ATP downregulates P2X<sub>7</sub> and inhibits osteoclast formation in RAW cells

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Hiken, Jeffrey F., and Thomas H. Steinberg. ATP downregulates P2X<sub>7</sub> and inhibits osteoclast formation in RAW cells. Am J Physiol Cell Physiol 287: C403–C412, 2004. First published April 7, 2004; 10.1152/ajpcell.00361.2003.—Multinucleated giant cells derive from fusion of precursor cells of the macrophage lineage. It has been proposed that the purinoreceptor P2X<sub>7</sub> is involved in this fusion process. Prolonged exposure of macrophages to ATP, the ligand for P2X<sub>7</sub>, induces the formation of plasma membrane pores and eventual cell death. We took advantage of this cytolytic property to select RAW 264.7 (RAW) cells that lacked P2X<sub>7</sub> function by maintaining them in ATP (RAW ATP-R cells). RAW ATP-R cells failed to fuse to form multinucleated osteoclasts in response to receptor activator nuclear factor-κB ligand, although they did become positive for the osteoclast marker enzyme tartrate-resistant acid phosphatase, and upregulated expression of other osteoclast marker genes. RAW ATP-R cells and wild-type RAW cells expressed similar amounts of P2X<sub>7</sub> protein, but little P2X<sub>7</sub> was present on the surface of RAW ATP-R cells. After ATP was removed from the medium of RAW ATP-R cells, the cells reexpressed P2X<sub>7</sub> on the cell surface, regained sensitivity to ATP, and formed multinucleated osteoclasts. These results suggest that P2X<sub>7</sub> or another protein that is downregulated in concert with P2X<sub>7</sub> is involved either in the mechanics of cell fusion to form osteoclasts or in a signaling pathway proximal to this event. These results also suggest that P2X<sub>7</sub> may be regulated by ligand-mediated internalization and that extracellular ATP may regulate the formation of osteoclasts and other multinucleated giant cells.

IN SEVERAL SITUATIONS, MONONUCLEAR phagocytes fuse to form multinucleated giant cells (2, 28). These include the formation of immune giant cells, foreign body giant cells, and osteoclasts. Although the mechanism of giant cell formation is not understood, several plasma membrane (PM) molecules have been implicated in this process, including SIRP-α, CD47, CD44, intercellular adhesion molecule-1, leukocyte function-associated antigen-1, and E-cadherin (15, 19, 22, 25). P2X<sub>7</sub>, a PM receptor for ATP, has also been implicated in giant cell formation. For example, J774 mouse macrophage clones selected for high expression of P2X<sub>7</sub> spontaneously fuse to form multinucleated giant cells, whereas clones selected for low P2X<sub>7</sub> expression do not (4). In addition, concanavalin A-induced formation of multinucleated giant cells from human monocytes is blocked by an inactivating monoclonal antibody directed against P2X<sub>7</sub> (7). Although these studies support a role for P2X<sub>7</sub> in giant cell multinucleation, a recent report showed that a P2X<sub>7</sub>-null mouse is able to form multinucleated osteoclasts in vitro and in vivo (16). Thus the role of P2X<sub>7</sub> in osteoclast formation remains unclear.

The only known physiologically relevant ligand for P2X<sub>7</sub> is ATP. In the presence of divalent cations, millimolar levels of ATP are required for receptor activation (24). Transient activation of P2X<sub>7</sub> with ligand opens a cation channel. However, P2X<sub>7</sub> displays an unusual property in that repeated or prolonged activation of the receptor with ligand is associated with the formation of a membrane pore that allows for the bidirectional passage of molecules up to ~900 Da in size (24, 26). The P2X<sub>7</sub> channel/pore closes on removal of ATP, and macrophages exposed to millimolar levels of ATP for ~10–15 min generally recover to functionally normal cells (24). Opening of the P2X<sub>7</sub> pore in macrophages by prolonged incubation with ATP leads to cell lysis (31).

One well-established model for macrophage fusion and multinucleation is the formation of osteoclast-like cells by induction of RAW macrophage-like cells with receptor activator nuclear factor-κB ligand (RANKL), a key regulator of osteoclast differentiation and function (14). We took advantage of the cytolytic effect of prolonged ATP exposure to select RAW mouse macrophages that lack P2X<sub>7</sub> pore-forming activity to further examine the role of extracellular ATP and P2X<sub>7</sub> in osteoclast formation.

MATERIALS AND METHODS

Differentiation of osteoclast-like cells from RAW cells. RAW cell media contained 90% DMEM with 2 g/l sodium bicarbonate, 1 mM sodium pyruvate, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 10% fetal bovine serum (not heat inactivated). On day 0, RAW cells were scraped from 100-mm tissue culture dishes, seeded into multi-well plates (24-well: 10,000 cells in 1 ml of media; 6-well: 50,000 cells in 3 ml of media), and grown overnight. On day 1, soluble mouse RANKL (a generous gift from Jose Moreno and Mehrdad Tondravi) was added to a final concentration of 100 ng/ml. Cells were fed on day 3 of RANKL induction.

Dye uptake assay. Media (0.2 ml) containing 0.5 mg/ml lucifer yellow or 2 μM YO-PRO-1 (Molecular Probes, Eugene, OR), either with 2 mM ATP or without ATP, were added to RAW cells growing in 24-well plates. Cells were incubated 15 min at 37°C before being washed three times with DMEM and viewed by phase and fluorescence microscopy.

Tartrate-resistant acid phosphatase staining. Cells were fixed for 10 min in 4% paraformaldehyde before being stained for tartrate-resistant acid phosphatase (TRAP) by using a leukocyte acid phosphatase kit, according to the manufacturer’s instructions (Sigma Diagnostics, St. Louis, MO; kit no. 387-A).

Generation of ATP-resistant RAW cells. Frozen 1-ml aliquots of RAW cells of the same passage were thawed for each experiment where ATP-resistant RAW cells were generated. RAW cells growing at ~70% confluence in 100-mm tissue culture dishes were fed with 10 ml of media containing 2 mM ATP (Roche Applied Science, Indianapolis, IN). ATP was freshly added to media from frozen stocks of 100 mM ATP prepared in DMEM and adjusted to pH 7.5. After overnight incubation (16–20 h), ~95% of cells were killed. Cells were fed with media and freshly added 2 mM ATP after the initial
overnight incubation and every other day thereafter. After an additional 3–5 days, cells were scraped and seeded into multiwell plates in media, either with 2 mM ATP (RAW ATP-R) or without ATP (RAW ATP-S). After overnight incubation, cells were induced with RANKL, as needed.

P2 receptor agonists/antagonists. Stock solutions of adenosine, AMP, ADP, UTP, and 2′,3′-O-(4-benzoylbenzoyl)-ATP (bzATP) (from Calbiochem, San Diego, CA) were prepared in DMEM and adjusted to approximately pH 7. AMP-Methylene-ATP, 2-methylthio-ATP, and pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate (PPADS) were from Sigma-Aldrich (St. Louis, MO). Osteoclast number in cultures incubated with P2 agonists/antagonists was assessed by counting detectable multinucleated cells in a low-power field (×4).

Real-time PCR. RAW, RAW ATP-R, and RAW ATP-S cells were either untreated or treated for 3 days with RANKL Total RNA was isolated by using TRIzol reagent (Invitrogen, Carlsbad, CA). DNase I-treated RNA (2 μg) was reverse transcribed by using random hexamer primers and SuperScript II reverse transcriptase (Invitrogen). cDNA corresponding to 10 ng of total RNA was used for real-time PCR analysis by using SYBRgreen I dye chemistry and the GeneAmp 5700 sequence detection system (Perkin Elmer Life Sciences). Primers corresponded to carbonic anhydrase II, cathepsin K, matrix metalloproteinase-9, nuclear factor of activated T cells 1, TRAP, and GADPH. PCR analysis by using SYBRgreen I dye chemistry and the GeneAmp 5700 system (Perkin Elmer Life Sciences) was set up with 0.2 μM of each primer, 10 μl of cDNA, 0.5 U of AmpliTaqGold DNA polymerase (Roche), and 10 μl of 2× SYBRgreen I master mix. Cycling conditions were 1 cycle of 94 °C for 15 s, 40 cycles of 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. After the PCR reaction, the specificity of the annealing step was verified by melting curve analysis. The presence or absence of the desired PCR product was confirmed by 1% agarose gel electrophoresis. The expression levels of each gene were normalized to the expression levels of the housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase, GAPDH).

RESULTS

RAW 264.7 cells form multinucleated osteoclast-like cells and display P2X7 pore-forming activity. In the presence of RANKL, RAW cells fuse to form multinucleated giant cells that express TRAP activity, characteristic of osteoclasts (14). In our hands, very little cell fusion occurred on days 0–3 of RANKL induction (Fig. 1B). By day 4 of RANKL induction, however, cell fusion and an osteoclast-like morphology were clearly evident (Fig. 1D). The resultant giant cells had upwards of ~100 nuclei, usually distributed around the cell periphery. Thus a large amount of cell fusion occurred between days 3 and 4 after addition of RANKL. These multinucleated cells also expressed TRAP activity (Fig. 1).

P2X7 is a receptor for extracellular nucleotides that is expressed predominantly on leukocytes. This receptor is associated with an unusual pore-forming activity in response to high concentrations of its ligand, ATP. Opening of these pores, which allows the nonspecific passage of molecules up to ~900 Da, can be monitored by the uptake of fluorescent dyes (24, 26). We asked whether ATP-induced pore formation occurred in RAW cells and in RAW cell-derived osteoclast-like cells by monitoring uptake of the membrane-impermeant fluorescent dyes lucifer yellow (457 Da) or YO-PRO-1 (375 Da). Cells were incubated in medium containing 0.5 mM lucifer yellow in the presence or absence of 2 mM ATP at 37°C for 15 min and viewed by epifluorescence microscopy (Fig. 2). In the absence of extracellular ATP, RAW cells did not take up lucifer yellow, but cells incubated in medium containing ATP demonstrated diffuse cytosolic staining with the fluorescent dye (Fig. 2, A
and B). RANKL-treated RAW cells that had undergone osteoclastic differentiation also became permeable to lucifer yellow in the presence, but not in the absence, of extracellular ATP (Fig. 2, C and D).

RAW cells maintained in 2 mM ATP lose P2X7 activity. Prolonged opening of P2X7-associated pores ultimately leads to cell lysis. We took advantage of this property to select RAW cells that lacked P2X7 activity (Figs. 3 and 4). When RAW cells were incubated overnight in 2 mM ATP, ~95% of the cells died. The surviving cells, when maintained in 2 mM ATP, proliferated nearly normally but were resistant to permeabilization by ATP (RAW ATP-R cells, Fig. 4, A and B). This resistance to ATP permeabilization was reversible, because the cells regained their sensitivity to ATP permeabilization after overnight incubation without ATP (RAW ATP-S cells, Fig. 4, C and D).

RAW ATP-R cells fail to fuse efficiently when induced by RANKL but upregulate expression of osteoclast markers. We next asked whether RAW ATP-R cells formed multinucleated osteoclasts when incubated in medium containing RANKL. RAW ATP-R cells incubated in ATP maintained their resistance to ATP permeabilization on each of days 0–4 of RANKL induction (data not shown). In contrast to RAW cells, RAW ATP-R cells failed to fuse efficiently to form multinucleated cells after being incubated with RANKL for 4 days (Fig. 5D). While some multinucleation was observed, the number of osteoclasts formed was reduced ~90% (Table 1), and those that did form contained far fewer nuclei. RAW ATP-S cells, which had regained ATP responsiveness after overnight withdrawal of ATP, efficiently formed multinucleated cells after 4 days in RANKL (Fig. 5F).

Despite the lack of multinucleation, RANKL-treated RAW ATP-R cells still demonstrated TRAP activity (Fig. 5D). The presence of TRAP activity in these cells led us to ask whether the expression of other osteoclast markers was similarly upregulated during RANKL induction of RAW ATP-R cells. We used real-time PCR to quantitate the expression of five different osteoclast marker genes and found that they were similarly upregulated in RANKL-treated RAW, RAW ATP-S, and RAW ATP-R cells (Fig. 6). The increase in expression of these markers ranged from ~5-fold for carbonic anhydrase to ~300-fold for matrix metalloproteinase-9 and TRAP (Fig. 6). These results suggest that RAW ATP-R cells may be blocked in their ability to fuse, as opposed to their ability to undergo osteoclast differentiation program per se. Further evidence supporting this notion was seen when ATP was withdrawn from RAW ATP-R cells on day 3 of RANKL induction. Withdrawal of ATP on day 3 of induction, a time at which little cell fusion is apparent in RAW cells, allowed formation of multinucleated osteoclast-like cells by day 4 (Fig. 7).

Effect of ATP degradation products and other nucleotides on RANKL-induced fusion. Many cells, including macrophages, express ecto-nucleotidases that degrade ATP to ADP, AMP, and adenosine. It is possible that the block of RANKL-induced fusion seen in RAW ATP-R cells was due to the effects of one of these degradation products on other P2 or P1 receptors, rather than a loss of P2X7 function. We therefore induced RAW cells with RANKL in the presence of 1 mM ADP, AMP, or adenosine (Table 1). Neither AMP nor adenosine inhibited multinucleated osteoclast-like formation. ADP at a concentration of 1 mM killed the RAW cells, but, at 100 μM, had no effect on RANKL-induced fusion. Similarly, the P2Y agonist UTP (1 mM) had no effect on fusion (Table 1). Nor did the P2X1 and P2X3 agonists αβ-methylene ATP (10 μM) and 2-methylthio-ATP (10 μM) show an appreciable effect on RANKL-induced fusion (Table 1).

The ATP analog bzATP has become a widely used agonist for P2X7, because it generally shows greater potency at P2X7 than ATP. However, the kinetic properties of P2X7 can be affected significantly by its buffer environment (13). For example, NaCl and serum decrease the potency of bzATP at P2X7 receptors. We tested the dose response of RAW perme-

Fig. 1. Receptor activator nuclear factor-κB ligand (RANKL)-induced RAW cell osteoclast-like differentiation. RAW cells were grown on glass coverslips in the absence or presence of 100 ng/ml RANKL, fixed with paraformaldehyde, and stained for tartrate-resistant acid phosphatase (TRAP) activity (without counterstain). Little fusion was seen in the absence of RANKL (A and C), or after 3 days of induction with RANKL (B). D: by 4 days of RANKL induction, large multinucleated cells had formed. TRAP staining (dark pigment) was present in RAW cells induced 3 days with RANKL (B); however, staining was not as intense or uniform as in cells induced for 4 days (D). Scale bars = 75 μm.
abilization to lucifer yellow in response to ATP and bzATP in whole media, because we were limited in our ability to alter conditions of the RAW cell osteoclast differentiation model. The RAW cells began to permeabilize in response to ATP at a concentration of 2 mM and to bzATP at a concentration of 1 mM (data not shown). When 2 mM ATP was replaced with 100 μM bzATP on day 0 of RANKL induction, RAW ATP-R cells formed multinucleated osteoclast-like cells by day 4 (Table 1). In contrast, when the 2 mM ATP was replaced with 1 mM bzATP, osteoclast formation was blocked (Table 1). Thus the ability of bzATP to block osteoclast formation correlated with its ability to induce permeabilization. This result is consistent with P2X7 loss of function mediating the failure of RAW ATP-R cells to fuse in response to RANKL.

Effect of PPADS on RANKL-induced fusion. PPADS is a nonselective P2 receptor antagonist, with the exception that it is only weakly effective at P2X4 receptors (IC50 > 300 μM) (20). The IC50 of PPADS on cloned mouse P2X7 receptors is ~10 μM when measured in NaCl-free buffer (3). As with bzATP, the presence of NaCl and BSA has been shown to decrease PPADS antagonist potency at P2X7 (13). Consistent with this observation, RAW cell osteoclast differentiation proceeded in the presence of 10 μM PPADS in NaCl and serum-containing medium (Table 1). By contrast, 100 μM PPADS was sufficient to inhibit RAW cell osteoclast formation (Table 1).

P2X7 receptor is not expressed at the surface of RAW ATP-R cells. We next sought to determine the mechanism by which RAW ATP-R cells had become resistant to ATP-mediated pore formation. First we analyzed lysates from RAW ATP-R cells by Western blotting to determine whether downregulation of P2X7 protein expression accounted for the loss of P2X7 func-

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Fig. 2. RAW cells and RAW osteoclast-like cells express P2X7 activity. RAW cells and RANKL-generated RAW osteoclast-like cells were incubated in medium containing the fluorescent dye lucifer yellow in the presence (+) or absence (−) of 2 mM ATP for 15 min, washed, and examined by phase and fluorescence microscopy. B and D: diffuse cytoplasmic fluorescence in RAW cells and RAW osteoclast-like cells was seen only in the presence of ATP. A: little fluorescence was detected in uninduced RAW cells in the absence of ATP. Lucifer yellow was detected in large endocytic vesicles along the periphery of some RAW osteoclast-like cells in the absence of ATP (for example, arrows in C). D: in addition, dye uptake by unfused RAW cells was evident in RANKL-induced samples in the presence of ATP. Scale bars = 100 μm.
tion in these cells. RAW, RAW ATP-R, and RAW ATP-S cells all expressed similar levels of P2X7 protein (Fig. 8A, top).

Because P2X7 protein was present in RAW ATP-R cells, we asked whether internalization of P2X7 could account for the lack of activity. We assayed PM localization by surface biotinylation. Cells were incubated in medium containing 0.5 mg/ml NHS-LC-biotin for 4 h at 4°C. Unreacted biotin was quenched with 50 mM Tris, and cells were lysed in buffer containing 1% Nonidet P-40. Cell surface proteins were affinity purified from total cell lysates by using streptavidin-conjugated beads and analyzed by Western blotting. While P2X7 protein was present at the surface of RAW cells, expression was dramatically reduced at the surface of RAW ATP-R cells (Fig. 8A, middle). Furthermore, RAW ATP-S cells, which had regained their P2X7 function, expressed a similar level of P2X7 protein at their surface as RAW cells (Fig. 8A, middle). The surface expression of the known PM protein, Na-K-ATPase (α-subunit), was similar for RAW, RAW ATP-R, and ATP-S cells, indicating that the biotinylation reaction was similarly efficient for all samples (Fig. 8A, bottom). These results indicate that RAW ATP-R cells lack P2X7 function because they do not express the receptor at their cell surface. Furthermore, these results suggest that localization of the P2X7 receptor may be regulated by its ligand.

We subjected RAW, RAW ATP-R, and RAW ATP-S cells to subcellular fractionation as an alternate method for assessing P2X7 PM expression. Membrane pellets from a low-speed spin of cell homogenates (13,000 g) were resuspended in buffer, layered on top of a 1.12 M sucrose cushion, and centrifuged at 77,000 g. The PM fraction (Fig. 8B) floats on top of the sucrose buffer, while the M/N fraction pellets (Fig. 8B). In addition, the supernatant from the initial low-speed spin was centrifuged at 175,000 g to yield a HSP (Fig. 8B). In agreement with the surface biotinylation experiment, the subcellular fractionation indicates that the level of P2X7 expression at the PM of RAW ATP-R cells is dramatically reduced compared with RAW and RAW ATP-S cells (Fig. 8B). Note that 25 μg of protein were loaded per lane on gels for the subcellular fractionations, which represented ~10 and 2% of the total PM and HSP yields, respectively. Therefore, Fig. 7B underrepresents the difference between PM and HSP expression by about fivefold. Densitometry of the representative blots shown in Fig. 8B indicated that...
Fig. 5. RAW ATP-R cells fail to fuse in response to RANKL. RAW, RAW ATP-R, and RAW ATP-S cells were incubated in the presence (B, D, and F) or absence (A, C, and E) of 100 ng/ml soluble RANKL for 4 days. Cells were then fixed and stained for TRAP activity. Untreated cells (Con) did not display multinucleation, whereas RANKL-treated RAW cells (B) formed large multinucleated cells that stained positive for TRAP activity (dark pigment). D: RANKL-treated RAW ATP-R cells failed to form large multinucleated cells but did develop staining for TRAP activity. F: RAW ATP-S cells, which had regained ATP sensitivity, formed multinucleated giant cells after RANKL induction, similar to those seen for RAW cells. Scale bars = 75 μm.

Table 1. Effect of ATP degradation products and P2 agonists/antagonist on RAW cell fusion

<table>
<thead>
<tr>
<th>Osteoclast No., %RAW</th>
<th>Raw</th>
<th>RAW ATP-R</th>
<th>ATP degradation product*</th>
<th>AMP (1 mM)</th>
<th>ADP (100 μM)</th>
<th>P2 agonist*</th>
<th>UTP (1 mM)</th>
<th>αβ-meATP (10 μM)</th>
<th>2-meSATP (10 μM)</th>
<th>bzATP (100 μM)</th>
<th>bzATP† (1 μM)</th>
<th>P2 antagonist†</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAW</td>
<td>100</td>
<td>10±3</td>
<td>adenosine (1 mM)</td>
<td>95±7</td>
<td>129±6</td>
<td>AMP (1 mM)</td>
<td>104±2</td>
<td>103±12</td>
<td>93±3</td>
<td>94±8</td>
<td>9±4</td>
<td>PPADS (10 μM)</td>
</tr>
<tr>
<td>RAW ATP-R</td>
<td>10</td>
<td></td>
<td>AMP (1 mM)</td>
<td>104±2</td>
<td></td>
<td>AMP (1 mM)</td>
<td>104±2</td>
<td>103±12</td>
<td>93±3</td>
<td>94±8</td>
<td>9±4</td>
<td>PPADS (10 μM)</td>
</tr>
</tbody>
</table>

Values are averages ± SD. ATP-R, RAW cells with ATP; αβ-meATP, αβ-methylene-ATP; 2-meSATP, 2-methylthio-ATP; bATP, N-(4-benzoylbenzoyl)-ATP; PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate. Cells were plated on day 0, receptor activator nuclear factor-κB ligand was added on day 1, and osteoclast no. was assessed on day 4. *Additions were made on day 0; †ATP (2 mM) in RAW ATP-R cell media was replaced with 100 μM or 1 mM bzATP on day 0. ‡PPADS was added on day 3.

Fig. 6. RANKL-induced RAW ATP-R cells upregulate expression of osteoclast markers. Total RNA was isolated from RAW, RAW ATP-R, and RAW ATP-S cells that were either untreated or treated for 3 days with RANKL. Real-time PCR was performed by using primers specific to matrix metalloproteinase-9 (MMP-9), nuclear factor of activated T cells 1 (NFATc1), carbonic anhydrase, cathepsin K, TRAP, and GADPH. Results were normalized to GADPH levels, and fold enhancement was calculated by dividing the RANKL-induced value by the uninduced value. Data are from a representative experiment and are presented as averages ± SD.
P2X7 was also loaded on gels as a P2X7 molecular weight marker. Lysate from human embryonic kidney cells stably transfected with rat P2X7, or antibody directed against the middle domain of P2X7, was subjected to Western blotting with anti-P2X7 antibody. Similar amounts of P2X7 were seen in total cell lysates of all samples.

Fig. 8. P2X7 is not expressed on the cell surface in RAW ATP-R cells.

A

B

Fig. 8. P2X7 is not expressed on the cell surface in RAW ATP-R cells. A: RAW, RAW ATP-S, and RAW ATP-R cell surface proteins were covalently labeled with biotin, as described in MATERIALS AND METHODS. A portion of total cell lysate was subjected to Western blotting with anti-P2X7 antibody (top). Biotinylated (surface) proteins were affinity purified from another portion of total cell lysate and then subjected to Western blotting with anti-P2X7 antibody (middle) or antibody directed against the α-1 subunit of Na-K-ATPase (bottom). Lysate from human embryonic kidney cells stably transfected with rat P2X7 was also loaded on gels as a P2X7 molecular weight marker (P2X7 marker). Similar amounts of P2X7 were seen in total cell lysates of all samples (top), whereas the amount of biotinylated P2X7 in RAW ATP-R cells was dramatically reduced. Blotting with antibody targeting the known plasma membrane protein Na-K-ATPase indicates that biotin labeling of cell surface proteins was similarly efficient for all samples (bottom). B: subcellular fractionation of RAW, RAW ATP-S, and RAW ATP-R cells. Membranes were separated into plasma membrane (PM), high-speed pellet (HSP), and mitochondrial/nuclear (M/N) fractions, as described in MATERIALS AND METHODS. Equal amounts of protein were loaded per lane (25 μg), corresponding to ~10, 2, and 1% of the total yield of PM, HSP, and M/N fractions, respectively. STDS, molecular weight standards; ATP-S, RAW ATP-S; ATP-R, RAW ATP-R.

the band representing P2X7 in the RAW cell PM fraction was approximately equivalent to the band representing P2X7 in the HSP fraction (data not shown). This suggests that approximately five times more P2X7 were localized intracellularly than on the surface of RAW cells.

Assessment of P2X7 localization by immunofluorescence. We analyzed localization of P2X7 in RAW cells and in RAW ATP-R cells using immunofluorescence with a polyclonal antibody raised against the last 20 amino acids of the intracellular COOH-terminal tail. While we knew from our biotinylation and subcellular fractionation experiments that P2X7 was expressed at the surface of RAW cells (Fig. 8), the receptor was difficult to discern at the PM by immunofluorescence. Instead, what appeared to be vesicular punctae appeared throughout the cytoplasm (Fig. 9). This staining was eliminated by preadsorbing the antibody with peptide antigen (data not shown). The P2X7 labeling in RAW ATP-R cells appeared similar to that seen in RAW cells (Fig. 9). By immunofluorescence, the receptor showed no obvious colocalization with markers for early endosomes (early endosome antigen-1), endoplasmic reticulum (bip), Golgi (GM-130), or lysosomes (lamp-2) (data not shown).

**DISCUSSION**

In this study, we examined the effect of extracellular ATP and of P2X7 loss of function on the formation of multinucleated osteoclast-like cells. Incubation of RAW mouse macrophages in ATP caused reversible downregulation of P2X7 receptors and loss of P2X7 pore formation. These RAW ATP-R cells failed to form multinucleated osteoclast-like cells in response to RANKL (Fig. 5). Removal of ATP restored cell surface expression of P2X7, ATP-induced pore formation, and the ability to form multinucleated cells. The functional and pharmacological data presented here suggest that the lack of P2X7 activity was involved in the failure to multinucleate. Other cell surface proteins, such as leukocyte function-associated antigen-1, intercellular adhesion molecule-1, SIRP-α, CD47, CD44, CD9, and CD81, have been implicated in the process of macrophage multinucleation (12, 15, 25, 27). We cannot exclude the possibility that other PM molecules whose expression or function was altered in RAW ATP-R cells are involved in multinucleation. Regardless of the potential involvement of multiple molecules in the inhibition of multinucleation in our RAW cell model, our results point to a possible role for extracellular ATP in modulating osteoclast multinucleation and, hence, bone turnover. Indeed, extracellu-
lar ATP has recently been shown to promote NF-κB nuclear translocation in mouse and rabbit osteoclasts in a RANKL-independent manner (18).

Our results support the observations of Chiozzi et al. (4), who found a correlation between P2X<sub>7</sub> expression and giant cell formation in macrophages, and the more recent study of Gartland et al. (8), in which an inactivating P2X<sub>7</sub> monoclonal antibody prevented osteoclast formation from human peripheral blood monocytes. In contrast to these findings, Ke et al. (16) reported that a P2X<sub>7</sub> null mouse formed multinucleated osteoclasts. Thus the role of P2X<sub>7</sub> in multinucleation of mononuclear phagocytes remains unclear.

One possible explanation for the above findings is that another receptor, possibly also a member of the P2X family, compensates for loss of P2X<sub>7</sub> function in the knockout mouse. ATP-induced pore formation, although primarily ascribed to P2X<sub>7</sub> among P2X receptors, has more recently been suggested to occur with at least P2X<sub>2</sub> and P2X<sub>4</sub> (17, 29). We have identified P2X<sub>2</sub>, P2X<sub>4</sub>, and P2X<sub>6</sub> protein in RAW cells, although biotinylation studies so far have not revealed regulation of cell surface expression of these receptors by ATP (preliminary data). Additionally, the RAW cell line may differ from primary bone marrow osteoclast precursors in an inability to utilize a possible compensatory mechanism for the loss of P2X<sub>7</sub> function.

Another possibility is that P2X<sub>7</sub> associates with or regulates the function of a molecule required for cell fusion, but that this molecule can function in the absence of P2X<sub>7</sub>. This molecule could physically associate with P2X<sub>7</sub> in the PM, in which case P2X<sub>7</sub> internalization induced by extracellular ATP (as in our RAW ATP-R cells) might cause internalization of this molecule and inhibit multinucleation by this means. This molecule could also be downstream of P2X<sub>7</sub> in a signaling cascade, in which case P2X<sub>7</sub> downregulation by extracellular ATP or binding of P2X<sub>7</sub> by an inhibitory monoclonal antibody might inhibit activation of this molecule.

While RAW ATP-R cells did not fuse efficiently in response to RANKL, the cells did become positive for the osteoclast marker enzyme TRAP (Fig. 5) and upregulated expression of four other osteoclast marker mRNAs to an extent similar to RAW and RAW ATP-S cells (Fig. 6). Furthermore, when ATP was withdrawn from RAW ATP-R cells after 3 days of induction, the cells still fused by day 4 (Fig. 7). These results indicate that RANKL was able to promote osteoclast differentiation in the presence of ATP and in the absence of P2X<sub>7</sub> pore-forming activity, and that extracellular ATP suppressed only later events (occurring between days 3 and 4), leading to cell fusion and multinucleation. Our results, therefore, suggest that P2X<sub>7</sub> is involved either in the mechanics of cell fusion or in a signaling pathway proximal to this event.

Our model system selected RAW cells that lacked P2X<sub>7</sub> activity and that were, consequently, resistant to the permeabilizing effect of ATP. Maintaining RAW ATP-R cells in 2 mM ATP did not appear to commit them to a nonosteoclastic pathway of terminal differentiation, or result in a global toxicity to the cells, because fusion of RAW ATP-R cells proceeded when ATP was withdrawn as late as 3 days of RANKL induction.

Resistance to ATP-induced pore formation in RAW ATP-R cells was reversible, because the cells regained their sensitivity to ATP-induced pore formation after overnight withdrawal of ATP (RAW ATP-S cells, Fig. 4). RAW, RAW ATP-R, and RAW ATP-S cells all expressed similar quantities of P2X<sub>7</sub>. However, ATP-resistant RAW cells lacked P2X<sub>7</sub> pore-forming activity because the receptor was not expressed at the cell surface (Fig. 8). After removal of ATP from the cells, both P2X<sub>7</sub> cell surface expression and P2X<sub>7</sub> pore formation appeared. These findings suggest that ligation of P2X<sub>7</sub> regulates P2X<sub>7</sub> activity via receptor internalization and that a latent pool of P2X<sub>7</sub> can appear at the cell surface after withdrawal of the nucleotide. Thus P2X<sub>7</sub> expression and activity appear to be regulated by receptor ligation.

The majority of P2X<sub>7</sub> protein was localized intracellularly in RAW cells. The subcellular fractionation experiment shown in Fig. 8B provided a rough estimate of fivefold more P2X<sub>7</sub> expressed intracellularly than on the cell surface (see RESULTS). Consistent with this finding, immunofluorescence of P2X<sub>7</sub> in RAW cells suggested that the receptor is localized mostly in intracellular vesicles (Fig. 9). Several factors may account for why P2X<sub>7</sub> was difficult to detect at the PM by immunofluorescence. The receptor may have been too diffuse in the PM for detection. It is also possible that cell surface P2X<sub>7</sub> was lost during permeabilization with saponin, or that the COOH-terminal epitope was masked at the PM due to homomerization or interaction with other proteins.

The apparent low level of PM P2X<sub>7</sub> expression suggests that surface localization of the receptor may be an important mechanism for regulation of its activity. There were no clearly discernable differences in immunofluorescence localization of P2X<sub>7</sub> between RAW cells and RAW ATP-R cells (Fig. 9). However, because the proportion of P2X<sub>7</sub> expressed at the surface was apparently small, it would be difficult to follow the
fate of internalized P2X7. The similar level of P2X7 expression seen in RAW and RAW ATP-R cells by immunofluorescence and Western blotting suggests that the receptor is not, overall, degraded more rapidly in the RAW ATP-R cells.

A number of recent studies have examined the localization of P2X7. P2X7 expression in human peripheral blood monocytes is predominantly intracellular, but surface expression increases as the cells mature in vitro into macrophages (9–11). Mutations and polymorphisms that affect cell surface expression have been identified in the COOH-terminal tail of P2X7 (1, 6, 23, 30). Adriouch et al. (1) identified a Pro-451 to Leu natural polymorphism present in C57BL/6 and DBA/2 mouse strains that results in reduced P2X7 surface expression on T cells and concomitant reduction of ATP-induced phosphatidylserine exposure and ethidium bromide uptake. Smart et al. (23) defined a "pore-enabling domain" in the distal COOH-terminal tail of P2X7 (amino acids 551–582), where various truncations and point mutations result in loss of surface immunoreactivity and consequent loss of P2X7 channel and pore-forming activity in transfected HEK cells. The same group subsequently discovered a human polymorphism within this domain (Ile-568 to Asn), where heterozygotes displayed half-normal P2X7 surface expression and reduced P2X7 function (30). Transfection of the mutant P2X7 into HEK cells indicated a trafficking defect that resulted in the absence of surface expression. The pore-enabling domain overlaps with a previously identified putative functional domain of P2X7, the "LPS binding domain" (573–590) (5). Mutation of a dibasic motif in the LPS binding domain results in a trafficking defect in P2X7 that prevents surface expression (6). These studies point to surface localization of P2X7 as a potential mode of regulation of receptor function. It will be of interest to determine whether binding of ATP to P2X7 leads to posttranslational modifications or changes in the complement of proteins bound to the domains defined by the above studies and subsequent regulation of receptor localization.

In summary, these studies show that RAW cells maintained in extracellular ATP lose P2X7 surface expression and function and fail to form multinucleated osteoclasts in response to RANKL. They suggest that P2X7 is involved in the multinucleation process and that ligand-sensitive localization of P2X7 may underlie a mechanism by which ATP regulates bone remodeling by a local and transient effect on osteoclast formation.

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