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Rolipram, a Phosphodiesterase 4 Inhibitor, Stimulates Osteoclast Formation by Inducing TRANCE Expression in Mouse Calvarial Cells

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Phosphodiesterase (PDE) 4 is an enzyme that degrades intracellular cAMP. In the present study, the effect of rolipram, a specific phosphodiesterase (PDE) 4 inhibitor, on osteoclast formation was investigated. Rolipram induced osteoclast formation in cocultures of mouse bone marrow cells and calvarial osteoblasts. This activity was not observed in the absence of calvarial osteoblasts, suggesting that calvarial osteoblasts are likely target cells of rolipram. Osteoclast formation by rolipram was completely blocked by the addition of osteoprotegerin (OPG), a soluble decoy receptor for the osteoclast differentiation factor, TNF-related activation-induced cytokine (TRANCE, identical to RANKL, ODF, and OPGL). Northern blot analysis revealed the effect of rolipram to be associated with the increased expression of TRANCE mRNA in mouse calvarial osteoblasts. Collectively, these data indicate that PDE4 inhibitor up-regulates the TRANCE mRNA expression in osteoblasts, which in turn controls osteoclast formation.

Key words: Osteoblast, Osteoclast, cAMP, Phosphodiesterase 4 inhibitor, TRANCE

INTRODUCTION

Osteoclasts are multinucleated cells that resorb bone (Väänänen *et al.*, 2000), which are derived from hemopoietic precursor cells when incubated in contact with osteoblastic/stromal cells (Takahashi et al., 1988). It is well known that TNF-related activation-induced cytokine (TRANCE, also known as RANKL, ODF, or OPGL) is essential for osteoclastic differentiation in the presence of macrophage-colony stimulating factor (M-CSF). TRANCE is up-regulated in osteoblastic/stromal cells by osteotropic hormones or cytokines, such as parathyroid hormone (PTH), 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), interleukin (IL)-11 and prostaglandin E_2 (PGE_2) (Wong *et al.*, 1997; Suda *et al.*, 1999). The action of TRANCE is blocked by osteoprotegerin (OPG), which functions as a soluble decoy receptor (Takahashi *et al.*, 1999).

Intracellular cyclic phosphodiesterases (PDEs) are critical regulators of cyclic nucleotide homeostasis, and catalyze the hydrolysis of the 3', 5'-cyclic nucleotides, cAMP and cGMP to their corresponding nucleotide 5'-monophosphates (Essayan *et al.*, 2001). Thus, PDEs are critical determinants of the biological process mediated by these important second messengers. At present, the PDE superfamily is known to consist of 11 separate families, which are classified according to their substrate selectivity, inhibitor sensitivity and sequence. In many cases, these families are comprised of multiple subtypes (Essayan *et al.*, 2001). A number of inhibitors displaying various degrees of selectivity for different types of PDE have been developed.

Recently, the application of PDE inhibitors to the treatment of metabolic bone diseases has been attempted. In particular, the cAMP-specific PDE4 inhibitors have been shown to exert therapeutic effects against bone loss in some animal osteopenia models by promoting osteoblast differentiation (Miyamoto *et al.*, 1997; Waki *et al.*, 1999) Therefore, PDE4 inhibitors are considered to be promising candidates for anti-osteoporosis drug therapies. However, their effects on osteoclast formation remain to be elucidated.

In the present study, the effect of the PDE4 inhibitor, rolipram, on osteoclast formation was investigated. Rolipram strongly induced osteoclast formation in mouse cocultures of bone marrow cells and calvarial osteoblasts. Additional studies were performed to identify the mechanism by which rolipram induces osteoclastogenesis.

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MATERIALS AND METHODS

Cells

Primary calvarial osteoblasts were isolated from the calvariae of neonatal ddY mice (Japan SLC Inc., Shizuoka, Japan) using conventional methods (Suda *et al.*, 1997). ST-2, a mouse stromal cell line, and ATDC-5, a mouse chondrocytic cell line, were kindly provided by Prof. Masamichi Takami (Showa University, Tokyo, Japan). All cells were cultured in α-MEM/10% FBS at 37°C in 5% CO_2.

Osteoclast formation assay

Cocultures of mouse bone marrow cells and calvarial osteoblasts were performed as previously described (Suda *et al.*, 1997). Briefly, bone marrow cells were obtained by flushing tibiae from 4 to 6-week-old ddY male mice. Mouse bone marrow cells (1×10^5 cells/well) and calvarial osteoblasts (5×10^3 cells/well) were cocultured in the presence or absence of rolipram (5 μM) in 96-well plates for 6 days. Alternatively, bone marrow cells were cultured in α-MEM/10% FBS, supplemented with 5 ng/mL M-CSF, for 12 h to separate adherent and nonadherent cells. The nonadherent cells were then harvested and cultured with 30 ng/mL M-CSF in the presence or absence of 5 μM rolipram. After 3 days of culturing, the floating cells were removed and the attached cells used as osteoclast precursors (pOC). To generate osteoclasts, osteoclast precursors were cultured in 96-well plates (2×10^4 cells/well) with 30 ng/mL M-CSF and 300 ng/mL TRANCE in the presence or absence of 5 μM rolipram for 3 days. The cells were fixed and stained with tartrate-resistant acid phosphatase (TRAP) as described previously (Suda *et al.*, 1997). TRAP-positive cells with more than three nuclei were counted as multinucleated cells (MNCs).

RT-PCR analysis

Total RNA (1 μg) was reverse-transcribed using Superscript II (Invitrogen, CA, USA) according to the manufacturer's protocols. Aliquots of the obtained cDNA pool were subjected to PCR amplification with Go Taq DNA polymerase (Promega Co., WI, USA). The primers used for the mouse PDE4 subtypes, calcitonin receptor (CTR), cathepsin K and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) in this study are as follows: PDE4A, 5'-ggaactcacacacctgtcg-3' (forward), 5'-gttcttgtgctaagaggtcc-3' (reverse); PDE4B, 5'-tggaaatcctggctgccat-3 (forward), 5'-tccacagaagctgtgtgct-3' (reverse); PDE4C, 5'-tggtatcag-agtaggattcc-3' (forward), 5'-ctctgtgtaaaccttggctg-3' (reverse); PDE4D, 5'-cggaactcgctctgatgt-3' (forward), 5'-acagaggcg-ttgtgcttg-3' (reverse); CTR, 5'-tttcaagaaccttagctgccagag-3' (forward), 5'-caaggcacggacaatgttgagaag-3' (reverse); cathepsin K, 5'-cttccaatacgtgcagcaga-3' (forward), 5'-acg-caccaatatcttgcacc-3' (reverse); and GAPDH, 5'-gaaggt-cggtgtgaacggatttggc-3' (forward), 5'-catgtaggccatgaggtc-caccac-3' (reverse). The PCR program was as follows: 32 (all mouse PDE4 subtypes, CTR and cathepsin K) or 28 (GAPDH) cycles, after an initial denaturation step at 94°C for 3 minutes, then denaturation at 94°C for 30 seconds, annealing at 58°C for 45 seconds and extension at 72°C for 60 seconds, with a final extension at 72°C for 10 minutes.

Northern blot analysis

Calvarial osteoblasts were cultured in 60-mm diameter dishes with various concentrations of rolipram for the indicated periods, and then subjected to total RNA isolation using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. Total RNA (20 μg) was subjected to electrophoresis in 1.2% agarose-formaldehyde gels, transferred on nylon membrane filters (Hybond N^+, Amersham Biosciences, Buckinghamshire, UK), and hybridized with ^{32}P-labeled cDNA probes. cDNAs encoding the TRANCE and GAPDH cloned by the polymerase chain reaction (PCR) were used as probes. After the final washing of the membrane filter, it was exposed to X-ray film (BioMax, Kodak, Rochester, NY) at -70°C.

Statistical analysis

Statistical analysis was performed using Students *t*-test, with a p value of less than 0.05 was considered significant. All data are presented as the mean +S.D. of triplicate determinations.

RESULTS

PDE4 inhibition by rolipram induces osteoclast formation in mouse coculture system

The effect of rolipram, a selective PDE4 inhibitor, was examined on osteoclast formation using a coculture system of mouse bone marrow and calvarial cells. As shown in Fig. 1A and B, rolipram induced the formation of TRAP-positive MNCs. To determine whether the stimulatory effect of rolipram correlated with the expressions of the osteoclast-specific calcitonin receptor (CTR) and cathepsin K mRNA, total RNA was prepared and analyzed by RT-PCR. Fig. 1C shows the RT-PCR analyses of the CTR and cathepsin K mRNA expressions when treated with or without rolipram. The levels of CTR and cathepsin K were increased in the cultures treated with rolipram. Together, these results demonstrated that PDE4 inhibition by rolipram strongly induces osteoclasts in the mouse coculture system.

Rolipram does not stimulate osteoclast formation in the absence of calvarial osteoblasts

To determine the type of cells that respond to rolipram

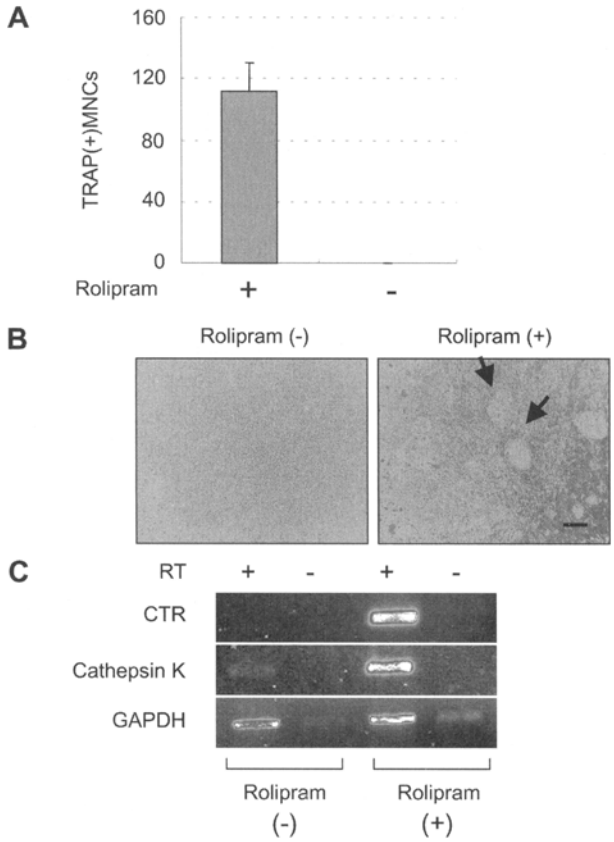

Fig. 1. Effect of rolipram, a PDE4 inhibitor, on the osteoclast formation in co-cultures. Mouse bone marrow and calvarial cells were cocultured for 6 days in the absence(-) or presence(+) of 5 μM rolipram. (A) TRAP-positive cells with more than three nuclei were counted as multinucleated cells (MNCs). Data are expressed as the mean+SD of triplicate cultures. (B) photographs of TRAP-stained osteoclasts induced by 5 μM rolipram (*right panel*). Vehicles were used as negative controls (*left panel*). (C) osteoclast-specific mRNA expression. Total RNA was prepared and reverse transcribed in the absence (-RT) or presence (+RT) of reverse transcriptase, and the cDNA amplified using specific primers designed for the mouse calcitonin receptor (CTR), cathepsin K and GAPDH genes. Bar, 200 μm.

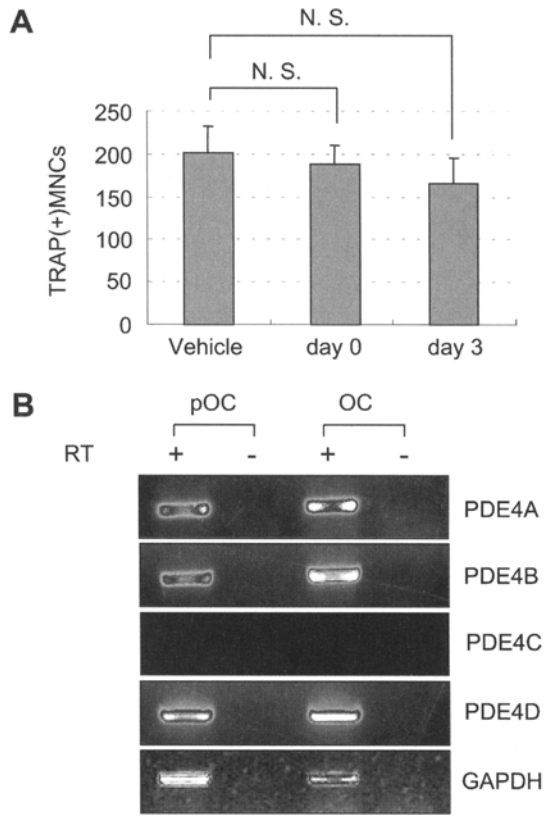

Fig. 2. (A) Effect of rolipram, a PDE4 inhibitor, on the osteoclast formation in bone marrow cells in the presence of M-CSF and TRANCE. Mouse bone marrow cells were cultured with M-CSF for 3 days. The osteoclast precursors were then treated with M-CSF and TRANCE for an additional 3 days. Rolipram was treated at day 0 or 3. TRAP-positive cells with more than three nuclei were counted as multinucleated cells (MNCs). Data are expressed as the mean+SD of triplicate cultures. N.S.=non significant. (B) RT-PCR analysis of PDE4 subtype mRNA. Total RNA was prepared from osteoclast precursors (pOC) and purified mature osteoclasts (OC). Templates for PCR were synthesized in the absence (-RT) or presence (+RT) of reverse transcriptase.

in cocultures, mouse bone marrow cells and calvarial osteoblasts were cultured separately in the presence or absence of rolipram. In the bone marrow cells, rolipram failed to stimulate osteoclast formation in the presence of M-CSF and TRANCE (Fig. 2A), suggesting that bone marrow cells are unlikely target cells of rolipram.

The PDE4 family consists of 4 subfamilies, A, B, C, and D, with each family member being encoded on separate genes localized to 3 chromosomes (Conti and Jin, 1999). To investigate the expression patterns of the PDE4 subtypes, osteoclast precursors were generated from mouse bone marrow cells by treatment with M-CSF (see Materials and methods). No TRAP-positive or multinucleated cells were observed in the osteoclast precursors preparation (Data not shown). From the RT-PCR using primers recognizing the unique sequences in the relevant mouse genes, products corresponding to the predicted size of PDE4A (276 bp), PDE4B (534 bp) and PDE4D (434 bp) were found in the osteoclast precursors (Fig. 2B). When the osteoclast precursors differentiated into osteoclasts after 3 days of culturing with M-CSF and TRANCE, no dramatic changes were observed in the expression patterns of the PDE4 subtypes (Fig. 2B).

Induced TRANCE expression in calvarial osteoblasts is involved in osteoclast formation by rolipram

Since rolipram appeared not to induce osteoclast formation by directly acting on bone marrow cells, the involvement of mouse calvarial cells was also examined. First, RT-PCR was performed to determine the mRNA expressions of the PDE4 subtypes in mouse calvarial

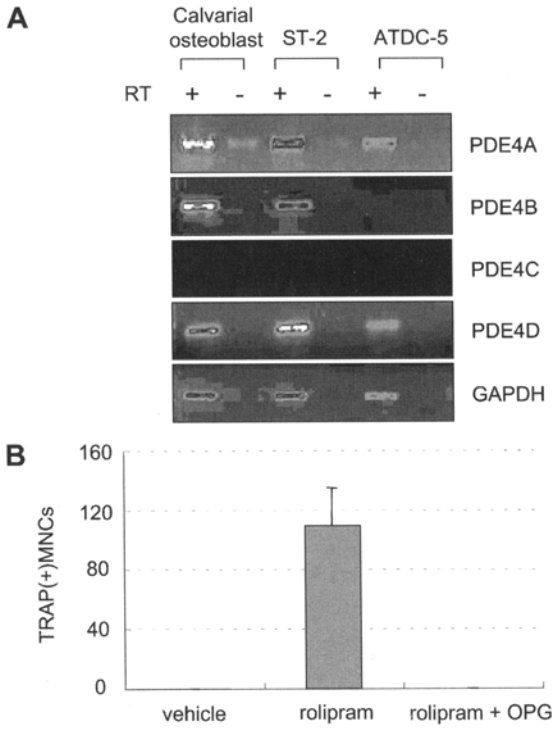

Fig. 3. (A) RT-PCR analysis of PDE4 subtype mRNA. Total RNA was prepared from calvarial osteoblast, ST2, and ATDC-5. Templates for PCR were synthesized in the absence (-RT) or presence (+RT) of reverse transcriptase. (B) Involvement of TRANCE for osteoclast formation induced by rolipram. Mouse bone marrow cells and calvarial osteoclasts were cocultured with or without 5 μM rolipram in the presence or absence of OPG (100 μg/mL) for 6 days. Data are expressed as the mean+SD of triplicate cultures.

cells, and compared these with the expression patterns in ST-2, a mouse bone-marrow stromal cell line able to differentiate into osteoblasts, and ATDC5, a mouse chondrogenic cell line. As shown in Fig 3A, calvarial osteoblasts expressed PDE4A, B and 4D, but not 4C. Similar results were also obtained with ST-2, but not with ATDC-5.

When the cocultures were treated with rolipram in the presence of OPG, a decoy receptor for TRANCE, the osteoclast formation was completely inhibited, indicating the involvement of TRANCE (Fig. 3B). To verify if PDE4 inhibition by rolipram was able to induce TRANCE gene expression, mouse calvarial osteoblasts were treated with rolipram for the indicated times. Northern blot analysis demonstrated that rolipram induced TRANCE mRNA with a peak at 3 h, which returned almost to the control levels within 24 h of treatment (Fig. 4A). To determine the dose dependence of rolipram-induced TRANCE mRNA expression, mouse calvarial osteoblasts were treated with various concentrations of rolipram for 3 h. As shown in Fig. 4B, rolipram treatment dose-dependently stimulated TRANCE mRNA expression with a maximum at 10 μM.

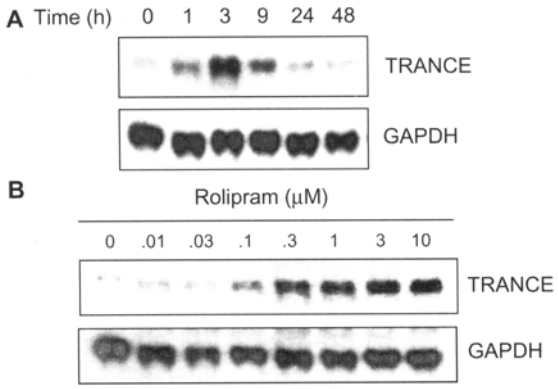

Fig. 4. Rolipram stimulates TRANCE expression in mouse calvarial cells. (A) Mouse calvarial cells were treated with 5 μM rolipram for the indicated times, or (B) with the indicated concentrations of rolipram for 3 h. Total RNA was extracted and analyzed by Northern blot using probes for TRANCE and GAPDH.

These results suggest that the rolipram-stimulated osteoclast formation was attributable to the up-regulation of TRANCE mRNA in calvarial osteoblasts.

DISCUSSION

PDE4 inhibitors are known to elevate the levels of intracellular cAMP by inhibiting the breakdown of cAMP by PDE4. In the present study, rolipram, a PDE4 inhibitor, was found to induce osteoclast formation in a mouse coculture system (Fig. 1). The effect of rolipram on osteoclastogenesis was revealed to correlate with the expression of TRANCE mRNA in mouse calvarial osteoblasts (Fig. 4).

PDE4 inhibitors have recently been reported to stimulate bone formation and systemic bone-mass *in vivo* (Kinoshita *et al.*, 2000). Consistent with this observation, several lines of evidence have shown that PDE4 inhibitors enhanced the differentiation of osteoblasts *in vitro* (Tsutusmimoto *et al.*, 2002; Wakabayashi *et al.*, 2002). In this study, however, rolipram was demonstrated to stimulate osteoclast formation. (Fig. 1). Our results seem to be contradictory to previous reports where PDE inhibitors have been shown to have anabolic effects (Kinoshita *et al.*, 2000; Miyamoto *et al.*, 1997; Waki *et al.*, 1999).

Interestingly, the biological activities of PDE4 inhibitors are very similar to those of parathyroid hormone (PTH). PTH induces osteoclast formation in a mouse coculture system by induction of the expression of TRANCE *via* a cAMP-mediated signaling pathway (Kondo *et al.*, 2002; Fu *et al.*, 2002), while once daily, or less, injections of PTH to animals are also known to produce a net anabolic effect (Tam *et al.*, 1982). However, continuous exposure to PTH leads to a coupled increase in bone formation and resorption, with a net loss of bone mass (Tam *et al.*, 1982). Similarly, Kinoshita *et al.* reported that PDE4

inhibitor increased the bone mass in experimental animals administered those drugs by daily injections. Therefore, the continuous administrations of PDE4 inhibitors may induce bone loss due to enhanced osteoclastogenesis. Thus, the application of PDE4 inhibitors for the treatment of osteoporosis needs to be further analyzed with regard to the conditions of administration.

The PDE4 family consists of four distinct genes, termed PDE4A, 4B, 4C and 4D, each of which encodes multiple variants generated by alternate splicing and/ or from transcriptional units (Houslay *et al.*, 1998). Numerous reports have indicated that these variants differ in their regulation, subcellular localization, protein-protein interaction and function (Houslay and Adams, 2003). Herein, the mouse calvarial cells were shown to express PDE4A, B and D at the mRNA level (Fig. 3A). Since the PDE4 inhibitors developed so far are nonselective, the question of distinct or overlapping functions of the PDE4s on osteoclast formation was unable to be addressed at this time. Further genetic strategy for PDE4 gene inactivation would contribute to improve our understanding of the function of the different PDE4 proteins in calvarial osteoblasts.

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