an enhanced chemiluminescence detection kit (Amersham Biosciences, Bukinghamshire, UK).

Prostaglandin E_2 (PGE_2) enzymeimmunoassay. Calvarial osteoblasts were treated with PTH (100 nM) or rolipram (10 μM) for 3 h. PGE_2 levels were measured using a commercially available PGE_2 enzyme-immunoassay kit (Amersham Biosciences, Bukinghamshire, UK) according to the manufacturer's protocol.

Results

COX-2 expression is induced by rolipram and PTH in osteoblasts

PTH induces COX-2 mRNA expression and PGE_2 production in osteoblasts via cAMP-mediated pathways [14]. Thus, we first confirmed the effects of PTH on COX-2 mRNA expression using mouse calvarial osteoblasts. 100 nM PTH increased COX-2 mRNA expression in a time-dependent manner, with a maximum 1 h after treatment (data not shown). Because rolipram, a PDE4 inhibitor, appears to increase intracellular cAMP by blocking the hydrolysis of cAMP, the effect of rolipram on COX-2 mRNA expression was also investigated. Rolipram induced COX-2 mRNA expression in calvarial osteoblasts, and maximum expression occurred 1 h after treatment (Fig. 1a, left). The induction of COX-2 mRNA by rolipram was highly dose-dependent, indicating a rolipram-specific response (Fig. 1a, right). To further confirm the induction of COX-2 mRNA by PTH or rolipram, a reporter gene construct containing the human COX-2 promoter was transiently transfected into osteoblasts and assayed for luciferase activity. Luciferase activity measured in vehicle-treated cells transfected with human COX-2 promoter vector was designated as 1. Compared to the vehicle, treatment with PTH or rolipram resulted in an increase in luciferase activity, suggesting the stimulative effect of rolipram on COX-2 mRNA expression, as well as PTH in osteoblasts (Supplementary Fig. 1).

COX-2 expression is regulated via both transcriptional and post-transcriptional mechanisms [15]. Thus, high COX-2 mRNA expression levels are not always well correlated with high COX-2 protein levels. To determine if COX-2 mRNA expression stimulated by rolipram or PTH is followed by up-regulated COX-2 protein expression, we investigated the expression of COX-2 protein, as well as PGE_2 production, which requires the induction of COX-2 activity. Immunoblot analysis revealed increased COX-2 protein expression initially 1 h after treatment with either rolipram or PTH, and relatively high levels were sustained after 3 h (rolipram; Fig. 1b) or 6 h (PTH; Supplementary Fig. 2). We then measured the PGE_2 concentration in the conditioned medium of osteoblasts incubated with rolipram or PTH. Both significantly increased PGE_2 in the culture medium (Fig. 1c), suggesting

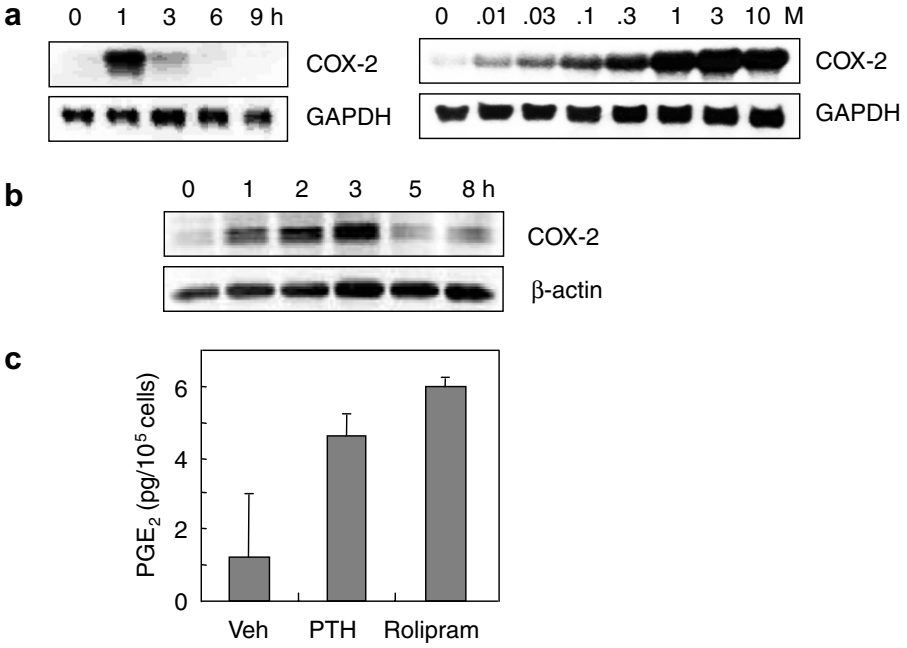

Fig. 1. Effects of rolipram and PTH on COX-2 expression. (a) Osteoblasts were treated with 10 μM rolipram for the indicated time intervals (left), or various concentrations of rolipram for 1 h (right). COX-2 mRNA expression was determined using a Northern blot assay. (b) Osteoblasts were treated with 10 μM rolipram for the indicated time intervals. COX-2 protein expression was determined using an immunoblot analysis. (c) Osteoblasts were treated with 10 μM rolipram or 100 nM PTH for 3 h after which PGE_2 concentrations in the conditioned media were determined by EIA.

that rolipram and PTH both produce PGE_2 by up-regulating COX-2 mRNA and protein expression in osteoblasts.

Possible involvement of PKA, ERK, and p38 MAPK in COX-2 mRNA expression induced by rolipram and PTH

We previously suggested that the PKA, ERK, and p38 MAPK signaling pathways are involved in cAMP-induced gene expression in osteoblasts [12]. Thus, the roles of the PKA, ERK, and p38 MAPK signaling cascades in COX-2 expression were examined to identify the signaling pathways used by rolipram to induce COX-2 mRNA expression. Immunoblot analysis using specific antibodies against phospho-ERK and phospho-p38 MAPK indicated that rolipram activated the ERK and p38 MAPK pathways in osteoblasts (Fig. 2a). Furthermore, a MAPK/ERK kinase (MEK) inhibitor, PD98059, and a p38 MAPK inhibitor, SB203580, decreased the COX-2 mRNA expression levels induced by rolipram (Fig. 2b). Likewise, pretreatment of osteoblasts with a PKA inhibitor, H89, also decreased the COX-2 mRNA expression levels induced by rolipram (Fig. 2b). These results suggest that rolipram regulates COX-2 mRNA expression in osteoblasts by activating the PKA, ERK, and p38 MAPK signaling pathways.

Because PTH stimulates COX-2 mRNA expression via cAMP accumulation [14], we also assessed the effects of H89, PD98059, and SB203580 on the COX-2 mRNA expression induced by PTH in osteoblast cells. All of these inhibitors decreased COX-2 mRNA expression levels (Fig. 2c), suggesting that the PKA, ERK, and p38 MAPK signaling pathways are involved in COX-2 mRNA expression induced by both PTH and rolipram.

Crosstalk between PKA, ERK, and p38 MAPK pathways activated by rolipram or PTH

We further examined the potential crosstalk between the PKA and MAPK pathways using H89, PD98059, and SB203580 in rolipram-treated osteoblasts. Pretreatment with PD98059 or H89 completely abolished phosphorylated ERK, indicating that phosphorylation of ERK by rolipram is largely dependent on MEK and PKA activation (Fig. 3a, left and middle). In contrast, pretreatment with H89 increased the phosphorylation of p38 MAPK, suggesting the negative control of p38 MAPK by the PKA pathway (Fig. 3a, right). Unexpectedly, pretreatment with SB203580 significantly increased p38 MAPK activation, which indicates a negative feedback control of the p38 MAPK pathway (Fig. 3a, right). When we investigated signaling pathway crosstalk in PTH-treated osteoblasts, we obtained similar results as with rolipram-treated osteoblasts (Fig. 3b). These data indicate dual patterns of crosstalk between the PKA and MAPK pathways in rolipram- or PTH-activated osteoblasts, i.e., positive regulation of ERK and negative regulation of p38 MAPK via the PKA pathway.

Possible involvement of COX-2 in TRANCE and OPG mRNA expression induced by rolipram and PTH

Rolipram induces TRANCE mRNA expression in osteoblasts, resulting in increased osteoclast formation in

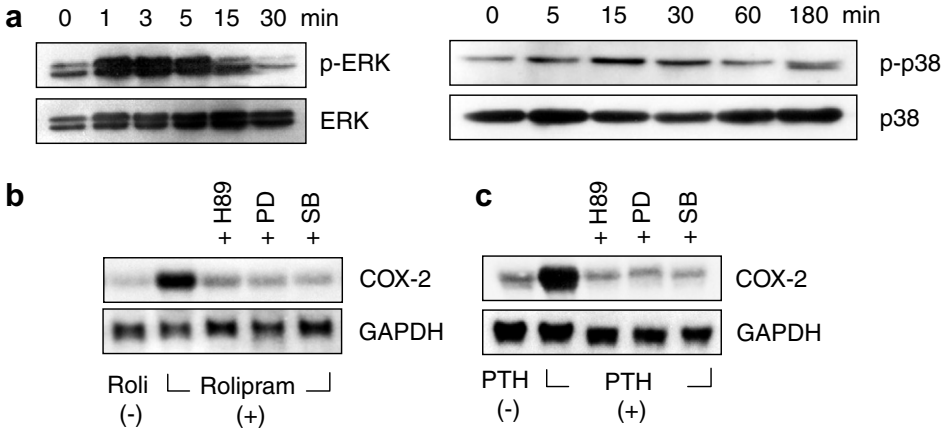

Fig. 2. Involvement of the PKA, ERK, and p38 MAPK pathways in COX-2 mRNA expression induced by rolipram or PTH. (a) Osteoblasts were treated with 10 μM rolipram for the indicated time intervals, and the activation of the ERK and p38 MAPK pathways was determined using an immunoblot analysis with anti-phospho ERK, ERK, phospho-p38, or p38 MAPK antibodies. (b,c) Osteoblasts were preincubated in the absence or presence of 30 μM H89, 50 μM PD98059, or 50 μM SB203580 for 30 min, and then treated with (+) or without (−) 10 μM rolipram (b) or 100 nM PTH (c) for 1 h. COX-2 mRNA expression was determined using a Northern blot assay.

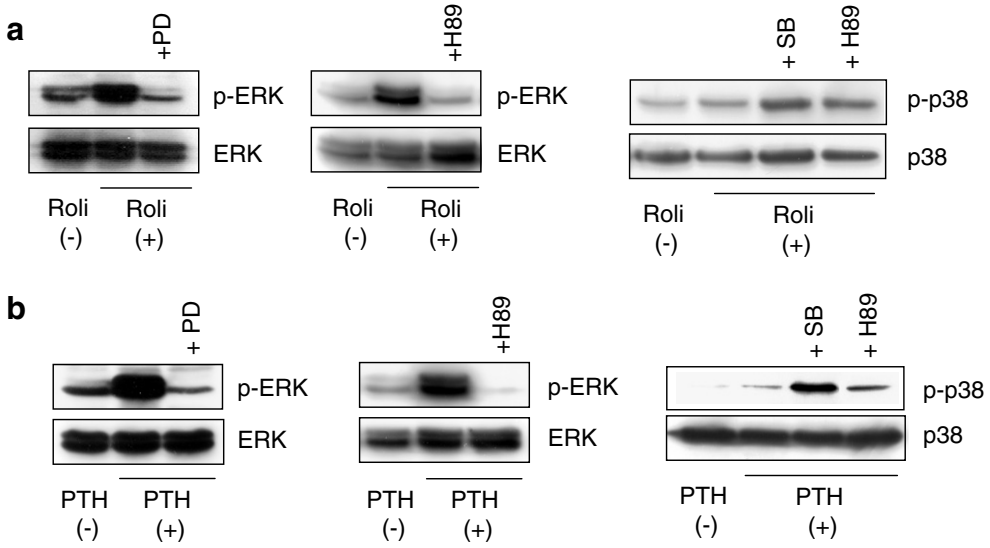

Fig. 3. Pattern of crosstalk between the PKA and MAPK signaling pathways activated by rolipram or PTH in osteoblasts. (a,b) Osteoblasts were preincubated in the absence or presence of 30 μM H89, 50 μM PD98059, or 50 μM SB203580 for 30 min, and then treated with (+) or without (−) 10 μM rolipram (a) or 100 nM PTH (b) for 5 min. Phospho-ERK, ERK, phospho-p38 MAPK, and p38 MAPK were detected by immunoblotting using specific antibodies.

co-cultures of mouse bone marrow cells and osteoblasts [12]. To explore the possibility that COX-2 induction by rolipram is involved in osteoclastogenesis, we investigated the effect of NS398, a COX-2-specific inhibitor, on osteoclast formation in co-cultures. Rolipram stimulated TRAP-positive osteoclast formation in co-cultures, but NS398 significantly suppressed the induction of osteoclast formation by rolipram (Fig. 4a).

Because COX-2-dependent PGE_2 induction in osteoclast precursors seems to be required for osteoclast differentiation [16], it is possible that NS398 suppressed osteoclast formation by inhibiting COX-2 activity in osteoclast precursors, not in osteoblasts. Thus, the effect of NS398 on TRANCE mRNA induction by rolipram was investigated in osteoblasts using a northern blot assay. Treatment with 10 μM rolipram increased TRANCE mRNA expression in osteoblasts, which was decreased by pretreatment with NS398 (Fig. 4b, left).

Because OPG also acts as a regulator of osteoclast formation by blocking the binding of TRANCE to its cognate signaling receptor TRANCE-R [5,6], we examined the involvement of COX-2 in OPG mRNA expression in rolipram-stimulated osteoblasts. Rolipram decreased OPG mRNA expression in osteoblasts, but pretreatment with NS398 reversed this effect (Fig. 4b, right).

Taken together, these results suggest that COX-2 mediates OPG mRNA expression, as well as that of TRANCE, in rolipram-stimulated osteoblasts. When we further inves-

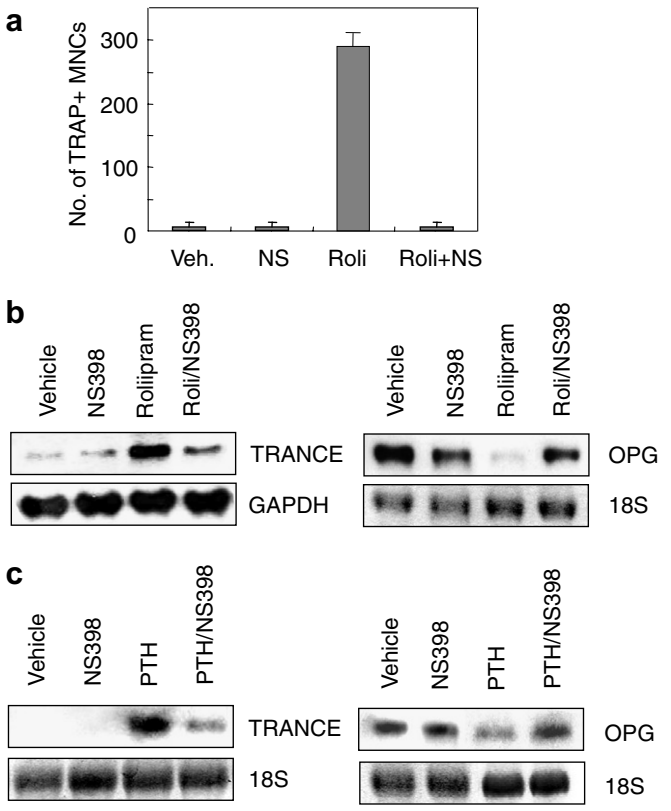

Fig. 4. Involvement of COX-2 in osteoclast formation induced by rolipram or PTH. (a) Mouse bone marrow cells and calvarial osteoblasts were co-cultured with or without 10 μM of rolipram in the presence or absence of 5 μM of NS398 for 6 days. Cells were then fixed and stained for TRAP. Data are expressed as means + SD of triplicate cultures. (b,c) Osteoblasts were treated with or without 10 μM rolipram (b) or 100 nM PTH (c) in the presence or absence of 5 μM NS398. TRANCE (left) and OPG (right) mRNA expression was examined using a Northern blot assay.

tigated the effect of NS398 on PTH-stimulated cells, similar results were obtained in TRANCE and OPG mRNA expression in osteoblasts (Fig. 4c), suggesting the similarity of rolipram- and PTH-induced signals.

Discussion

PTH-dependent osteoclast formation is inhibited by COX-2 inhibitors, and osteoclast formation is reduced by more than 50% in bone marrow culture or osteoblast/spleen co-culture from COX-$2^{-/-}$ mice [17]. Therefore, COX-2 is critical for PTH induction of osteoclast differentiation. The suppression of PTH-dependent osteoclast formation by COX-2 inhibitors was attributable not only to decreased TRANCE mRNA expression, but also increased OPG mRNA expression, resulting in a reduced TRANCE/OPG ratio (Fig. 4c). Given that the balance between TRANCE and OPG expression in bone is considered particularly important for the regulation of osteoclast differentiation [18], our results support the conclusions of an earlier study, which showed that COX-2 is required for maximal formation of osteoclast-like cells by PTH *in vivo* [17].

The PKA, ERK, and p38 MAPK pathways were also involved in COX-2 mRNA expression induced by PTH in osteoblasts (Fig. 2c). Specifically, PKA regulated the ERK and p38 MAPK pathways either positively or negatively (Fig. 3b). It is noteworthy that the alternative ERK and p38 MAPK pathways were involved in COX-2 mRNA expression induced by PTH, in addition to the well-known PKA pathways [14].

Rolipram, a PDE4 inhibitor, induces osteoclast formation and TRANCE mRNA expression in osteoblasts in a manner similar to that of PTH [12]. Given that PTH rapidly stimulates PDE4 activity in osteoblast cells, and PDE4 acts as a negative regulator of cAMP in PTH-treated osteoblasts [19], it is reasonable to expect a PDE4 inhibitor to replace the biological action of PTH by elevating intracellular cAMP in osteoblasts. Indeed, rolipram induced COX-2 expression via a transcriptional mechanism (Fig. 1a), which in turn regulated TRANCE and OPG mRNA, in manner similar to that of PTH (Fig. 4). Furthermore, the signaling pathways and their crosstalk patterns activated by rolipram were similar to those activated by PTH (Figs. 2 and 3). Thus, the PDE4 inhibitor appears to mimic the effects of PTH in osteoblasts.

Although PTH induces osteoclast formation in a mouse co-culture system, it has biphasic effects on osteoblast function that greatly depend on the intermittent vs. continuous administration of the hormone [20]; continuous exposure to PTH leads to a coupled increase in bone formation and bone resorption, with a net loss of bone mass. In contrast, injecting animals with PTH once daily produces a net anabolic effect *in vivo*. Thus, PTH was recently approved and is widely used as an anabolic bone therapy, despite its low cost efficacy and risk of side effects. Interestingly, it has been reported that rolipram, a selective PDE4 inhibitor, increases systemic bone mass in mice [21]. Other PDE4 inhibitors have also shows therapeutic effects against bone loss in some animal osteopenia models [22,23]. Therefore, PDE4 inhibitors are potential anabolic anti-osteoporosis drugs that could replace PTH. Further analysis of PDE4 inhibitors on osteoblasts will contribute to the development of new anti-osteoporosis drugs.

In conclusion, a PDE4 inhibitor induces COX-2 mRNA and protein expression, which in turn regulates TRANCE and OPG mRNA in osteoblasts. The PKA, ERK, and p38 MAPK pathways regulate the COX-2 mRNA expression induced by the PDE4 inhibitor; PKA is the central regulator of the ERK and p38 MAPK pathways. These effects of the PDE4 inhibitor on osteoblasts are similar to those of PTH, raising the possibility that PDE4 inhibitor could be used as a mimic of PTH in the regulation of osteoblast function.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.12.174.

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