Intercellular Calcium Signaling Occurs between Human Osteoblasts and Osteoclasts and Requires Activation of Osteoclast P2X7 Receptors

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Signaling between osteoblasts and osteoclasts is important in bone homeostasis. We previously showed that human osteoblasts propagate intercellular calcium signals via two mechanisms: autocrine activation of P2Y receptors, and gap junctional communication. In the current work we identified mechanically induced intercellular calcium signaling between osteoblasts and osteoclasts and among osteoclasts. Intercellular calcium responses in osteoclasts required P2 receptor activation but not gap junctional communication. Pharmacological studies and reverse transcriptase-PCR amplification demonstrated that human osteoclasts expressed functional P2Y1 receptors, but, unexpectedly, desensitization of P2Y1 did not block calcium signaling to osteoclasts. We also found that osteoclasts expressed functional P2X7 receptors and showed that pharmacological inhibition of these receptors blocked calcium signaling to osteoclasts. Thus these studies show that calcium signaling between osteoblasts and osteoclasts occurs via activation of P2 receptors, but that different families of P2 receptors are required for calcium signaling in these two cell types. Intercellular calcium signaling among bone cells is therefore amenable to pharmacological manipulation that will specifically affect only bone-forming or bone-resorbing cells. P2 receptors may be important drug targets for the modulation of bone turnover.

Osteoclasts are the cells responsible for bone resorption, whereas osteoblasts deposit new bone throughout a lifetime. Bone resorption and formation are coordinated, and in adult life are maintained in a balance, so that no significant bone loss occurs. In later life, especially in women after menopause, osteoclast activity is increased relative to osteoblast activity, and this unbalanced cellular activity causes increased relative bone resorption, and in turn, bone loss and osteoporosis. Both endocrine and paracrine factors modulate osteoclast activity, including calcitropic, growth, sex, and adrenal hormones as well as cytokines, growth factors, electrolytes, and mechanical forces. Most of these paracrine and endocrine factors affect osteoclast activity indirectly, acting on osteoclasts. Thus, bone-resorptive signals must be transmitted from osteoclasts to osteoclasts via mechanisms of cell-to-cell communication between the two lineages.

We have previously shown that mechanical stimulation of human osteoclasts in vitro generates a calcium signal that is communicated to other osteoclasts (1, 2). The propagation of this signal involves two different mechanisms. One is the autocrine action of ATP on plasma membrane purinergic receptors of the P2Y subtype, and the other involves the passage of a soluble messenger through gap junctions, leading to influx of extracellular calcium.

Recent studies have revealed the presence of nucleotide receptors in osteoclasts. ATP can act on two different classes of receptors. The P2Y receptors are G-protein-coupled and stimulate phospholipases, subsequently activating the inositol 1,4,5-triphosphate pathway releasing calcium from intracellular stores. Members of the P2X family are non-selective cation channels permeable to Na⁺, K⁺, Ca²⁺, and H⁺. Receptors from both classes have been identified on bone-resorbing cells (3, 4), as determined both by pharmacological profiles and effector functions. Thus, ATP induces both a non-selective cation current (P2X-mediated) and release of calcium from intracellular stores (P2Y-mediated) (3). P2Y2 mRNA has been demonstrated in giant cells from a human osteoclastoma by RT-PCR, although the significance of this finding is unclear because P2Y2-mediated responses were not present as UTP failed to increase intracellular calcium concentration (Ca²⁺). (5, 6). Other investigators have identified nucleotide-mediated responses in osteoclastic cells. ATP has been shown to cause a transient decrease in intracellular pH, possibly related to activation of P2X cation channels (7). This response might favor formation of osteoclastic resorption pits and increased bone resorption. Supporting this hypothesis, ATP induces osteoclast activation and resorption in rat osteoclasts (8) and in giant cells from human osteoclasts (9). ATP can also modulate osteoclast activity in vitro (10). P2Y1 receptors are widely distributed in bone and contribute to osteoclast activation. Recent studies have shown that P2Y1 receptors mediate ATP-promoted signaling in osteoclasts (11). P2Y1 receptor activation has been shown to lead to calcium mobilization and changes in intracellular pH (12). P2X7 receptors are expressed in human osteoclasts (13). ATP can cause calcium mobilization and changes in intracellular pH in human osteoclasts through activation of P2X7 receptors (14). P2X7 receptors are involved in calcium signaling between osteoclasts and osteoblasts (15). P2X7 receptors are also expressed in human osteoclasts (16). ATP can cause calcium mobilization and changes in intracellular pH in human osteoclasts through activation of P2X7 receptors (17).

1 Naemsch, L. N., Dixon, S. J., and Sims, S. M. (2001) J. Biol. Chem. 276, 39107–39114

2 The abbreviations used are: RT, reverse transcriptase; [Ca²⁺], intracellular calcium concentration; PBS, phosphate-buffered saline; TRAP, tartrate-resistant acid phosphatase activity; TBS, Tris-buffered saline; BzATP, benzoylbenzyl-ATP; OB, osteoblasts; OC, osteoclasts; M-CSF, macrophage colony stimulating factor; α-ATP, oxidized ATP; RANKL, receptor activator of NF-κB ligand; ATPγS, adenosine 5’-O-(thiotriphosphate).
osteoclastoma (6). The effect on osteoclast formation is, however, biphasic, because low concentrations increase osteoclast formation while high concentrations decrease mouse osteoclast formation (8).

In this study we have investigated the propagation of calcium transients from primary osteoblasts to osteoclasts from human bone marrow and among osteoclasts. This intercellular signal propagation might represent a mechanism by which signals initiated by mechanical stimulation of bone cells, primarily osteocytes, are diffused through the bone tissue to surface osteoblasts and in turn to osteoclasts, thus regulating bone remodeling. We report herein that a calcium signal can be communicated from osteoblasts to osteoclasts and among osteoclasts. Whereas calcium signals among osteoclasts involve activation of P2Y receptors, we found unexpectedly that P2X nucleotide receptors, probably of the P2X7 subtype, are required for osteoclast calcium signals activation.

EXPERIMENTAL PROCEDURES

Cells—Human bone marrow (5–10 ml) was obtained from healthy volunteers (aged 20–34) by puncture of the posterior iliac spine. The marrow material was collected in a 50-ml tube, containing 15 ml of heparinized (100 units/ml) minimum essential medium. The mononuclear fraction of the cells was then isolated by centrifugation on a Lymphoprep gradient. The cells were plated in T-75 culture flasks with 10 × 10⁶ cells/flask in RPMI 1640 medium supplemented with glutamine (Invitrogen, Grand Island, NY) and 30% horse serum (Sigma Chemical Co., St. Louis, MO). The culture was incubated in a humidified atmosphere of 5% CO₂ and 37 °C. Initially and at every medium change, the adherent cell population was transferred to minimum essential medium and 10% fetal calf serum (Invitrogen) and maintained by completely changing the medium once a week. After 3–4 weeks of culture, the osteoblastic cells reached 80% confluence and were used to co-culture with the tartrate-resistant acid phosphatase-positive giant cells (osteoclastic cells). The non-adherent cells were counted, and 5% osteoblastic cells were added to the cell suspension. The cells were then replated in 6-well plates, each well containing a 25-mm no. 1 glass coverslip, at 2 × 10⁶ osteoblastic cells/well. The co-culture was maintained in RPMI 1640 medium supplemented with glutamine (Invitrogen) and 30% horse serum (Sigma). Initially and at every medium change 10⁻⁸ M 1,25(OH)₂ vitamin-D₃ (Roche Molecular Biochemicals) and 20 ng/ml human RANKL (PeproTech), to promote osteoclast differentiation and maturation. A similar method has been shown to be able to produce osteoclasts from human periph-

Calcium Imaging—Measurement of the intracellular calcium concentration was done using the calcium indicator dye fura-2. Co-cultures of osteoblastic and osteoclastic cells in monolayers adherent to non-coated glass coverslips were incubated at 37 °C in medium containing 5 μM fura-2/AM for 30 min and then incubated for an additional 20 min in medium without dye. Coverslips were affixed to a Teflon chamber and mounted in a PDM-2 open perfusion microincubator (Medical Systems Corp., Greenvale, NY) maintained at 37 °C with superfused O₂ on a Zeiss Axiovert 35 inverted microscope (Carl Zeiss Inc., Thornwood, NY). Imaging was performed with the Metamorph/Metafluor system (Universal Imaging, West Chester, PA) with excitation wavelengths of 340 and 380 nm for acquiring ratio images of fura-2. Probes were adjusted at a concentration of 1 mM was added throughout the experiment to prevent dye leakage from the cytoplasm of the cells (15). Ratio images were calibrated using buffers of known calcium concentrations (Molecu-

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RNase treated using an RNase-free DNase set also from Qiagen. Total RNA was extracted from the osteoclasts using the RNaseasy Mini kit from Qiagen. The RNA was DNase-treated using an RNase-free DNase set also from Qiagen.

RT-PCR—Almost pure osteoclast cultures were used as described above. After 2 weeks of culture, total RNA was extracted from the osteoclasts using the RNaseasy Mini kit from Qiagen. The RNA was DNase-treated using an RNase-free DNase set also from Qiagen.

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The PCR reactions were set up using 5 μl of the cDNA template previously synthesized. The PCR was performed in a PTC-100 programmable thermal controller (MJ Research, Inc.). Initially, the template was denatured for 2 min at 94 °C, followed by 40 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C (P2Y1)/53 °C (P2X7) for 30 s, elongation at 72 °C for 1 min, and ended with a prolonged elongation at 72 °C for 7 min. Finally, the PCR products were separated in a 2% agarose gel, the P2Y1 product at a length of 685 bp and the P2X7 product at a length of 399 bp.

Histochemical Staining of TRAP—Osteoclasts were cultured on glass slides and treated as described above in the RNA extraction section. After 2 weeks of culture, the flask was removed from the slide, and the slide was washed briefly in PBS and then fixed in 70% ethanol for 5 min. Then the cells were incubated for 15 min in TRAP solution (naphthol-as-bi-phosphate, N,N-methylformamid, paraarosulam, sodium nitrite, and tartrate in Michaelis buffer, pH 5.0), washed with tap water, and then counterstained with Meyers hematoxylin for 10 s. Finally, preparations were mounted in aqueous mounting medium.

Immunocytochemistry—Osteoclasts were cultured on glass slides and treated as described above. They were fixed in ethanol for 10 min and incubated for 15 h with P2X7 receptor antibody, developed in rabbit secondary antibody (Jackson ImmunoResearch), diluted 1:50 with TBS containing 2% bovine serum albumin. After incubation the slides were washed 3 times down to 1 μl, and then the cells were washed briefly with PBS and then fixed in 70% ethanol for 5 min. Then the cells were incubated for 15 min in TRAP solution (naphthol-as-bi-phosphate, N,N-methylformamid, paraarosulam, sodium nitrite, and tartrate in Michaelis buffer, pH 5.0), washed with tap water, and then counterstained with Meyers hematoxylin for 10 s. Finally, preparations were mounted in aqueous mounting medium.

RESULTS

Intercellular Calcium Waves Can Be Communicated Bi-directionally between Osteoblasts and Osteoclasts—We have previously shown that human osteoblastic cells can communicate intercellular calcium waves by two mechanisms: one involving autocrine action of ATP and one dependent on gap junctional communication (2). In this study, we asked whether the intercellular calcium signal, which can be elicited in osteoblast networks, is also communicated to osteoclasts, the other major cell type effecting bone remodeling. Co-cultures of human osteoblastic and osteoclastic cells were loaded with the calcium indicator fura-2, and a single osteoblastic cell was stimulated mechanically with a glass micropipette. The stimulated cell showed an initial increase in [Ca$^{2+}$], spreading to adjacent osteoblasts and, with a 10- to 15-s time lag, to adjacent osteoclasts (n = 34) (Fig. 2). Interestingly, when one osteoclast was mechanically stimulated, [Ca$^{2+}$], rapidly increased in this cell, and a calcium wave was initiated and propagated to adjacent osteoclasts as well as osteoclasts (n = 48 of 52 experiments), in a similar fashion as it occurred with mechanical stimulation of one osteoblast (Fig. 3). Hence, intercellular calcium waves can be elicited by mechanical perturbation of either osteoblasts or osteoclasts and can be communicated bi-directionally between cells of the two lineages, as well as among cells of each lineage.

Osteoclast Intercellular Calcium Waves Require Activation of P2 Receptors but Do Not Require Gap Junctional Communication—We next asked whether propagation of calcium signals to osteoclasts required either gap junctional communication or activation of P2 receptors. We used the gap junction inhibitor heptanol to test whether gap junctional communication is required for the propagation of osteoclast-initiated calcium waves. First, a single cell in the fura-2-loaded monolayer was mechanically stimulated to confirm the ability of the cells to propagate a calcium signal upon mechanical stimulation. After 5 min incubation with heptanol (3.5 mM), another cell was mechanically stimulated. Despite the inhibition of gap junctional communication, the stimulated osteoclast was still able to initiate a calcium pulse to the neighboring osteoclasts (n = 5) (Fig. 4). Thus, gap junctional communication is not required for the propagation of intercellular calcium signals generated by mechanical perturbation of osteoclasts (Table I).

In similar experiments we used the P2 receptor antagonist suramin to determine whether P2 receptors were required for calcium signal propagation to osteoclasts, because they are in osteoblast-to-osteoblast signaling (2). One single osteoblast in a fura-2-loaded monolayer of an osteoblast-osteoclast co-culture was stimulated mechanically, and a calcium signal was propagated to neighboring osteoblasts and osteoclasts as described above. We then added suramin in a final concentration of 100 μM, and after a short incubation period, we stimulated an osteoblast mechanically. No calcium waves were seen, either to osteoblasts or osteoclasts, in five of six experiments (Table I). Thus, P2 receptors are involved in the propagation of intercellular calcium signals to osteoclasts.

Human Osteoclasts Express Functional P2Y1 Receptors—Having demonstrated the involvement of a P2Y receptor superfamily member in osteoclast calcium wave propagation, we next functionally characterized the member of this receptor superfamily that may be present in our human osteoclast preparations, by measuring nucleotide-induced calcium transients in osteoblast-osteoclast monolayers, as summarized in Table II. We have previously shown that UTP and ATP induce increases in [Ca$^{2+}$] in human osteoblasts, consistent with the expression of P2Y2 receptors in the osteoclasts. Monolayers of osteoblast-osteoclast co-cultures were loaded with fura-2, mounted on the stage of the calcium imaging system, and the effect of different nucleotides on the osteoclast [Ca$^{2+}$] was tested. ATP was able to increase the [Ca$^{2+}$] in all experiments (n = 57) at concentrations of 100 μM and 1 mM. Response to ATP stimulation was seen with ATP concentrations down to 1 μM. A maximum increase in [Ca$^{2+}$] of 400 nM was seen with a final ATP concentration of 100 μM.

The same experiments were performed with ADP, UDP, UTP, and benzoylbenzyl-ATP (BzATP). Neither UDP (100 μM)
Intercellular calcium signaling occurs among human osteoclasts cultured with RANKL and M-CSF, as described under “Experimental Procedures.” The numbers on each panel indicate time in seconds after mechanical stimulation. OC, osteoclast; the scale bar shows intracellular calcium concentrations in micromolar.

The gap junction inhibitor heptanol does not affect the propagation of a calcium signal to osteoclasts in response to mechanical stimulus. The left-hand panel shows a monolayer of osteoclastic cells before stimulation. In the middle panel, one single osteoclast is stimulated mechanically with a glass micropipette, and a calcium wave is generated to neighboring osteoclasts (as shown in the right-hand panel), even in the presence of heptanol. Osteoclasts are obtained by the RANKL and M-CSF method as described in the text.

nor UTP (100 μM) had an effect on the [Ca^{2+}] in the osteoclasts (n = 5), but UTP resulted in increases in [Ca^{2+}] in the osteoblasts as expected. The absence of UDP- and UTP-induced calcium transients indicates that the primary human osteoclastic cells lack functional P2Y2, P2Y4, or P2Y6, even though P2Y2 mRNA has been identified in human osteoclasts by other investigators (5). In contrast, ADP (100 μM) increased [Ca^{2+}] in osteoclastic cells (29 of 31 experiments) (Fig. 5). Based on nucleotide specificity of the different purinergic receptors, this pharmacological profile is most consistent with the presence of functional P2Y1 receptors on these cells. Osteoblasts sometimes exhibited a weak calcium response after addition of ADP, again consistent with P2Y2 expression in these cells.

We next confirmed the expression of P2Y1 receptors by RT-PCR amplification of mRNA from human osteoclasts. For these experiments, monocultures of human osteoclasts were produced from human bone marrow mononuclear cells by stimulation with RANKL and M-CSF. RT-PCR using primers specific for the P2Y1 coding sequence and total RNA extracted from isolated osteoclastic cells as a template revealed a clear 685-bp product corresponding to P2Y1 (Fig. 6).

Desensitization of P2Y Receptors Does Not Inhibit Osteoclast Intercellular Calcium Signaling—P2Y receptors, like many other G-protein-coupled receptors, undergo desensitization after ligand binding. Accordingly, we found that a second addition of ADP to osteoclasts was not accompanied by a cytosolic calcium transient (n = 5) (Fig. 5). We used this homologous desensitization to ask whether activation of P2Y receptors on human osteoclasts was required for the propagation of intercellular calcium waves to these cells. After demonstrating the ability to propagate waves from osteoblasts to osteoclasts, we desensitized the osteoclast P2Y1 receptors with ADP and saw an increase in [Ca^{2+}]. Surprisingly, when another mechanical stimulation was applied, intercellular calcium signaling to osteoclasts was unperturbed by the desensitization (Table I). Thus, although osteoclasts express functional P2Y1 receptors, these receptors were not required for the communication of calcium waves from osteoblasts to osteoclasts.

In separate experiments we also used ATP to desensitize P2Y receptors. In all cases both osteoblasts and osteoclasts responded to ATP stimulation with an increase in [Ca^{2+}]. Subsequent mechanical stimulation of an osteoblast resulted in a calcium transient in the stimulated cell. Whereas intracellular calcium did not increase in neighboring osteoblasts, as expected, neighboring osteoclasts propagated a calcium wave as they did before ATP desensitization (Table I). Thus, P2Y receptors are required for intercellular calcium signaling among osteoblasts but not for calcium waves among osteoclasts.

**Table I**

| Addition | Signaling to OB | Signaling to OC |
|----------|----------------|----------------|
| Heptanol | + (5/5)        | -              |
| Suramin  | - (1/6)        | -              |
| ATP pretreatment | - (14/14) | -              |
| ADP pretreatment | + (10/11)  | +              |
| α-ATP pre-treatment | + (11/12) | - (2/18)        |
| BzATP pretreatment | + (13/13)   | + (11/13)      |

* In a previous study we have performed similar experiments in cultures of human osteoblastic cells, showing that ATP inhibited fast waves, not slow waves. ADP did not inhibit calcium signaling. Heptanol only inhibited slow calcium signaling between the osteoclastic cells (2).

**Table II**

| Nucleotide | OB response | OC response |
|------------|-------------|-------------|
| ATP        | ++          | ++          |
| UTP        | +           | –           |
| ADP        | +           | +           |
| BzATP      | –           | –           |
| α-ATP + BzATP | –          | –           |

FIG. 3. Intercellular calcium signaling occurs among human osteoclasts cultured with RANKL and M-CSF, as described under “Experimental Procedures.” The numbers on each panel indicate time in seconds after mechanical stimulation. OC, osteoclast; the scale bar shows intracellular calcium concentrations in micromolar.

FIG. 4. The gap junction inhibitor heptanol does not affect the propagation of a calcium signal to osteoclasts in response to mechanical stimulus. The left-hand panel shows a monolayer of osteoclastic cells before stimulation. In the middle panel, one single osteoclast is stimulated mechanically with a glass micropipette, and a calcium wave is generated to neighboring osteoclasts (as shown in the right-hand panel), even in the presence of heptanol. Osteoclasts are obtained by the RANKL and M-CSF method as described in the text.
Osteoclasts Express Functional P2X7 Receptors, Which Are Required for Osteoclast Calcium Signaling—The pharmacological studies revealed that, although P2Y1 receptors are present in osteoclasts, they are not involved in propagation of mechanically induced calcium waves among osteoclasts. Because several P2X receptors, namely P2X2, P2X4, and P2X7, have been detected in osteoclastic cells, we then asked whether members of this class of P2 receptors may be responsible for inter-osteoclast calcium waves. We first tested for the presence of functional P2X7 receptors in the osteoblast-osteoclast co-cultures using BzATP, a P2X agonist with relative specificity for P2X7. BzATP (100 μM) increased [Ca^{2+}]_i of osteoclasts in all experiments (n = 9) (Fig. 5) but had no effect on cells of the osteoblastic lineage. When we preincubated cell monolayers with the P2X7 receptor antagonist, oxidized ATP (oATP), the BzATP-induced calcium increase was blocked (n = 4) (Fig. 5). These experiments demonstrated that osteoclasts but not osteoblasts expressed functional P2X7 receptors and showed that osteoclast P2X7 activity could be inhibited by oATP.

Activation of P2X7 receptors with ATP results in formation of a plasma membrane pore that allows molecules <900 Da to traverse the plasma membrane. To confirm P2X7 activity in the osteoclastic cells, we assessed ATP-induced uptake of Lucifer Yellow in the co-cultures by incubation with Lucifer Yellow in the presence of 100 μM BzATP for 10 min. In the presence of BzATP, Lucifer Yellow uptake occurred in osteoclastic, but not osteoblastic cells, demonstrating ATP-induced pore formation selectively in osteoclasts. In the absence of the agonist, cytoplasmic dye uptake of Lucifer Yellow was not seen (Fig. 7). Thus, human osteoclasts express functional P2X7 receptors in the plasma membrane, as do monocytes and macrophages.

We then determined the expression of P2X7 mRNA and protein by osteoclasts. As described above, human osteoclast mononuclear cells induced by RANKL were used to selectively detect the presence of P2X7 in these cells. P2X7 mRNA was clearly detected by RT-PCR using primers specific for the P2X7 coding sequence of total osteoclast RNA (Fig. 8). Immunohistochemical staining using a Cy3-labeled anti-P2X7 antibody confirmed the presence of this receptor on the surface of osteoclastic cells (Fig. 9) but only faint staining in the osteoblastic cells.

Osteoblast-Osteoclast Intercellular Calcium Signaling Requires Functional P2X Receptors—We next asked whether P2X7 receptors mediate intercellular calcium signaling between osteoblasts and osteoclasts. After an initial mechanically induced wave, we incubated cells in medium containing 300 μM oATP, which did not result in an increase in the [Ca^{2+}]_i. A second mechanical stimulation was applied to an osteoblast, and a calcium signal was propagated to surrounding osteoblasts in 11 of 12 experiments. In contrast, in only 2 of 18 experiments the signal was propagated to a single osteoclast in the field of view, whereas in 16 experiments the signal was not propagated to osteoclasts at all. Thus, P2X7 receptors seem to be responsible for the propagation of intercellular calcium signaling between osteoblasts and osteoclasts (Table 1).

To verify that the addition of oATP did not affect the cells P2Y1 response, we first added oATP to the cell culture. As expected, the intracellular calcium concentration was not affected. Subsequently we added ADP to stimulate P2Y1 receptors, and the osteoclasts showed an increase in intracellular calcium concentration (n = 3; Fig. 5). Thus oATP did not inhibit the P2Y1-mediated response. We also confirmed that the cal-

Fig. 5. P2 receptor agonist and antagonist responses on intracellular calcium concentrations in cultures of human osteoclastic cells. a, ADP is added to a monolayer of osteoclastic cells to desensitize P2Y1 receptors. A second ADP addition induces no increase in intracellular calcium concentration, supporting the desensitization of the P2Y1 receptor. In contrast, the response to BzATP is not affected by ADP treatment, indicating that ADP does not desensitize P2X7 receptors. b, osteoclastic cells treated with BzATP without any pretreatment respond with an increase in intracellular calcium concentration. A subsequent addition of BzATP was now unable to increase calcium, indicating the ability of oATP to block P2X7 receptor activation. In contrast, adding ADP after oATP, cells increased in intracellular calcium concentration, showing that the response of ADP binding to P2Y1 receptors is not affected by oATP action. The scale bar shows intracellular calcium concentrations in micromolar. Osteoclasts were obtained from human bone marrow, and osteoclast phenotype was induced by RANKL and M-CSF as described in the text. BzATP, benzoyl-benzyl-ATP; oATP, oxidized ATP.

Fig. 6. Human osteoclasts express P2Y1 mRNA as assessed by RT-PCR. Human osteoclasts were formed in vitro by culturing bone marrow mononuclear cell precursors in medium containing RANKL and M-CSF as described, and RT-PCR was performed as described. Control experiments were performed without the addition of reverse transcriptase (−RT) or with P2Y1 plasmid as template.

Required for Osteoclast Calcium Signaling

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FIG. 7. Benzyl-benzoyl-ATP permeabilizes the plasma membrane of human osteoclasts to fluorescent dyes. RANKL- and M-CSF-induced human osteoclasts were incubated in medium containing Lucifer Yellow with or without BzATP. In the presence of BzATP, Lucifer Yellow diffusely stained the cell cytoplasm, demonstrating the presence of functional P2X7-induced pores in these cells. The left panels show phase-contrast microscopy of osteoclast; right panels show Lucifer Yellow (LY) after excitation with 480-nm light.

P2X7 in human OC

FIG. 8. Human osteoclasts express P2X7 mRNA as assessed by RT-PCR. Human osteoclasts were formed in vitro by culturing bone marrow mononuclear cell precursors in medium containing RANKL as described, and RT-PCR was performed as described. Control experiments were performed without the addition of reverse transcriptase (−RT) or with RNA from the macrophage cell line U937 as positive control template.

Cium response elicited by P2X receptor activation was not subject to desensitization by ADP. Although, as mentioned above, incubation in medium containing ADP prevented a second ADP-induced calcium transient, ADP did not prevent a subsequent calcium transient in response to BzATP (Fig. 5).

Osteoclast-Osteoclast Calcium Signaling Also Requires P2X but Not P2Y Receptors—We finally wanted to determine the mechanism by which osteoclasts communicate calcium signals to each other. First, we examined the role of purinergic receptors by examining areas in the co-cultures with two or more osteoclastic cells in close proximity to each other. One single osteoclast was mechanically stimulated, and this generated the expected calcium response elicited by P2X receptor activation. The increase in [Ca²⁺]i, that rapidly propagated to the neighboring osteoclasts. To desensitize receptors of the P2Y type, ATP was added (100 μM to 1 mM), and an increase in [Ca²⁺]i was seen. The mechanical stimulation was repeated, and a calcium wave was again propagated to the neighboring osteoclasts (n = 8). The experiment was also done with ADP (100 μM), and no inhibition of the calcium signaling was seen either (n = 4; Table I). These results demonstrate that intercellular calcium signaling between osteoclasts does not depend on receptors of the P2Y type. By contrast, in the presence of oATP at a final concentration of 300 μM, mechanical stimulation of osteoclasts did not induce a calcium wave, because a wave was seen only in 16 of 18 experiments (Table I). Thus, P2X7 receptors are involved in the propagation of intercellular calcium signals to osteoclast upon mechanical stimulation of either osteoblasts or osteoclasts.

FIG. 9. Immunochemical detection of the P2X7 receptor. Immunochemical staining for P2X7 was performed in cultures of human bone marrow-derived osteoclastic cells induced by RANKL and M-CSF according to the method described in the text, and osteoblastic cells, for comparison. a, a single multinucleated osteoclast staining positively for the receptor; b, several mononuclear osteoclastic precursors staining positively for the receptor as well; c, a culture of osteoblastic cells only showing very vague staining for the P2X7 receptor.

DISCUSSION

These studies demonstrate that intercellular calcium signaling can occur between osteoblasts and osteoclasts and among osteoclasts. In addition, they reveal that the mechanism by which osteoclasts respond to calcium signals is different from the mechanisms we previously identified for calcium signaling among osteoblasts. Whereas osteoblast intercellular calcium signaling is propagated either by gap junctional communication or by autocrine activation of P2Y2 receptors, intercellular calcium signaling to osteoclasts requires activation of P2X7 receptors. Thus, these studies not only demonstrate that intercellular calcium signaling is a mechanism by which bone-forming and -resorbing cells can directly communicate, but they also reveal that different molecular mechanisms are responsible for this form of communication between and among the two major bone cell types.

In these studies, summarized in Table II, we found that human osteoclastic cells respond to nucleotide stimulation with an increase in intracellular calcium concentration. The cells express both purinergic receptors of the P2Y and P2X subclasses, as determined by nucleotide affinity. The pharmacological profiles of the nucleotide receptors as well as the presence of P2Y1 and P2X7 mRNA and P2X7 protein is consistent with expression of P2Y1 and P2X7 on osteoclasts, but other P2 receptors, especially other P2X receptors, may also be present. We also found that human osteoblasts-osteoclast networks can propagate calcium transients in response to mechanical stimulation and that propagation of these signals involves the action of ATP or other nucleotides on membrane-bound purinergic receptors. These calcium signals can also be transmitted between osteoblasts by P2X7 receptors. Finally, we show that prolonged ATP stimulation of the osteoclastic cells induces
permeabilization of the plasma membrane, presumably via pore formation of the P2X7 receptor, a phenomenon previously described for macrophages (16), lymphocytes (17), and dendritic cells (18).

It is commonly believed that signaling from osteocytes through their processes in the canaliculi to surface osteoblasts or lining cells is the mechanism by which mechanical signals are diffused through the bone tissue and modulate bone cell activity. The propagation of calcium transients from osteoblasts to osteoclasts represents an additional mechanism of intercellular communication that allows signals to be transmitted from cells of the osteogenic lineage to bone-resorptive, osteoclastic cells. Our work demonstrated that osteoclasts utilize the autocrine action of ATP and specific purinergic receptors to propagate calcium signals to osteoclasts, and it is known that rat osteoclasts (3, 4) and human osteoclastoma giant cells (6) respond to ATP with an increase in intracellular calcium that rat osteoclasts (3, 4) and human osteoclastoma giant cells (6) respond to ATP with an increase in intracellular calcium activities. The propagation of calcium transients from osteoclastic cells. Our work demonstrated that osteoblasts utilize the autocrine action of ATP and specific purinergic receptors to propagate calcium signals to osteoclasts, and it is known that rat osteoclasts (3, 4) and human osteoclastoma giant cells (6) respond to ATP with an increase in intracellular calcium concentrations. Thus, P2 receptor-mediated signaling between osteoblasts and osteoclasts may represent a mechanism by which bone remodeling is controlled.

The effect of ATP on osteoclasts seems to be biphasic. At relatively low concentrations (0.2–2.0 μM), ATP increased osteoclast formation and resorption pit formation up to 5.6-fold in neonatal rat co-cultures of osteoblasts and osteoclasts, as well as in a model system based on mouse bone marrow (8). The slowly hydrolyzable ATP analog ATPγS can also induce bone resorption in human osteoclastoma giant cells at 10 μM (6). Conversely, at higher concentrations (20–200 μM), ATP reduces or even blocks osteoclast formation by a selective cytotoxic effect that does not alter osteoblast viability (8). This correlates well with other studies finding a cytotoxic effect of ATP in millimolar concentrations on other cells of hemopoietic origin, e.g. macrophages (19, 20). Osteoclasts are part of the mononuclear phagocyte system with precursors present in both bone marrow and in the circulating blood and share precursors with macrophages (21). In macrophages, the P2X7 receptor is involved in the control of cell death (22). In the present study, we detected the presence of this receptor in osteoclastic cells. This may be a mechanism for high dose ATP-induced cell death in osteoclasts. The presence of multiple P2Y and P2X receptors in osteoclasts may allow generation of different responses to ATP and perhaps to other nucleotides, depending upon ligand environment via paracrine or autocrine secretion of nucleotides may be important for osteoblast modulation of osteoclast function. Furthermore, the cell type-specific roles of P2Y2 and P2X7 receptors in osteoblasts and osteoclasts, respectively, and their unique pharmacological profiles in theory allows selective modulation of signal diffusion to either osteoblasts or osteoclasts by pharmacological agents, thus offering novel therapeutic strategies for modulation of bone turnover.

Factors that might affect the degree of bias are the lack of cell-matrix interaction, the relatively non-physiological stimulus of cell poking, and the limitations of pharmacological methods. Though a lot of effort has been put into culturing cells close to “in vivo” osteoclasts and osteoblasts as possible, in vitro cultured cells still lack some of the exact phenotypic characteristics and stimuli present in vivo.

In conclusion, we provide evidence for a novel mechanism by which calcium signals can be transmitted from osteoblasts to osteoclasts by means of activation of P2X7 receptors. Although the ultimate physiological role of P2X7 receptor activation for osteoclast function remains to be determined, the ability to exchange short-range calcium signals in the bone microenvironment via paracrine or autocrine secretion of nucleotides may be important for osteoblast modulation of osteoclast function. Furthermore, the cell type-specific roles of P2Y2 and P2X7 receptors in osteoblasts and osteoclasts, respectively, and their unique pharmacological profiles in theory allows selective modulation of signal diffusion to either osteoblasts or osteoclasts by pharmacological agents, thus offering novel therapeutic strategies for modulation of bone turnover.

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