Prostaglandin E₂ (PGE₂) has been proposed to be a potent stimulator of bone resorption. However, PGE₂ itself has been shown to directly inhibit bone-resorbing activity of osteoclasts. We examined the role of PGE₂ in the function of mouse osteoclasts formed in vitro. Bone marrow macrophage osteoclast precursors expressed PGE₂ receptors EP1, EP2, EP3β, and EP4, and the expression of EP2 and EP4 was down-regulated during osteoclastic differentiation induced by receptor activator of NF-κB ligand and macrophage colony-stimulating factor. In contrast, functional EP1 was continuously expressed in mature osteoclasts. PGE₂ as well as calcitonin caused intracellular Ca²⁺ influx in osteoclasts. However, PGE₂ and 17-phenyltrinitol-PGE₂ (an EP1 agonist) failed to inhibit actin-ring formation and pit formation by osteoclasts cultured on dentine slices. When EP4 was expressed in osteoclasts using an adenovirus carrying EP4 cDNA, both actin-ring and pit-forming activities of osteoclasts were inhibited in an infectious unit-dependent manner. Treatment of EP4-expressing osteoclasts with PGE₂ further inhibited their actin-ring and pit-forming activities. Such inhibitory effects of EP4-mediated signals on osteoclast function are similar to those that are calcitonin receptor-mediated. Thus, osteoclast precursors down-regulate their own EP2 and EP4 levels during their differentiation into osteoclasts to escape inhibitory effects of PGE₂ on bone resorption.

Osteoclasts are bone-resorbing multinucleated cells derived from the monocyte-macrophage lineage (1–3). The differentiation and activation of osteoclasts are tightly regulated by osteoblasts or bone marrow-derived stromal cells (4–6). Osteoblasts express two cytokines essential for osteoclast differentiation; they are receptor activator of NF-κB ligand (RANKL)³ and macrophage colony stimulating factor (M-CSF)⁴. RANKL stimulates osteoclast precursors in a cell-to-cell contact-dependent manner and synergistically with M-CSF stimulates the differentiation of mouse bone marrow-derived macrophages (BMMΦ), osteoclast precursors, into osteoclasts (25, 26). We have recently shown that the synergistic effect of PGE₂ on

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† The abbreviations used are: RANKL, receptor activator for NF-κB; M-CSF, macrophage colony-stimulating factor; PGE₂, prostaglandin E₂; PKA, protein kinase A; PKC, protein kinase C; CT, calcitonin; ifa, infectious unit; 1α,25(OH)₂D₃, 1α,25-dihydroxyvitamin D₃; BMMΦ, bone marrow-derived macrophages; F-actin, filamentous actin; α-MEM, α-modified minimum essential medium; FBS, fetal bovine serum; 1,25(OH)₂D₃, 1α,25-dihydroxyvitamin D₃; interleukin 11 (IL-11), IL-1, and lipopolysaccharide (7, 9). Osteoclast precursors differentiate into mature osteoclasts in the presence of RANKL and M-CSF (10, 11). Mature osteoclasts as well as osteoclast precursors express RANK, a receptor of RANKL. RANKL also induces bone-resorbing activity of osteoclasts (4, 7, 8).

Bone-resorbing osteoclasts form unique cellular structures such as clear zones and ruffled borders toward the bone surface (12). The clear zone, also called the “actin ring,” consists of a ring-like alignment of filamentous actin (F-actin) dots and surrounds the ruffled border, from which protons and lysosomal enzymes are secreted into the resorption lacunae (13). Calcitonin (CT), a bone resorption-inhibiting hormone, disrupts actin rings and inhibits the pit-forming activity of osteoclasts cultured on bone or dentine slices (14, 15). Osteoclasts express abundant CT receptors, which are coupled to Gαs and Gqα proteins (16). We have previously shown that signals mediated by adenosine 3′,5′-cyclic monophosphate (cAMP)-dependent protein kinase A (PKA) play important roles in CT-induced inhibition of pit formation and actin ring formation by osteoclasts cultured on dentine slices (17).

Prostaglandin E₂ (PGE₂) has diverse biological activities in a variety of tissues (18). The actions of PGE₂ in the target cells are mediated by four different G protein-coupled receptor subtypes, EP1, EP2, EP3, and EP4 (19). The EP subtypes differ in tissue distribution, ligand binding affinity, and coupling to intracellular signaling pathways. The signal of EP1 predominantly increases intracellular Ca²⁺ and activates protein kinase C (PKC) (19). EP2 and EP4 activate Gqα followed by increases in adenylate cyclase activity, adenosine cAMP production, and PKA activity in the target cells. In contrast, EP3 acts via Gαq to inhibit cAMP generation.

PGE₂ has been proposed to be a potent stimulator of bone resorption involved in inflammatory diseases such as rheumatoid arthritis (20–23). However, the mechanism of PGE₂-induced bone resorption has not yet been clearly explained. Like other osteotropic factors, PGE₂ stimulates expression of RANKL in osteoblasts (21, 24). Among PGE₂ receptor subtypes, EP4 has been shown to mainly mediate PGE₂-induced RANKL expression in osteoblasts (24). In addition, PGE₂ directly and synergistically with RANKL and M-CSF stimulates the differentiation of mouse bone marrow-derived macrophages (BMMΦ), osteoclast precursors, into osteoclasts (25, 26). We have recently shown that the synergistic effect of PGE₂ on

TRAP, tartrate-resistant acid phosphatase; IBMX, 3-isobutyl-1-methylxanthine; RT, reverse transcription; Ad-LacZ, adenovirus carrying β-galactosidase cDNA; Ad-EP4, adenovirus carrying EP4 cDNA.
RANKL-induced osteoclast differentiation is mediated through EP2 and EP4. Transforming growth factor-β-activated kinase 1 (TAK1) acts as an adapter molecule linking PKA-induced signals and RANKL-induced signals in osteoclast precursors (27). Thus, PGE2 stimulates osteoclastic bone resorption through two pathways, the induction of RANKL expression by osteoblasts and direct enhancement of RANKL-induced osteoclast differentiation of the precursors.

Paradoxically, PGE2 has been shown to inhibit bone-resorbing activity of osteoclasts when added to osteoclasts cultured on bone or dentine slices (28, 29). Lerner et al. (30) also reported that PGE2 transiently inhibited bone resorption and the release of lysosomal enzymes in mouse calvarial cultures. This inhibitory effect of PGE2 on bone resorption may be due to a direct activation of adenylate cyclase in mature osteoclasts, thereby mimicking the effect of CT. Thus, the role of PGE2 in the function of mature osteoclasts is still a matter of controversy.

In the present study we explored the role of PGE2 in osteoclast function. Osteoclast precursors of BMMφ expressed EP1, EP2, EPβ3, and EP4, but mature osteoclasts expressed only EP1. PGE2 affected neither the actin ring-forming nor the resorption pit-forming activity of osteoclasts cultured on dentine slices. Forced expression of EP4 in osteoclasts suppressed both activities of osteoclasts. The inhibitory effects of the EP4 expression on osteoclast function were quite similar to those of CT. These results suggest that the down-regulation of EP2 and EP4 in osteoclast precursors during osteoclastic differentiation is an important event for escaping the inhibitory effect of PGE2 on bone resorbing activity.

**Experimental Procedures**

**Chemicals**—Recombinant human RANKL and M-CSF (Leukoprol) were purchased from PeproTech EC Ltd. (London) and Kyowa Hakko Kogyo Co. (Tokyo), respectively. PGE2, bacterial collagenase, and Pronase E were obtained from Sigma. 3-Isobutyl-1-methylxanthine (IBMX) and 17-phenylethynyl-PGE2 were from BIOMOL Research Laboratory Inc. (Plymouth Meeting, PA). 1α,25(OH)2D3 was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Ectoncin, a synthetic analogue of calcitonin, was kindly provided by Asahi Kasei Pharma (Tokyo). Fura Red AM, fluo-4 AM, and Pluronic F127 were from Molecular Probes Inc. (Eugene, OR). All other chemicals were of analytical grade.

**Cultures of Bone Marrow-derived Macrophages**—BMMφ were prepared as osteoclast precursors as described previously (31). Briefly, bone marrow cells were obtained from tibiae of 5–8-week-old male ddY mice by flushing femur cavities. Animals were anaesthetized at 56°C for 2–4 h. BMMφ were prepared for 2 days with M-CSF (50 ng/ml). The adherent cells, most of which expressed macrophage-specific antigens such as Mac-1, F4/80, were used as BMMφ.

**Survival Assay of Purified Osteoclasts**—Osteoclasts were generated in co-cultures of mouse primary osteoblasts and bone marrow cells in collagen gel-coated dishes as described previously (32). Primary osteoblasts and bone marrow cells were co-cultured in α-MEM supplemented with 10% FBS in 100-mm tissue culture dishes (Corning Inc., Corning, NY) precoated with type I-collagen gel (Nitta Gelatin, Osaka, Japan) in the presence of 1α,25(OH)2D3 (10−6 M). After the cells were cultured for 7 days, all cells were recovered from the dishes by treatment with 0.2% collagenase. The purity of osteoclasts in this preparation was about 95%. To purify osteoclasts the crude osteoclast preparation was plated in 100-mm tissue culture dishes. After the cells were cultured for 6 h, osteoclasts were removed by treatment of cells with phosphate-buffered saline (PBS(−)) containing 0.001% Pronase E and 0.02% EDTA for 5 min. The purity of osteoclasts in this preparation was about 95%. For the osteoclast survival assay, purified osteoclasts were further incubated for the indicated periods in the presence or absence of test chemicals and stained for tartrate-resistant acid phosphatase (TRAP, a marker enzyme of osteoclasts) as described. TRAP-positive multinucleated cells containing more than three nuclei were counted as viable osteoclasts.

**Assay of cAMP Production**—To measure the amount of cAMP produced, cells cultured in 48-well plates (BMMφ, 8 x 104/well; purified osteoclasts, 2 x 104 cells/well) were preincubated for 5 min at 37°C in α-MEM containing 1 mM IBMX, then incubated for 5 min at 37°C with [3H]cAMP (10−10 M) or PGE2 (10−6 M). Cells were washed with ice-cold
phosphate-buffered saline containing 1 mM IBMX and then lysed. The content of intracellular cAMP was determined using a cAMP enzyme immunoassay kit (Amersham Biosciences) according to the manufacturer's instructions. The results were expressed as the mean ± S.D. of quadruplicate cultures.

Measurement of Intracellular Ca$^{2+}$ Influx—The effects of PGE$_2$ and calcitonin on intracellular Ca$^{2+}$ influx in mature osteoclasts and in BMMø were measured using a confocal laser scanning microscope (LSM510, Carl Zeiss, Jena, Germany) according to the methods described previously (27). Osteoclasts or BMMø were incubated in a glass-bottom dish (ASAHI TECHNO GLASS Corp., Tokyo) for 6 h. Cells were incubated in the presence of 5 μM fluo-4 AM, 5 μM Fura Red AM, and 0.05% pluronic F127 for 30 min in serum-free Dulbecco's modified Eagle's medium (Sigma). Cells loaded with these dyes were washed twice with α-MEM and postincubated in α-MEM containing 10% FBS. Cells were further washed three times with Hank's balanced salt solution and then excited at 498 nm, and emission at 505–530 nm for fluo-4 and 600–680 nm for Fura Red were acquired simultaneously at 2-s intervals. The ratio of the fluorescence intensity of the fluo-4 to Fura Red was calculated to estimate intracellular Ca$^{2+}$ concentrations in single cells.

Construction of Adenovirus—A pBluescript vector containing the entire open reading frame of EP4 was a kind gift from Dr. S. Narumiya (Kyoto University, Japan). The EP4 cDNA was inserted into the ApaI-XbaI site of a p-shuttle vector (BD Clontech Laboratories, Inc. Palo Alto, CA), and the DNA fragment was inserted into the PI-SceI/-uiSite of a pAdenoX vector (BD Clontech Laboratories, Inc.). Two micrograms of the pAdenoX vector containing EP4 cDNA linearized by PacI was transduced using PEF transfection reagents (Promega Corp., Madison, WI). Amplified crude viral stocks were purified by CsCl gradient ultracentrifugation and used for the infection. Adenovirus carrying β-galactosidase cDNA (Ad-LacZ) was also generated and used for the control infection. The infectious units (ifu) were determined using an Adeno-X rapid titration kit (BD Clontech Laboratories, Inc.).

Infection of Osteoclasts with Adenovirus Carrying EP4 cDNA—Infection of osteoclasts with Ad-LacZ or adenovirus carrying EP4 cDNA (Ad-EP4) was carried out according to the methods described previously (33). Primary osteoclasts and bone marrow cells were co-cultured for 4 days in collagen gel-precotted dishes (100-mm diameter). Co-cultures were then incubated for 1 h with 2 ml of α-MEM containing the indicated ifu of recombinant adenoviruses and 10% FBS and further incubated with 15 ml of α-MEM with 10% FBS in the presence of 1α, 25(OH)$_2$D$_3$ (10$^{-8}$ M) and 1α, 25(OH)$_2$D$_3$ (10$^{-5}$ M) for an additional 48 h.

Statistical Analysis—For the determination of cAMP production, data were expressed as the mean ± S.D. of quadruplicate cultures. For the real-time PCR analysis, osteoclast survival assay, and pit forming assay, representative data were expressed as the mean ± S.D. of three determinations. All experiments were independently repeated three times, and similar results were obtained. Statistical analyses were performed using Student's t test.

RESULTS

Expression of PGE$_2$ Receptor Subtypes in Osteoclast Precursors and Mature Osteoclasts—We first analyzed the expression of PGE$_2$ receptor subtypes in BMMø, osteoclast precursors, and mature osteoclasts. Purified osteoclasts were prepared from co-cultures grown on collagen gel-coated dishes as described under “Experimental Procedures.” Total RNA was extracted from BMMø and purified osteoclasts, and cDNA was synthesized from the total RNA. The expression of EP1, EP2, EP3α, EP3β, EP3γ, EP4, CT receptor, and glyceraldehyde-3-phosphate dehydrogenase mRNAs was detected by PCR in the presence (+) or absence (−) of reverse transcriptase. B, effects of PGE$_2$, 1α, 25(OH)$_2$D$_3$, or RANKL on expression of EP2 and EP4 mRNAs in BMMø. BMMø prepared from bone marrow cells were cultured with or without PGE$_2$ (10$^{-5}$ M), 1α, 25(OH)$_2$D$_3$ (10$^{-7}$ M), or RANKL (100 ng/ml) in the presence of M-CSF (50 ng/ml) for 24 h or cultured with or without RANKL (100 ng/ml) in the presence of M-CSF for 72 h. Total RNA was extracted from these cultures. The expression levels of EP2 mRNA (upper panel) and of EP4 mRNA (lower panel) were determined by quantitative real-time RT-PCR as described under “Experimental Procedures.” The ratio of the expression in the culture treated with PGE$_2$, 1α, 25(OH)$_2$D$_3$, or RANKL to that in the control culture was calculated. Results are expressed as the mean ± S.D. of triplicate cultures. * and #, Significantly different between the treated and the control culture; * p < 0.01; # p < 0.05.

Expression of EP4 mRNA but not EP2 mRNA in BMMø (Fig. 1B). Treatment of BMMø with RANKL induced osteoclast differentiation of BMMø in the presence of M-CSF. Expression of both EP2 and EP4 mRNAs was significantly down-regulated in BMMø treated with RANKL for 24 h (Fig. 1B). Parallel experiments showed that the purified osteoclast preparation and the BMMø culture treated with RANKL for 72 h contained more than 95 and 65% TRAP-positive cells, respectively (Fig. 1C). The BMMø culture treated with RANKL for 24 h contained less than 2% TRAP-positive cells (data not shown). These results suggest that EP2 and EP4 are down-regulated in osteoclasts during osteoclastic differentiation.

Function of EP Subtypes Expressed in Osteoclast Precursors and Mature Osteoclasts—We next examined the effects of PGE$_2$ on cAMP production in BMMø and mature osteoclasts in com-
comparison with those of CT. PGE₂ (10⁻⁶ M) but not CT (10⁻⁷ M) significantly stimulated cAMP production in BMMφ (Fig. 2A, *upper panel*). In contrast, CT (10⁻⁶ M) but not PGE₂ (10⁻⁶ M) stimulated cAMP production in purified osteoclasts (Fig. 2A, *lower panel*). These results suggest that functional EP2 and EP4 are expressed in osteoclast precursors but not in mature osteoclasts.

EP1 has been shown to be coupled to Ca²⁺ signals, which induce the formation of inositol 1,4,5-trisphosphate and diacylglycerol (18). CT receptors activate not only Gαq but also Gαq, the signals of which cause an increase in cytosolic Ca²⁺. We next examined whether EP1 is functional in mature osteoclasts and BMMφ. Treatment of osteoclasts with PGE₂ at 10⁻⁵ M sharply increased the intracellular Ca²⁺ influx in osteoclasts (Fig. 2, *B* and *C*). PGE₂ at 10⁻⁶ M, a concentration that was sufficient to activate adenylate cyclase in BMMφ, did not induce clear Ca²⁺ influx in osteoclasts (data not shown). Ca²⁺ influx in osteoclasts was also induced by the addition of CT (10⁻¹⁰ M) (Fig. 2D). PGE₂ at 10⁻⁵ M increased the Ca²⁺ influx in BMMφ as well (Fig. 2E). These results suggest that functional EP1 is expressed in both mature osteoclasts and BMMφ.

**Role of EP1 in Osteoclast Function**—We previously showed that purified osteoclasts spontaneously died within 48 h due to apoptosis (32). Not only RANKL but also CT was shown to stimulate the survival of osteoclasts (35). We next examined the effects of PGE₂ on the survival of osteoclasts in comparison with those of RANKL and CT (Fig. 3). Purified osteoclasts gradually died during the incubation for 36 h, and RANKL (100 ng/ml) and CT (10⁻⁶ M) significantly promoted the survival of osteoclasts (Fig. 3, *A* and *B*). However, PGE₂ (10⁻⁶ M) showed no effect on the survival (Fig. 3, *A* and *B*). The effect of a higher concentration of PGE₂ (10⁻⁵ M) on the survival of osteoclasts was also examined because PGE₂ at the concentration of 10⁻⁵ M increased the intracellular Ca²⁺ influx in osteoclasts and BMMφ (Fig. 3C). The survival of osteoclasts was not supported
Down-regulation of EP2/4 in Mouse Osteoclasts

In the present study we examined the role of PGE2 in the function of mouse osteoclasts formed in vitro. BMMφ osteoclast precursors expressed EP1, EP2, EP3β, and EP4, and expression of EP2 and EP4 was down-regulated during the differentiation of BMMφ into osteoclasts. In contrast, functional EP1 was continuously expressed in mature osteoclasts. PGE2 as well as CT induced intracellular Ca2+ influx in osteoclasts. However, CT but not PGE2 inhibited formation of actin rings and resorption pits by osteoclasts cultured on dentine slices. When EP4 was expressed in osteoclasts using adenovirus Ad-EP4, the actin ring- and pit-forming activities of osteoclasts were strongly inhibited in an ifu-dependent manner. PGE2 further inhibited both of these activities in osteoclasts infected with Ad-EP4. Thus, mouse osteoclasts formed in vitro expressed functionally active EP1 but not EP2 and EP4. However, EP1-mediated signals did not appear to regulate the bone-resorbing activity of osteoclasts.

RT-PCR analysis showed that the level of expression of EP2 and EP4 mRNAs in mature osteoclasts was much lower than that in BMMφ (Fig. 1). It was reported that PGE2 suppressed

**FIG. 4. Effect of PGE2 on actin ring formation and pit formation by osteoclasts.** Crude osteoclast preparations were cultured on dentine slices in the presence or absence of PGE2 (10−7 to 10−5 M), 17-phenyltrinol-PGE2 (an EP1 agonist, 10−6 and 10−5 M), or CT (10−10 M). After cells were cultured for 48 h the dentine slices were recovered. A, some dentine slices were processed for F-actin staining (upper panels, bar = 25 μm). The other slices were processed for Mayer's hematoxylin staining to visualize resorption pits (lower panels, bar = 100 μm). B, percentages of resorbed area on dentine slices. Percentages of resorbed area relative to the total surface were determined using an image analysis system. The values were expressed as the mean ± S.D. of triplicate cultures. * Significantly different from the control cultures; p < 0.01.

**FIG. 5. Effect of forced expression of EP4 in osteoclasts on the cAMP production.** Co-cultures grown on collagen gel-coated dishes (100-mm diameter) were infected with either Ad-LacZ or Ad-EP4 on day 4 at a dose of 109 ifu. A, expression of EP4 in purified osteoclasts infected with Ad-LacZ (lane 1) or Ad-EP4 (lane 2). Osteoclasts were purified on 35-mm dishes. Total RNA was extracted from purified osteoclasts and used for RT-PCR with an EP4 receptor-specific primer that hybridized to both endogenous and exogenous EP4 mRNAs. B, effect of PGE2 on cAMP production in osteoclasts infected with Ad-LacZ or Ad-EP4. Purified osteoclasts were preincubated for 5 min with IBMX (1 μM) and incubated for 5 min with PGE2 (10−6 M). The amount of intracellular cAMP was determined by enzyme-linked immunosorbent assay. The values were expressed as the mean ± S.D. of quadruplicate cultures. * Significantly different from the control culture infected with Ad-LacZ; p < 0.01. # Significantly different from the control culture infected with Ad-EP4; p < 0.05. G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

by either PGE2 or 17-phenyltrinol-PGE2 (an EP1 agonist) even at 10−5 M. These results suggest that EP1-mediated signals do not affect the osteoclast survival.

When osteoclasts were cultured for 48 h on dentine slices, the osteoclasts formed actin rings and resorption pits on the slices (Fig. 4, A and B). Treatment of osteoclasts with CT (10−7 M) strongly inhibited their actin ring- and pit-forming activities. In contrast, neither PGE2 (10−2 to 10−5 M) nor 17-phenyltrinol-PGE2 (an EP1 agonist, 10−5 M) affected actin ring formation or pit formation by osteoclasts cultured on dentine slices (Fig. 4, A and B). Thus, mature osteoclasts expressed functional EP1, but EP1-mediated signals did not appear to induce appreciable effects on osteoclast function in our assay systems.

**Inhibition of Osteoclast Function by Forced Expression of EP4—** Our final question was why osteoclast precursors lose EP2 and EP4 during the differentiation into osteoclasts; that is, what are the functional consequences of the disappearance of EP2 and EP4 in osteoclasts. To address this question, EP4 was expressed in osteoclasts using Ad-EP4 (Fig. 5). RT-PCR analysis showed that osteoclasts purified from the co-cultures that had been infected with Ad-EP4 at 109 ifu expressed high levels of EP4 mRNA, but those infected with Ad-LacZ did not (Fig. 5A). To examine whether EP4 expressed in the infected osteoclasts is functional, the concentration of intracellular cAMP was determined in Ad-EP4- and Ad-LacZ-infected osteoclasts in the presence or absence of exogenous PGE2 (Fig. 5B). Intracellular cAMP levels were significantly higher in osteoclasts infected with Ad-EP4 than in those infected with Ad-LacZ even in the absence of exogenously added PGE2 (Fig. 5B). PGE2 (10−6 M) significantly increased cAMP production in Ad-EP4-infected osteoclasts but not in Ad-LacZ-infected osteoclasts. These results suggest that the EP4 expressed in osteoclasts is functionally active.

We finally examined the effects of the forced expression of EP4 on the function of osteoclasts (Fig. 6). Crude osteoclast preparations from co-cultures infected with Ad-EP4 or Ad-LacZ were placed on dentine slices, and the cells were further cultured for 48 h in the presence or absence of PGE2 or CT. Similar numbers of TRAP-positive cells were observed on the slices cultured with cells expressing Ad-EP4 and Ad-LacZ (Fig. 6A). However, the number of actin rings formed in EP4-expressing osteoclasts was markedly decreased. When PGE2 (10−6 M) was added to the culture, actin rings in osteoclasts expressing Ad-LacZ remained unchanged, but those in osteoclasts expressing Ad-EP4 disappeared completely (Fig. 6A). The pit-forming activity of osteoclasts was not affected by Ad-LacZ but was markedly inhibited by Ad-EP4 infection in an ifu-dependent manner (Fig. 6B and C). The addition of PGE2 (10−6 M) to the cultures of osteoclasts infected with Ad-LacZ had no inhibitory effects on the pit-forming activity (Fig. 6B and C). However, PGE2 significantly suppressed the pit-forming activity of osteoclasts infected with Ad-EP4 at 1 × 108 ifu. CT (10−10 M) completely inhibited the pit-forming activity of osteoclasts infected with either Ad-LacZ or Ad-EP4 (Fig. 6C). Thus, EP4-mediated signals were similar to those mediated by CT receptors in osteoclasts.

**DISCUSSION**

In the present study we examined the role of PGE2 in the function of mouse osteoclasts formed in vitro. BMMφ osteoclast precursors expressed EP1, EP2, EP3β, and EP4, and expression of EP2 and EP4 was down-regulated during the differentiation of BMMφ into osteoclasts. In contrast, functional EP1 was continuously expressed in mature osteoclasts. PGE2 as well as CT induced intracellular Ca2+ influx in osteoclasts. However, CT but not PGE2 inhibited formation of actin rings and resorption pits by osteoclasts cultured on dentine slices. When EP4 was expressed in osteoclasts using adenovirus Ad-EP4, the actin ring- and pit-forming activities of osteoclasts were strongly inhibited in an ifu-dependent manner. PGE2 further inhibited both of these activities in osteoclasts infected with Ad-EP4. Thus, mouse osteoclasts formed in vitro expressed functionally active EP1 but not EP2 and EP4. However, EP1-mediated signals did not appear to regulate the bone-resorbing activity of osteoclasts.

RT-PCR analysis showed that the level of expression of EP2 and EP4 mRNAs in mature osteoclasts was much lower than that in BMMφ (Fig. 1). It was reported that PGE2 suppressed
the expression of EP4 mRNA in mouse peritoneal macrophages in a cAMP/PKA-dependent manner (34). In our experiments PGE2 and 1α,25(OH)2D3 significantly decreased the expression of EP4 mRNA but not EP2 mRNA in BMMφ within 24 h (Fig. 1). On the other hand, expression of both EP2 and EP4 mRNAs were decreased in PGE4-treated BMMφ with PKC. These results suggest that osteotropic factors can modulate the expression of EP subtypes in osteoclast precursors, but down-regulation of EP2 and EP4 in osteoclasts is a consequence of the decision to differentiate into osteoclasts, probably for other appropriate purposes.

Osteoclasts expressed functionally active EP1 as well as CT receptors. Treatment of osteoclasts with PGE2 at 10^{-5} M or with CT sharply increased the intracellular Ca^{2+} influx to a similar extent (Fig. 2). Several studies have shown that intracellular calcium is involved in calcitonin-induced inhibition of osteoclast function (36–38). Zhang et al. (39) reported that the increase in cytosolic calcium induced by CT decreased tyrosine phosphorylation of Pyk2, which in turn disrupted actin rings in osteoclasts. Activation of protein kinase C but not PKA was also shown to mediate the inhibitory effect of CT on human osteoclast function (40). In our experiments actin ring formation and pit formation by osteoclasts were strongly inhibited by calcitonin but not by PGE2 or 17-phenyltrinol-PGE2, an EP1 agonist (Fig. 4). Survival of osteoclasts was also supported by CT but not by PGE2 or 17-phenyltrinol-PGE2 (Fig. 3). These results suggest that osteoclasts express functionally active EP1, but EP1-mediated signals do not play appreciable roles in osteoclast function, at least in our assay systems.

CT receptors are coupled to Go6 and Go4 proteins that activate cAMP/PKA and Ca^{2+}/PKC signals, respectively. Nicholson et al. (41) reported that forskolin, an activator of adenylate cyclase, induced a synergistic effect with CT in stimulating cAMP production in isolated rat osteoclasts and augmented the hypocalcemic response to CT in vivo. We also showed that cAMP/PKA signals were involved in the CT-induced inhibition of actin ring formation and pit formation by osteoclasts (17). Knaoka et al. (42) reported that CT inhibited nitric oxide-induced apoptosis of osteoclasts in vitro through cAMP/PKA signals. Thus, cAMP/PKA signals play important roles in the inhibition of osteoclast function and the promotion of osteoclast survival induced by CT.

One important question we addressed was why osteoclast progenitors lose EP2 and EP4 in the process of the differentiation into osteoclasts. Forced expression of EP4 in osteoclasts strongly inhibited actin ring formation and pit formation even in the absence of exogenous addition of PGE2, suggesting that EP4 expressed in osteoclasts may respond to PGE2 produced endogenously. In fact, the cAMP concentration in osteoclasts infected with Ad-EP4 was significantly higher than that in osteoclasts infected with Ad-LacZ (Fig. 5). The addition of PGE2 to the cultures of EP4-expressing osteoclasts enhanced cAMP production and inhibited the actin ring-forming activity and pit-forming activity of those osteoclasts. The EP4-induced inhibitory effects on the osteoclast function were similar to CT receptor-induced ones. These results further support the notion that cAMP/PKA but not Ca^{2+}/PKC signals inhibit osteoclast function. Down-regulation of EP2 and EP4 in osteoclast precursors during osteoclastic differentiation appears to be an important event for escaping the inhibitory effect of PGE2 on bone-resorbing activity (Fig. 7).

Several reports have indicated that PGE2 acts as a direct inhibitor of bone resorption in isolated rat, chick, and rabbit osteoclasts. Osteoclasts continuously express EP1, but the role of EP1 in osteoclast function is not known. When EP4 is expressed in osteoclasts, PGE2 directly inhibits the pit-forming activity of the osteoclasts through a cAMP/PKA-dependent mechanism. Thus, the EP4-mediated signals in osteoclasts are similar to those mediated by CT receptors. Thus, PGE2 becomes a potent bone resorption-stimulating factor as a result of the disappearance of EP2 and EP4 from mature osteoclasts. The down-regulation of EP2 and EP4 in osteoclast precursors during osteoclastic differentiation is an important event for escaping the inhibitory effect of PGE2 on bone resorption. CTR, calcitonin receptor.
osteoclasts (28, 29, 43). A pharmacological study using specific agonists for each EP subtype showed that isolated rat osteoclasts possess EP2 (44). Rabbit osteoclasts have been shown to express EP4 (29). We used mouse osteoclasts formed in vitro, but not authentic osteoclasts, in this study. Therefore, it is possible that authentic mouse osteoclasts may express EP2 and EP4 and decrease their function in response to PGE2. However, even if osteoclasts express EP2 and EP4, the expression level must be extremely low. PGE2 does not inhibit but rather stimulates bone resorption in a mouse organ culture system using fetal or newborn calvariae and long bones (45–48). Unlike PGE2, calcitonin completely inhibits not only osteotropic factor-induced bone resorption but also spontaneously occurring bone resorption in such organ culture systems. These results further support our conclusion that osteoclast precursors lose EP2 and EP4 to escape the inhibitory effects of PGE2 on bone resorption (Fig. 7).

We and others have shown that PGE2 synergistically enhanced RANKL-induced osteoclastic differentiation of BMMΦ through EP2 and EP4 (25–27). Recently, we found that TAK1 (transforming growth factor-β-activated kinase 1), a signal adapter molecule, plays important roles in cross-talk between cAMP-PKA signals and RANKL-induced signals in PGE2-enhanced RANKL-induced osteoclast differentiation (27).

Our results suggest that osteoclast precursors express EP2 and EP4, through which PGE2 synergistically enhances RANKL-induced osteoclast differentiation (Fig. 7). After the differentiation into osteoclasts is decided, osteoclasts would lose EP2 and EP4 to escape the inhibitory effects of PGE2. In addition, PGE2 strongly induces RANKL expression in osteoblasts through EP2/EP4-mediated signals. Thus, PGE2 is a potent bone resorption factor with multiple effects on osteoclast precursors and osteoblasts.

In contrast to the above in vitro studies, many in vivo studies have shown that the PGE series have stimulatory effects on bone formation (49–57). Sibonga et al. (52) and Keila et al. (53) independently reported that administration of PGE2 even into aged rats increased bone mass due to an increase in osteoblasts. The findings obtained from in vivo studies suggest that EP4 and EP2 are targets for therapeutic intervention in metabolic bone diseases. Further studies will be necessary to elucidate the molecular mechanism of PGE2-induced bone formation in vivo.

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23. Sibonga et al. (52) and Keila et al. (53) independently reported that administration of PGE2 even into aged rats increased bone mass due to an increase in osteoclasts over osteoblasts. It was also demonstrated that PGE2 and bipedal stance exercise synergistically prevented cancellous bone loss induced by ovarectomy in aged rats (54).

Local administration of PGE2 has been shown to increase alveolar bone loss induced by ovariectomy in aged rats (54). Local administration of PGE2 in aged rats increased bone mass due to an increase in osteoblasts. These results further support our conclusion that osteoclast precursors lose EP2 and EP4 to escape the inhibitory effects of PGE2 on bone resorption (Fig. 7).

We and others have shown that PGE2 synergistically enhanced RANKL-induced osteoclastic differentiation of BMMΦ through EP2 and EP4 (25–27). Recently, we found that TAK1 (transforming growth factor-β-activated kinase 1), a signal adapter molecule, plays important roles in cross-talk between cAMP-PKA signals and RANKL-induced signals in PGE2-enhanced RANKL-induced osteoclast differentiation (27).

Our results suggest that osteoclast precursors express EP2 and EP4, through which PGE2 synergistically enhances RANKL-induced osteoclast differentiation (Fig. 7). After the differentiation into osteoclasts is decided, osteoclasts would lose EP2 and EP4 to escape the inhibitory effects of PGE2. In addition, PGE2 strongly induces RANKL expression in osteoblasts through EP2/EP4-mediated signals. Thus, PGE2 is a potent bone resorption factor with multiple effects on osteoclast precursors and osteoblasts.

In contrast to the above in vitro studies, many in vivo studies have shown that the PGE series have stimulatory effects on bone formation (49–57). Sibonga et al. (52) and Keila et al. (53) independently reported that administration of PGE2 even into aged rats increased bone mass due to an increase in osteoclasts over osteoblasts. It was also demonstrated that PGE2 and bipedal stance exercise synergistically prevented cancellous bone loss induced by ovarectomy in aged rats (54).

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