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ORIGINAL ARTICLE

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## Phosphodiesterase 4 inhibitor rolipram potentiates the inhibitory effect of calcitonin on osteoclastogenesis

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**Abstract** To assess the combination effect of calcitonin and the phosphodiesterase 4 inhibitor rolipram on osteoclastogenesis, adherent cell-depleted bone marrow cells from mouse tibia and femur (ACD-BMCs), which were cultured in the presence of 25 ng/ml colony-stimulating factor 1 (CSF-1) and 100 ng/ml soluble receptor activator of NF- $\kappa$ B ligand (sRANKL), were utilized. Calcitonin inhibited formation of tartrate-resistant acid phosphatase-positive multinucleated cells, as mature osteoclasts, by 70% even at 20 pM, whereas rolipram (10  $\mu$ M) scarcely affected osteoclast formation; in contrast, the combination of both agents led to significant inhibition of multinucleation and pit formation ability of osteoclasts. The combined administration of calcitonin and rolipram attenuated calcitonin receptor mRNA expression in comparison to treatment with either agent alone, whereas expression of RANK and CSF-1 receptor mRNAs was unchanged. Alone, these agents scarcely elevated intracellular cyclic AMP (cAMP) concentration; however, combination treatment with both agents significantly increased cAMP concentration in osteoclast

progenitors and osteoclasts. The combination effect was abolished by H-89, an inhibitor of protein kinase A. It appears that rolipram inhibited hydrolysis of cAMP formed by calcitonin in cells and potentiated the inhibitory effect of calcitonin on osteoclastogenesis. The escape phenomenon following calcitonin treatment may also be prevented by concomitant treatment with the phosphodiesterase 4 inhibitor.

**Key words** calcitonin · phosphodiesterase 4 · rolipram · bone marrow cells · osteoclastogenesis

### Introduction

The signal pathways of parathyroid hormone and calcitonin (CT), which regulate calcium metabolism, involve cyclic AMP (cAMP) as an intracellular second messenger following stimulation of the receptors on osteoblasts and osteoclasts, respectively [1]. Therefore, inhibition of hydrolysis of cAMP produced in response to these hormones is expected to potentiate the action of these hormones on bone cells. Cyclic nucleotide phosphodiesterase (PDE) is classified into 11 isoenzyme families, which differ in their substrate specificity, affinity for cyclic nucleotides, and regulatory properties [2–5]. Among these families, PDE 4 isoenzyme is a cAMP-specific PDE; furthermore, its inhibitors display selective bronchodilator activity [6,7] and antiinflammatory action [8]. Moreover, we recently reported that among PDE inhibitors the PDE 4 inhibitor specifically increased osteoblast formation and decreased osteoclast formation in *in vitro* bone marrow culture; additionally, we identified a therapeutic effect of PDE 4 inhibitors against bone loss in some animal osteopenia models [9,10]. We also indicated that the effects of PDE 4 inhibitors on osteoblast and osteoclast formation synergistically inhibit hydrolysis of cAMP in the progenitor cells produced by prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) secreted by bone marrow stromal cells [11,12].

Osteoclast progenitor cells are stimulated and their maturation is promoted via membrane interaction with

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osteoblasts/stromal cells through the receptor activator of nuclear factor  $\kappa$ B (RANK), which is a receptor activator of the nuclear factor  $\kappa$ B ligand (RANKL) system [13,14]. Osteoclast formation and maturation are promoted by a production ratio of RANKL versus osteoprotegerin (OPG; a decoy receptor for RANKL secreted by stromal cells; also referred to as osteoclastogenesis inhibitory factor, OCIF) following stimulation of osteoblastic stromal cells by osteotropic factors such as  $1\alpha,25$ -dihydroxyvitamin  $D_3$  ( $1,25(OH)_2D_3$ ), para thyroid hormone (PTH), or interleukin 11 (IL-11) [15,16]. Therefore, osteoclastogenesis is regulated by interaction with stromal cells and stimulation involving several osteotropic factors. On the other hand, it is well known that CT directly inhibits osteoclast formation and its bone resorption activity [17–19].

This study examined the direct effect of CT on osteoclastogenesis and the combination effect of the PDE 4 inhibitor rolipram employing adherent cell-depleted bone marrow cells from mouse tibia and femur (ACD-BMCs), which were cultured in the presence of 25 ng/ml colony-stimulating factor 1 (CSF-1, or M-CSF) and 100 ng/ml soluble RANKL (sRANKL).

## Methods

### Materials and animals

Rolipram (Wako, Osaka, Japan), naphthol AS-MX phosphate sodium, fast red violet LB salt (Sigma, St. Louis, MO, USA), salmon calcitonin (CT; Bachem Feinchemikalien, Bubendorf, Switzerland), and *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinoline-sulfonamide (H-89; Seikagaku Kogyo, Tokyo, Japan) were purchased from the respective commercial sources. Recombinant human CSF-1 and recombinant human sRANKL were acquired from PeproTech (London, England). Male ddY mice (8 weeks of age; Nippon SLC, Hamamatsu, Japan) were used randomly in this study.

### Preparation of ACD-BMCs

Suspensions (2 ml) of bone marrow cells obtained from mouse tibiae and femurs were gently introduced into the Sephadex G-10 (Amersham Pharmacia Biotech, Bucks, U.K.) column system according to the method of Ly and Mishell [20]. Cells were infiltrated with the carrier; subsequently, the cell suspension was introduced to 8 ml of the medium, followed by incubation at 37°C for 45 min. Next, the eluted suspension of nonadherent bone marrow cells was collected and used as ACD-BMCs.

### Primary culture of ACD-BMCs

According to a method described by Niida et al. [21], ACD-BMCs were cultured to generate osteoclasts. Wells of 96-well plates were coated with 5  $\mu$ l of a solution containing

625 ng/ml CSF-1 and 2.5  $\mu$ g/ml sRANKL in fetal bovine serum (FBS) and dried for 1 h on a clean bench. The ACD-BMCs were prepared for  $1.5 \times 10^6$  cells/ml with alpha-minimum essential medium ( $\alpha$ -MEM) (pH 7.0) containing 15% FBS; subsequently, cell suspensions (125  $\mu$ l/well) were seeded into 96-well plates. At 3 days after culture, the medium was changed to  $\alpha$ -MEM containing 15% FBS, 25 ng/ml CSF-1, and 100 ng/ml sRANKL in the presence or absence of calcitonin and rolipram. The treatment was conducted for 2 days, after which cultures were fixed and stained in 0.1 mg/ml naphthol AS-MX phosphate sodium, 0.6 mg/ml fast red violet LB salt, and 50 mM sodium tartrate/0.1 M sodium acetate (pH 5.0) for tartrate-resistant acid phosphatase (TRAP). Samples were washed with distilled water, dried, and observed under a light microscope. Those cells positive for TRAP and exhibiting more than three nuclei were counted as mature osteoclasts.

The absence of stromal cells or osteoblast progenitors in the cell population was confirmed from bone-type alkaline phosphatase (ALP) mRNA expression. Moreover, ALP-positive cells or nodules were never observed as a result of treatment with or without rolipram under these conditions, whereas PDE 4 inhibitors, including rolipram, exerted an osteoblastic action on stromal or bone marrow cells [9–11].

### Pit assay

ACD-BMCs were plated on dentine slices and cultured in  $\alpha$ -MEM containing 15% FBS, 25 ng/ml CSF-1, and 100 ng/ml sRANKL for 6 days [22]. Further incubation consisted of 48-h treatment of cells on the slices in the presence or absence of calcitonin and rolipram. After culture, the slices were treated with 0.05 M ammonium hydroxide for 30 min, and cells were removed from the dentine slices. Backscattered electron images of the dentine slices were examined with a Hitachi S-2500CX scanning electron microscope [23].

### Reverse transcription-polymerase chain reaction assay

mRNAs were prepared from cultures utilizing a QuickPrep micro mRNA purification kit (Pharmacia Biotech, Uppsala, Sweden). Synthesis of cDNA from isolated mRNA was conducted with RNase H-reverse transcriptase and analyzed for expression of mouse RANK, CSF-1 receptor (CSF-1R), and calcitonin receptor (CT-R) transcripts by reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR was performed using primers per details described previously for  $\beta$ -actin [24]. Primers for mouse RANK were 5'-GCTTGCTGCATAAAGTCTG-T-3' and 5'-ACGT CCTAGAATCTCTGACT-3' (708 bp); primers for mouse CSF-1 receptor were 5'-AACAAGTTCTACAACTGGT GAAGG-3' and 5'-GAAGCCTGTAGTCTAAGCATC TGTC-3' (752 bp); primers for mouse calcitonin receptor were 5'-AGAATTCCTGCATCCACCTA-3' and 5'-TGAAAGCGTTGCACAGAGTA-3' (558 bp) (cited from GenBank sequence database); and primers for  $\beta$ -actin were

5'-TTCTACAATGAGCTGCGTGTGGC-3' and 5'-CTC(A/G)TAGCTCTTCTCCAGGGAGGA-3' (456bp), as reported by Waki et al. [24].

### Measurement of cyclic AMP

Intracellular cyclic AMP (cAMP) was measured with a cAMP EIA system (Amersham International, Little Chalfont, U.K.).

### Data analysis

Data were analyzed using Student's *t* test to compare the unpaired means of two sets of data. A *P* value of 0.05 indicated a significant difference between sets of data.

## Results

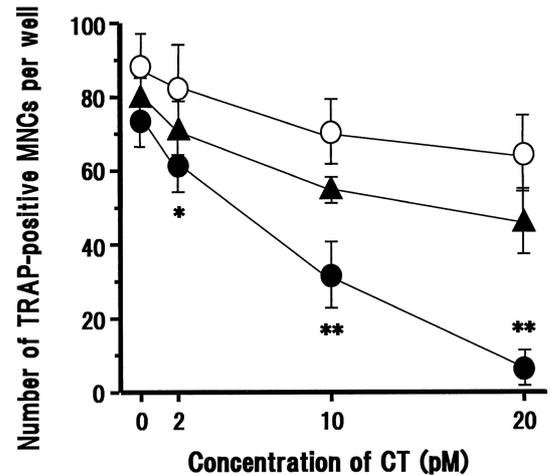
### Effect on osteoclast formation in culture of ACD-BMCs

ACD-BMCs were treated with CT (2–20pM) and/or rolipram (10μM) in the presence of 25ng/ml CSF-1 and 100ng/ml sRANKL; subsequently, the number of TRAP-positive MNCs was counted. As shown in Fig. 1, CT reduced the number of TRAP-positive MNCs by approximately 70% even at 20pM; furthermore, rolipram (10μM) alone also inhibited MNC formation somewhat. However, rolipram synergistically potentiated the inhibitory effect of CT on osteoclast formation. Figure 1 also exhibits abolition of the combination effect by an inhibitor of protein kinase A, H-89 (10μM).

Following combined treatment with CT (10pM) and rolipram (10μM), DNA content in the culture was unchanged (data not shown). In addition, numerous TRAP-positive mononuclear cells were present in the culture,

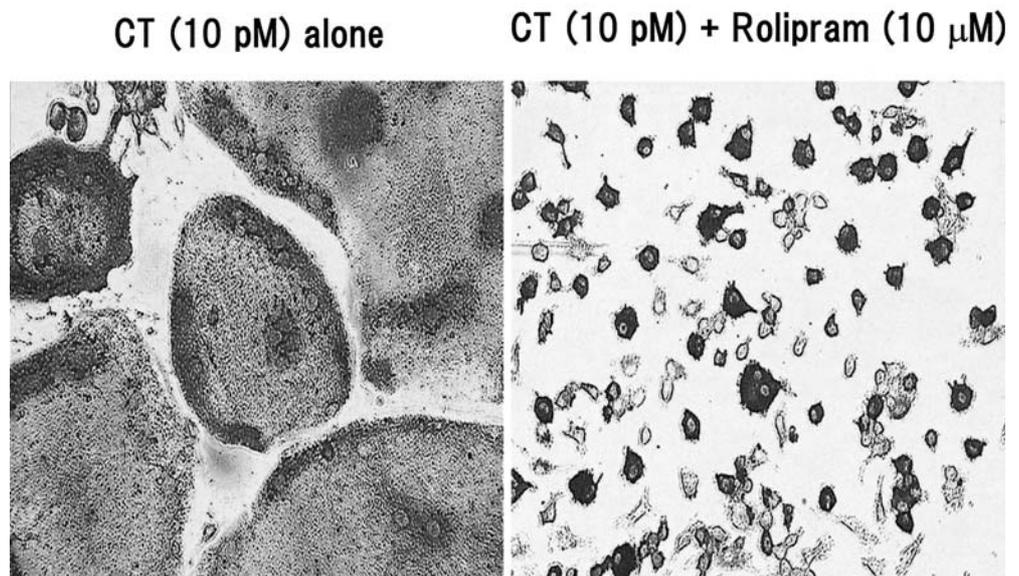
although TRAP-positive MNCs were observed after CT administration alone (Fig. 2).

Figure 3 illustrates the pit formation ability of osteoclasts after treatment with CT (10pM) and rolipram (10μM). Many lacunae were formed on the dentine slice of untreated controls and on samples treated with calcitonin alone; in contrast, combination treatment involving CT and rolipram abolished both osteoclast formation and lacunae resorption.

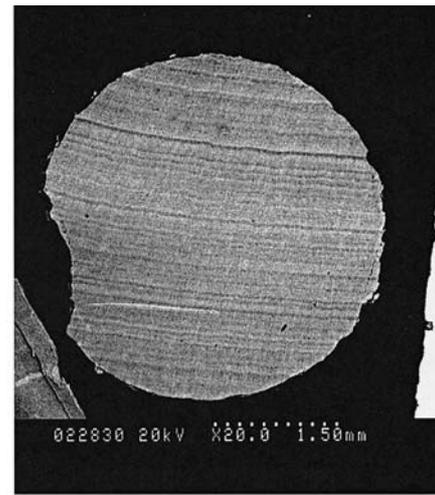
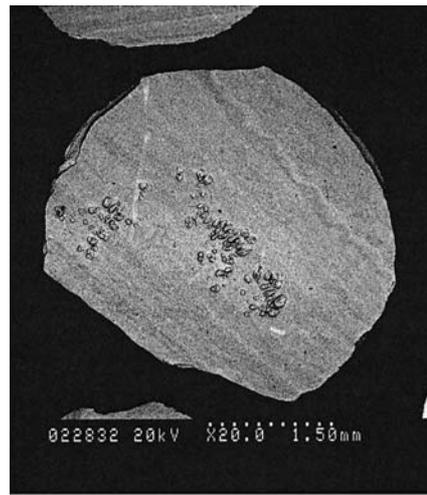


**Fig. 1.** Effect of rolipram on formation of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs) in adherent cell-depleted bone marrow cells (ACD-BMCs). Cells were cultured with 25 ng/ml colony-stimulating factor 1 (CSF-1) and 100 ng/ml soluble receptor activator of NF-κB ligand (sRANKL) (osteoclastogenesis conditions) for 5 days and the indicated concentrations of calcitonin (CT) in the absence or presence of 10μM rolipram and/or 10μM H-89 for the final 2 days. After culture, cells were fixed and stained for TRAP. ○, CT alone; ●, CT plus rolipram; ▲, CT plus rolipram plus H-89. Each value is the mean ± SD of six experiments. \*\*\*Significantly different from the CT-only cultures at *P* < 0.05 and 0.01, respectively

**Fig. 2.** Phase-contrast micrographs of ACD-BMC cultures. Cells were cultured with 10pM CT with (left) or without (right) 10μM rolipram under osteoclastogenesis conditions. After culture for 5 days, cells were fixed and stained for TRAP

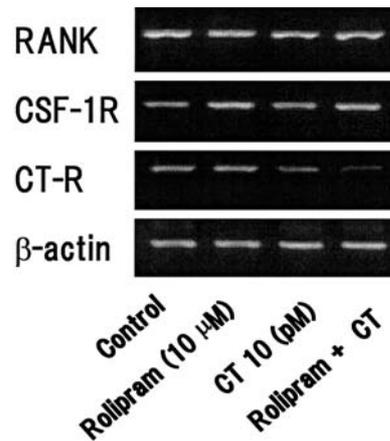


**Fig. 3.** Scanning electron micrographs of dentine slice cultured with ACD-BMCs. ACD-BMCs were cultured on a dentine slice with 25 ng/ml CSF-1, 100 ng/ml sRANKL, and 10 pM CT with (left) or without (right) 10  $\mu$ M rolipram. Backscattered electron images of dentine slices are presented



CT (10 pM) alone

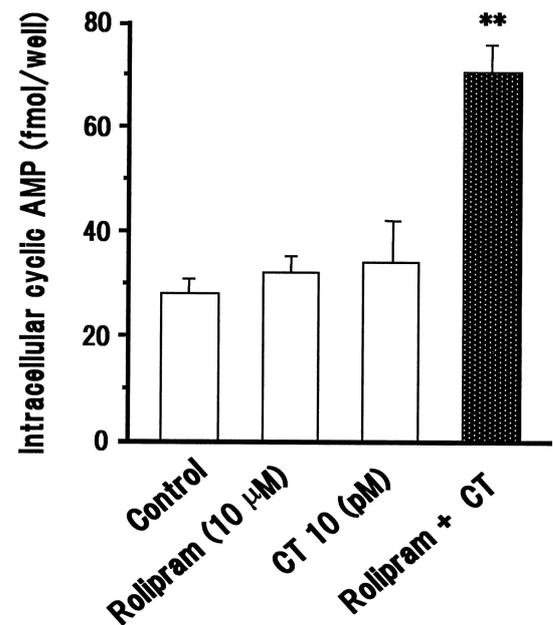
CT (10 pM) + Rolipram (10  $\mu$ M)



**Fig. 4.** mRNA expression of several factors for osteoclast differentiation in ACD-BMCs. ACD-BMCs were cultured for 5 days in the absence or presence of 10  $\mu$ M rolipram and/or 10 pM CT under osteoclastogenesis conditions; subsequently, expression of *RANK*, *CSF-1R*, *CT-R*, and  $\beta$ -*actin* mRNAs was analyzed via reverse transcription-polymerase chain reaction (RT-PCR). These experiments were conducted four times; standard results are represented

#### Analysis of mRNA expression

mRNAs of *RANK* and *CSF-1R* in ACD-BMCs were expressed from the early stage of culture with sRANKL and CSF-1; however, expression of *CT-R* mRNA increased with the culture period and maturation of osteoclasts [12]. CT (10 pM) slightly inhibited expression of *CT-R* mRNA, but not that of *RANK* and *CSF-1R* mRNAs; furthermore, expression of *CT-R* mRNA was markedly decreased upon combination with rolipram (10  $\mu$ M), which scarcely altered the expression of these mRNAs alone (Fig. 4).



**Fig. 5.** Effect of rolipram and CT on intracellular cyclic AMP (cAMP) production in ACD-BMCs. After culture for 3 days under osteoclastogenesis conditions, cells were treated with 10  $\mu$ M rolipram and/or 10 pM CT, and the concentration of intracellular cAMP was determined after 1 h. Each value is the mean  $\pm$  SD of six experiments. \*\*Significantly different from the control at  $P < 0.01$

#### Effect on intracellular cAMP concentration in osteoclasts

CT (10 pM) and/or rolipram (10  $\mu$ M) were introduced to osteoclasts, and intracellular cAMP concentration was measured for 1 h. Figure 5 demonstrates that intracellular cAMP concentration was little changed following addition of CT or rolipram alone; in contrast, cAMP levels increased significantly following combined treatment with CT and rolipram.

## Discussion

When ACD-BMCs were cultured in the presence of 25 ng/ml CSF-1 and 100 ng/ml sRANKL for 5 days to mature osteoclasts, the inhibitory effects of CT on osteoclast multinucleation and pit formation were synergistically potentiated by combination with the PDE 4 inhibitor rolipram (see Figs. 1–3). We previously reported that expression of PDE 4 isoenzyme among some PDE isoenzymes increased during culture of rat BMCs [11] and that the PDE 4 inhibitors enhanced osteoblastogenesis and diminished osteoclastogenesis, resulting in a therapeutic effect against bone loss in some animal osteopenia models [9,10]. Furthermore, we indicated that PDE 4 inhibitors promote osteoblast formation and inhibit osteoclast formation via enhancement of the action of cAMP in their progenitor cells produced by endogenous prostaglandin  $E_2$  (PGE<sub>2</sub>) [11,12]. Thus, the PGE<sub>2</sub> receptors are expressed on the plasma membrane of both osteoblasts and osteoclasts, but the signal pathways of parathyroid hormone and CT involve cAMP as an intracellular second messenger after stimulation of their receptors on osteoblasts and osteoclasts, respectively [1]. Therefore, PDE 4 inhibitors may exert a complex influence on bone metabolism *in vivo*.

This investigation confirmed that the PDE 4 inhibitor rolipram potentiates the inhibitory effect of CT on osteoclastogenesis through cAMP production in osteoclast progenitors. It has been reported that CT immobilizes and contracts osteoclasts away from the bone surface through the action of cAMP enhanced because of stimulation of CT-R [25]; however, osteoclasts continuously exposed to CT can escape the effects of CT as a result of rapid profound reduction in CT-R mRNA expression [26]. In this study, following 5-day CT exposure, its effect appeared to be weak (see Figs. 1, 3); however, it inhibited the expression of CT-R mRNA but not that of RANK or of CSF-1R mRNAs (see Fig. 4). These results may indicate the escape phenomena of osteoclasts from CT. On the other hand, in combination with rolipram, the number, particularly in the case of multinuclear cells, and the function of osteoclasts were significantly attenuated; additionally, CT-R expression decreased further. These findings suggest that addition of a PDE 4 inhibitor during CT treatment prevents the escape phenomena with respect to CT and inhibits osteoclastogenesis through the action of elevated cAMP in osteoclast progenitors.

Recently, several PDE 4 inhibitors have been developed for asthma and chronic obstructive pulmonary disease and have proceeded to late-phase clinical trial [27], although rolipram cannot be used because of its severe side effects, such as emesis [28]. Therefore, novel PDE 4 inhibitor drugs may be candidates for osteoporosis therapy consequent to their anabolic actions and potentiation of CT action.

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