P2X7 Receptor-Mediated Release of Cathepsins from Macrophages Is a Cytokine-Independent Mechanism Potentially Involved in Joint Diseases

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P2X<sub>7</sub> Receptor-Mediated Release of Cathepsins from Macrophages Is a Cytokine-Independent Mechanism Potentially Involved in Joint Diseases

Gloria Lopez-Castejon,* Jill Theaker, † Pablo Pelegrin,** ‡ Andrew D. Clifton, † Martin Braddock, † and Annmarie Surprenant*

The ATP-gated P2X<sub>7</sub> receptor (P2X<sub>7</sub>R) is a promising therapeutic target in chronic inflammatory diseases with highly specific antagonists currently under clinical trials for rheumatoid arthritis. Anti-inflammatory actions of P2X<sub>7</sub>R antagonists are considered to result from inhibition of P2X<sub>7</sub>R-induced release of proinflammatory cytokines from activated macrophages. However, P2X<sub>7</sub>Rs are also expressed in resting macrophages, suggesting that P2X<sub>7</sub>R may also signal via cytokine-independent mechanisms involved in joint disease. In this study, we examined P2X<sub>7</sub>-R function in resting human lung macrophages and mouse bone marrow-derived macrophages and found that ATP induced rapid release of the lysosomal cysteine proteases cathepsin B, K, L, and S and that was independent of the presence of the proinflammatory cytokines IL-1β and IL-18. Cathepsins released into the medium were effective to degrade collagen extracellular matrix. ATP-induced cathepsin release was abolished by P2X<sub>7</sub>-R antagonists, absent from P2X<sub>7</sub>-R<sup>−/−</sup> mouse macrophages, and not associated with cell death. Our results suggest P2X<sub>7</sub>-R activation may play a novel and direct role in tissue damage through release of cathepsins independently of its proinflammatory actions via IL-1 cytokines. The Journal of Immunology, 2010, 185: 2611–2619.

The ATP-gated P2X<sub>7</sub> receptor (P2X<sub>7</sub>R) can initiate the synthesis of the proinflammatory cytokines IL-1β and IL-18. However, no release of these cytokines occurs until danger-associated molecular patterns secondarily activate the inflammasome complex to initiate the processing and release of the bioactive cytokines (1–4). Extracellular ATP, released at sites of tissue damage, is a potent danger-associated molecular pattern molecule that exerts its effects by targeting the purinergic P2X<sub>7</sub> receptor (P2X<sub>7</sub>-R) in macrophages and microglia (5, 6). P2X<sub>7</sub>-Rs are primarily expressed on hematopoietic cells (5, 7, 8) and are activated by high concentrations of ATP (9). Its distinctive pharmacology and tissue localization, coupled to its unique downstream signaling to rapid processing and release of IL-1β and IL-18, has provided the basis for considering this receptor a key physiological danger sensor of the innate immune system (2, 5). Further support for this idea comes from studies using P2X<sub>7</sub>-R gene-deleted transgenic mice, which have demonstrated anti-inflammatory phenotypes and reduced pain sensitivity in models of neuropathic pain, arthritis, and other chronic inflammatory processes (10). Moreover, several highly selective and potent P2X<sub>7</sub>-R antagonists have been identified recently, showing antinociceptive actions in animal models and significant attenuation of joint destruction in a model of rheumatoid arthritis (RA) in rats (10, 11).

Virtually all studies to date into the therapeutic potential of targeting P2X<sub>7</sub>-R have focused on processing and release of IL-1β from activated monocytes, macrophages, and microglia (6, 12, 13). Nevertheless, P2X<sub>7</sub>-R activation has also been shown to signal to other cellular processes that are likely to play a role in inflammation. These include shedding of Fas-selectin and the low-affinity IgE receptor CD23 and the release of tissue factor CD142, matrix metalloproteinase (MMP) 9, and cathepsin B (12–14). However, it is not clear whether any of these additional actions of P2X<sub>7</sub>-R stimulation are independent of or a result of activation of the caspase-1/IL-1 inflammasome cascade or other PAMP-induced transcriptional signaling. Cathepsins are a family of lysosomal proteases known to play important roles in the development of both inflammatory, rheumatoid arthritis (RA), and noninflammatory, osteoarthritis (OA), joint destruction (18, 19). It had been thought that their site of action was intracellular, in acidic lysosomes, where they could then break down phagocytosed extracellular matrix proteins at low pH (19–21). However, in light of studies demonstrating a marked joint tissue acidosis in both patients with RA and OA (19, 22, 23) and presence of high levels of cathepsins in synovial fluid from patients with RA (18–20, 24), the extracellular roles for these cathepsins in joint destruction are now considered likely. Moreover, transgenic mice lacking specific cathepsins (cathepsins S and K) have demonstrated a reduced susceptibility to collagen-induced arthritis (25, 26).

The absence of studies showing whether P2X<sub>7</sub>-R plays a role in any of the actions of cathepsins in joint diseases led us to investigate this possibility. In this study, we find that P2X<sub>7</sub>-R activation, in both mouse bone marrow-derived macrophages (BMDMs) and human lung alveolar macrophages, releases sufficient amounts of lysosomal cathepsins into the extracellular medium within minutes to degrade extracellular collagen matrix in vitro. The mechanism of lysosomal release does not require initial PAMP-induced signaling and therefore is independent of IL-1β and is abolished by specific...
P2X7R antagonists but not anti–IL-1, anti–IL-6, or anti–TNF-α approaches or other drugs used in the treatment of RA and OA. Thus, our results suggest the possibility that P2X7R-induced cathepsin release may be important in joint disease pathology, and selective P2X7R antagonists may decrease bone and cartilage damage by this mechanism.

Materials and Methods

Preparation of BMDMs

Mice used in these study were C57BL/6 (P2X7R wild-type) and P2X7R-deficient (P2X7R−/−) mice in the C57BL/6 background. All experiments were carried out under the regulations of the U.K. Animal Scientific Procedures Act of 1986. BMDMs were prepared as previously reported (27) from leg bones of mice (8–10 wk of age) killed with an excess of isoflurane. Femurs and tibiae were removed, and the bone marrow flushed out. The bone marrow cells were resuspended in DMEM (Invitrogen, Paisley, U.K.) supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Osmolarity and pH values were 295–315 mOsm and 7.3, respectively, for these solutions. The loading buffer for fura 2-AM calcium measurement was composed of 0.2 M sodium acetate, 4 mM EDTA, and 4 mM DTT.

Preparation of human lung alveolar macrophages

Human lung tissue was flushed with PBS, and the resulting cell suspension was resuspended in RPMI 1640 (phenol-red free) supplemented with 2 mM glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin. Macrophages were allowed to adhere for 1 h in culture flasks at 37˚C in the presence of 5% CO2. After 5–9 d, the resulting BMDMs were detached with PBS containing 5 mM EDTA and 4 mg/ml lidocaïne, replated into 12-well plates or glass coverslips, and used the following day. The macrophage purity of these preparations was usually >95%. This was measured routinely by flow cytometry and immunocytochemistry using murine macrophage Ag F4/80.

Solutions

The standard physiological extracellular solution (PSS) was used for BMDMs and had the following composition: 147 mM NaCl, 10 mM HEPES, 13 mM glucose, 2 mM KCl, 2 mM CaCl2, and 1 mM MgCl2. The Ca2+-free extracellular solution had no CaCl2 and 1 mM BAPTA-AM. This was added to 6 mM MD-Glucose, and 5 mM EGTA. Cathepsin assay buffer was composed of 1.5 mM CaCl2, 1 mM MgCl2, 5 mM glycine, and 0.01% BSA. Agonists were prepared in PSS or BSS at concentrations indicated; antagonists were assayed for IL-1β content by sandwich ELISA using IL-1β capture and biotinylated Abs from R&D Systems. OD540 values were read in a microplate reader (Anthos Labtec Instruments, Salzburg, Austria) and compared with a standard of mouse IL-1β.

Imaging

BMDMs were plated on 13-mm glass coverslips from VWR International (Lutterworth, U.K.) and used the following day. Cells were washed once in PBS. Lysosomal membrane integrity was assessed by acridine orange labeling. Cells were incubated for 10 min in PSS containing 1 μg/ml acridine orange (Calbiochem) and washed once with PBS. Then, cells were incubated with or without 5 mM ATP in PSS at 37˚C for 10 min and fixed immediately in a 4% formaldehyde fixing solution for 30 min. After that cells were washed with PBS and mounted with Prolong Gold mounting medium (Invitrogen). To detect the intracellular cathepsin B, cells were activated with or without ATP as mentioned before and incubated with the Magic Red Cathepsin B substrate (ImmunoChemistry Technologies, Bloomington, MN) for 45 min in PSS and washed with PBS before fixation and DAPI staining as stated above. Images were acquired on a Delta Vision RT (Applied Precision, Issaquah, WA) restoration microscope using a 60×/1.42 Plan Apo or 100×/1.40 Uplan Apo objective and the 540-nm/565-nm and 405-nm/470-nm filter sets (Chroma 86000v2, Chroma Technology, Bellows Falls, VT). The images were collected using a Coolscope HQ (Photometric, Tucson, AZ) camera with a Z optical spacing of 0.2 μm. Raw images were then deconvolved using the Softworx software, and maximum intensity projections of these deconvolved images are shown in the Results.

Lactate dehydrogenase release

The presence of lactate dehydrogenase (LDH) in the medium was measured using the Cytotoxicity Detection kit (Roche) following the manufacturer’s instructions and expressed as the percent of total amount of LDH in the cells.

Fura 2 calcium assay

BMDMs were incubated with 4 μM fura 2-AM (Invitrogen) at 37˚C for 40 min. Before recording, fura 2-AM was removed and replaced with PSS. Fluorescence was recorded by an automatic fluorescence plate reader, FlexStation 3 (Molecular Devices, Sunnyvale, CA) over 150 s at 4-s intervals. The dual excitation for fura 2 was 340 nm/380 nm, and the emission was 510 nm. ATP (1 mM) was added into the wells automatically by the machine at designated time point. Data were acquired using SoftMax Pro 5 software (Molecular Devices), and the intracellular calcium level was expressed as the ratio of the emission intensities at 340 and 380 nm.

Cathepsin assays

Cells were incubated in standard physiological solution (PSS for BMDMs and BSS for alveolar macrophages) in the presence or absence of ATP at the concentrations and times indicated and supernatants collected. Where indicated, cells were preincubated for 10 min with 100 μM E-64 or 50 μM Ca-074-Me before adding ATP. Supernatants were incubated with cathepsin assay buffer and the different fluorogenic cathepsin substrates (40 μM Z-Phe-Arg-AMC (Z-FR-AMC), 40 μM Z-Arg-Arg-AMC (Z-RR-AMC), 10 μM (AC-Lys-Gln-Lys-Leu-Arg-AMC) from Bachem (Weil am Rhein, U.K.), and 5 μM (Z-LR)-Z-RH-110, from Calbiochem). Unless otherwise stated, Z-FR-AMC substrate was used to measure cathepsin activity in the supernatants. After activation in the assay buffer for 90 min at 30˚C, the end point activity was measured in either a Flexstation 3 microplate reader (Molecular Devices) with λex = 355 ± 60 nm and λem = 460 ± 35 nm or a Spectramax Gemini (Molecular Devices) at excitation/emission of 380/460 nm. Specificity of the cysteine cathepsin-dependent hydrolysis was checked by preincubating the samples with 100 μM cysteine protease inhibitor E-64 for 10 min.
Immuno-depletion

Immobilized Protein G Plus beads (Pierce, Rockford, IL) were washed three times with PBS, and equal amounts of 50% bead resin suspension were mixed with either a monoclonal anti-human cathepsin B Ab (mouse monoclonal, clone 155714 from R&D Systems), or PBS, and incubated on a shaker at 4 °C for 30 min. After washing three times with PBS, Ab-coated beads and controls were incubated with cathepsin B, macrophage supernatants, or buffer for 1 h at 4 °C on a shaker. The immune complexes were collected by centrifugation, and the supernatants were tested for cathepsin activity as described previously.

Zymography

BMDMs were activated with or without 5 mM ATP for 20 min, and supernatants were collected and concentrated as described before (see Protein assays by Western blots and ELISAs section). To detect the cysteine protease and MMP activity, concentrated supernatants were mixed with 10 μl 2× Tris-Glycine SDS Sample Buffer [0.5 M Tris-HCl (pH 6.8), 2% gycerol (v/v), 10% (w/v) SDS, 0.1% bromophenol blue], loaded onto 12% polyacrylamide gels with 0.1% gelatin (zymogram gel), and run for 90 min under a constant voltage of 125 V. For cysteine protease zymography, gels were washed and incubated for 30 min in renaturing buffer [50 mM NaAc (pH 5.5), 100 mM NaCl, 10 mM cystein, Triton X-100, 2.5% (v/v)], followed by incubation at 37 °C for 48 h in developing buffer [50 mM NaAc (pH 4.8), 20 mM cystein, 1 mM EDTA] (30). For MMP activity, detection gels were washed and incubated in renaturing buffer (2.5% Triton X-100) for 30 min, followed by incubation at 37 °C for 48 h in developing buffer (50 mM Tris, 0.2 M NaCl, 5 mM CaCl2, and 0.02% Triton X-100). Gels were stained with 0.5% Coomassie blue R-250 for 60 min and destained overnight. Zymograms were analyzed by densitometry analysis using National Institutes of Health ImageJ software (Bethesda, MD; http://rsb.info.nih.gov/ij/).

Data analysis and statistics

Average results are expressed as the mean ± SEM from the number of assays indicated. Data were analyzed using an unpaired Student t test to determine the difference between groups using Prism (GraphPad, San Diego, CA) and Kaleidagraph (Synergy, Reading, PA) software (*p < 0.05; **p < 0.001).

Results

ATP-induced release of cathepsins is mediated by P2X7R and is independent of macrophage priming

PAMP priming of macrophage with LPS and/or IFN-γ has been shown to increase mRNA levels for P2X7-R (31); such results have led to the general assumption that primed macrophages show increased functional expression of P2X7-R, although this assumption has not been directly tested. P2X-Rs are calcium-permeable ion channels; thus, the most upstream signaling events are membrane currents and simultaneous calcium influx (9). Therefore, we first assayed functional P2X7-R expression by measuring ATP-induced calcium transients in resting and LPS-primed BMDMs from wild-type and P2X7-R−/− mice (Fig. 1A). Contrary to our expectations, we found no differences in ATP-induced calcium transients between primed and unprimed BMDMs. ATP did not evoke sustained calcium increase in BMDMs from P2X7-R−/− mice; the initial transient calcium rise results from activation of phospholipase C via activation of G-protein–coupled P2Y receptors (32) and was abolished in the presence of the phospholipase C inhibitor U73122 (Fig. 1A).

Because functional expression of P2X7-R was equivalent in unprimed and LPS-primed BMDMs, we asked whether P2X-R coupled to cathepsin release in unprimed macrophages. Initial mRNA expression profiling showed that cathepsins B, L, S, D, and K were expressed in all samples with no significant differences between wild-type and P2X7-R−/− BMDM preparations, with cathepsin K being weakly expressed in all cases. Cathepsin E was highly expressed in human, but not mouse macrophages as previously reported (33), and the neutrophil-specific cathepsin G was absent in all samples (Fig. 1B). ATP stimulation induced the release of active cathepsin B, L, S, and K from unprimed macrophages as assayed by the use of specific fluorogenic substrates (Fig. 1C) (34–37). The activity of all of the released cathepsins was abolished in the presence of pan-cathepsin inhibitor E-64 (38) (Fig. 1C).

We also investigated whether ATP-induced IL-1β release from LPS-primed BMDMs was dependent on cathepsin by blocking cathepsin activity with E-64. In keeping with previous studies (5, 39), no IL-1β was present in supernatants of unprimed or LPS-primed BMDMs, whereas ATP induced large amounts of IL-1β release from LPS-primed (but not unprimed) BMDMs. The ATP-induced release of IL-1β was unaltered by cathepsin inhibition (Fig. 1D), indicating that cathepsin activity is not required for IL-1β release.

Functional characterization of ATP-induced cathepsin release from resting macrophages

Several purinoceptors are known to be expressed in both activated and resting macrophages (40, 41), but it is not known whether ATP-mediated cathepsin release is solely a function of P2X7-R ligation. Therefore, we examined properties of ATP-induced cathepsin activation from resting wild-type and P2X7-R−/− mouse BMDMs. In the absence of ATP, the lysosomal marker, acridine orange (42), as well as a specific cathepsin B fluorogenic substrate, showed the punctate intracellular distribution typical of lysosomes with no obvious differences between wild-type and P2X7-R−/− macrophages (Fig. 2A, 2B). In wild-type macrophages, ATP induced rapid cytoskeletal rearrangements (data not shown) (43), and lysosomal destabilization as indicated by the switch from punctate to diffused distribution of acridine orange (Fig. 2A) and cathepsin B fluorogenic substrate (Fig. 2B). ATP did not alter cell morphology (data not shown) or lysosomal distribution in P2X7-R−/− macrophages (Fig. 2A, 2B, right-most panels). Western blot analysis of protein levels of cathepsins B, S, and L in the supernatants showed large amounts of each of these proteins in response to ATP application in wild-type BMDMs but none in supernatants of P2X7-R−/− BMDMs, although intracellular levels of each of these cathepsins were similar in wild-type (data not shown) and knockout BMDMs (Fig. 3B).

The kinetics of extracellular cathepsin activity in response to ATP applied for up to 60 min to wild-type mouse BMDMs showed significant release of active cathepsins within 5 min, which increased approximately exponentially to plateau between 50 and 60 min; low levels of basal cathepsin release that showed a linear increase over time were also observed (Fig. 3A). ATP did not increase cathepsin release even during prolonged (60 min) stimulation from P2X-R knockout BMDMs, but there was a significant decrease in the levels of basal cathepsin release from the P2X7-R knockout BMDMs (Fig. 3A), suggesting low levels of constitutive P2X7-R activation may underlie much of the basal release of cathepsins from macrophages. Similar ATP-evoked release of cathepsins B, S, and L protein was observed from human lung alveolar macrophages, but no cathepsin proteins were detected in the supernatants from alveolar macrophages in response to ATP in the presence of the P2X7-R antagonist AZ11645373 (Fig. 4A, Supplemental Fig. 1A). Moreover, ATP concentration-response curves for extracellular cathepsin activity in human alveolar macrophage (Fig. 4B) revealed half-maximum values (EC50 = 0.36 mM; n = 6) that were the same as those obtained for human P2X7-R activation of membrane currents or calcium influx in heterologous expression systems and THP1 human monocyte cell lines (44, 45). Taken together, these results conclusively show that all destabilization of lysosomes and release of cathepsins induced by ATP result solely from activation of P2X7-R in both murine and human macrophages.

P2X7-R-induced cathepsin release uses different signaling pathway to that of IL-1β release

Several studies have shown that the rapid release of IL-1β from PAMP-primed macrophages and microglia after P2X-R activation

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is dependent on extracellular calcium (28, 46). In addition, potassium efflux in response to opening of the cationic P2X7R ion channel leads to marked intracellular potassium depletion, which is considered the key upstream signaling event required to directly activate the inflammasome complex, a conclusion based on the well-known observation that high extracellular potassium abolishes ATP-evoked caspase-1 activation and IL-1β release (39, 47). Therefore, we asked whether in resting macrophages similar signaling processes were involved with P2X7R-induced cathepsin release by measuring cathepsin activities in the supernatants from wild-type BMDMs. In the presence of calcium-free extracellular solution, the ATP-induced cathepsin release was significantly but only partially reduced (36 ± 8%), whereas incubation of cells in high extracellular potassium solution did not alter the ATP-evoked release of cathepsins (Fig. 3C). In addition, the calcium ionophore ionomycin was able to induce similar cathepsin release from BMDMs than P2X7R stimulation (Fig. 3D), validating the role of intracellular calcium in this process and confirming previous results in which P2X7R activation induces exocytosis of secretory lysosomes by an increase in intracellular calcium (12, 48).

However, in our model, P2X7R-dependent release of cathepsins was not entirely dependent on intracellular calcium (Fig. 3C), suggesting another parallel signaling pathway independent on calcium. Surprisingly, treatment of BMDMs with the potassium/proton ionophore nigericin was also able to induce similar cathepsin release compared with P2X7R stimulation (Fig. 3D), but potassium efflux

FIGURE 1. ATP-induced cathepsin release does not require PAMP priming and is independent of IL-1β. A, ATP-evoked calcium influx in mouse BMDM. ATP (1 mM) induced a sustained calcium increase in wild-type macrophages that was absent in P2X7R-deficient cells. LPS priming prior to ATP activation had no effect on the levels of calcium influx. The initial transient calcium increase present in P2X7R−/− BMDM was due to P2Y receptor activation and abolished in the presence of U73122. B, RT-PCR of cathepsin mRNA expression in wild-type and P2X7R-deleted mouse BMDMs and human lung macrophages. C, ATP-induced release of cathepsins from unprimed BMDMs. Cathepsin activity in the supernatants was measured after ATP (5 mM) stimulation using fluorogenic substrates specific for cathepsins B, L, S, and K. Incubation of these supernatants with the cystein-protease inhibitor E-64 (100 μM) completely blocked this activity; n = 10 for Z-FR-AMC substrate, n = 3 for the others. D, IL-1β release from BMDM after ATP (5 mM) application for 20 min. Incubation of cells with the cathepsin inhibitor E-64 (100 μM) did not affect the cytokine release; n = 3.
cannot account for this effect, as we have found that potassium depletion is not needed for this process (Fig. 3C). It has been shown that agents like nigericin or ammonium chloride induce an increase in lysosomal pH and subsequent exocytosis of lysosomes (49). In accordance with these results, NH4Cl also triggered cathepsin release in BMDM, and, surprisingly, this release was independent on intracellular calcium (Fig. 3E). Such alkalinization can well explain the calcium/potassium-independent release of cathepsin induced by nigericin and P2X7R activation.

Because cell death is known to occur in macrophages and fibroblasts upon prolonged P2X7R stimulation (39, 50), we measured LDH release levels in all our experiments. In BMDM supernatants, there was a small increase in the level of LDH released after 45–60 min of ATP stimulation (Supplemental Fig. 2A, 2B), time in which cathepsin release was maximum (Fig. 3A), suggesting that cathepsin release was not due to cell death. Furthermore, ATP induced cathepsin release was not affected in the presence of 5 mM glycine (Supplemental Fig. 3A) but as expected (29), glycine clearly inhibited LDH release from ATP-treated BMDMs (Supplemental Fig. 3A). This effect was not detected after 20 min of ATP application due to the low levels of LDH released (Supplemental Fig. 3A). Similar cell death (LDH release) and cathepsin release was obtained after BMDM ATP stimulation in BSS or PSS extracellular solutions containing glycine (Supplemental Fig. 3B), and stimulation of human lung macrophages with ATP in BSS solution induced lower levels of LDH release (>3%) than when glycine was removed from the solution (>5%), whereas levels of cathepsin, as detected by Western blot, were not affected (Supplemental Fig. 3C).

We next asked if the presence of proteases, such as cathepsins, in the extracellular space could potentially damage the cells integrity and was inducing the release of LDH over time. We measure the levels of LDH and cathepsin release in supernatants from BMDM after ATP stimulation in the presence of cathepsin inhibitors. We found that despite abolishing cathepsin enzymatic activity (Supplemental Fig. 3D), stimulation of BMDM with ATP in the presence of the broad cathepsin inhibitors E-64 and the more specific cathepsin B inhibitor Ca-074-Me did not affect the levels of LDH release or cathepsins present in the extracellular media (Supplemental Fig. 3D, 3E).

Thus, we can conclude the P2X7R-mediated release of cathepsins that occurs with stimulation periods used in our experiments is independent of the release from damaged or dying cells and is an active mechanism involving lysosome secretion by a calcium-dependent direct exocytosis and calcium-independent pH-dependent lysosome destabilization.

**Cathepsin B is an important contributor to P2X7R-induced cathepsin activity released from human macrophages**

We next asked whether one or more of the cathepsin enzymes we observed to be released (Fig. 4A) may be primarily responsible for the extracellular activities measured from human lung macrophages (Fig. 4B) in view of the therapeutic relevance of these cells. We found that the specific immunodepletion of cathepsin B with a monoclonal human cathepsin B Ab reduced enzymatic activity from supernatants of ATP-stimulated alveolar macrophages in a concentration-dependent fashion (Fig. 4C). The inhibition of enzymatic activity of the released cathepsins by cathepsin B immunodepletion paralleled the inhibition of purified cathepsin B by increasing concentrations of the anti-cathepsin B Ab (Fig. 4C). Quantitative pharmacological profiling of inhibition of enzymatic activity of ATP-induced cathepsins from human lung macrophage supernatants by cathepsin inhibitors was carried out.
and compared with compound potencies (IC$_{50}$ values) obtained for inhibition of purified cathepsin B. The potent cathepsin B inhibitor, Ca-074 (51), inhibited enzymatic activity from macrophage supernatants with the same IC$_{50}$ (1.2 nM) as for purified cathepsin B, whereas a highly specific cathepsin K inhibitor, inhibitor I, was inactive for either purified cathepsin B or supernatants from ATP-stimulated human lung macrophages after cathepsin B immunodepletion with increasing concentrations of a monoclonal anti-human cathepsin B Ab; $n = 3$.

Table I. Quantitative pharmacological characterization of potencies of cathepsin inhibitors at purified cathepsin B and ATP-stimulated lung macrophage supernatants

<table>
<thead>
<tr>
<th>Compound</th>
<th>Purified Cathepsin B (nM)</th>
<th>Macrophage Supernatant (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-64</td>
<td>2.7</td>
<td>4.1</td>
</tr>
<tr>
<td>Ca-074 (cathepsin B inhibitor)</td>
<td>1.1</td>
<td>1.2</td>
</tr>
<tr>
<td>Cathepsin K inhibitor I</td>
<td>Inactive</td>
<td>Inactive</td>
</tr>
<tr>
<td>AZ10573295</td>
<td>Inactive</td>
<td>Inactive</td>
</tr>
</tbody>
</table>

Compound potencies (IC$_{50}$ values) did not differ between inhibition of purified cathepsin B activity and macrophage supernatant cathepsin activity ($n = 4$ for all). The P2X$_7$R antagonist, AZ10573295, was inactive, thus showing it has no direct effect on cathepsin.

It was of therapeutic interest to examine whether other compounds in clinical use for treatment of inflammatory and non-inflammatory joint disease have anticephsin actions on P2X$_7$-R-mediated release. Anakinra (IL-1R antagonist), etanercept (anti-TNF-$\alpha$), sulphasalazine, prednisolone, diclofenac, methotrexate, and MAB206 (anti-IL-6) (53–56) were used at concentrations known to be maximally effective in other in vitro assays and/or ∼10-fold greater than therapeutic free plasma concentrations, but only the P2X$_7$-R antagonist inhibited ATP-induced cathepsin release (Fig. 5B). These results provide further evidence that P2X$_7$-R directly induces cathepsin release independently of IL-1 and other cytokines.

Cathepsin activity released by P2X$_7$-R stimulation is sufficient to degrade extracellular matrix

Finally, we asked whether P2X$_7$-R-induced cathepsin release was sufficient to exert physiological effects by assaying cathepsin gelatinolytic activity using cystein zymography. Low levels of constitutive gelatinolytic activity were detected from unstimulated wild-type mouse BMDMs supernatants; this increased by 50% upon 20-min stimulation with ATP (Fig. 6A, 6C). Constitutive gelatinolytic activity was significantly reduced in supernatants of P2X$_7$-R$^{-/-}$ BMDMs compared with the wild-type, and ATP induced no increase (Fig. 6A, 6C). The cysteine cathepsin gelatinolysis was observed as two bands, of the same size as cathepsin B proform (36 to 37 kDa) and mature single chain (28–30 kDa) (57, 58). We also analyzed MMP activity in the supernatants from the same experiments by specific MMP zymography. We detected two bands of activity corresponding to pro-MMP9 (92 kDa) and pro-MMP2 (72 kDa) that did not change in intensity upon ATP stimulation. There was no difference in the intensity of any of the bands from wild-type or P2X$_7$-R knockout BMDMs (Fig. 6B, 6D).

Fig. 1D), an inactive analog at P2X$_7$-R, did not alter ATP-induced cathepsin release (Fig. 5A).

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environment, whereas only one, cathepsin release, operates in the absence of an inflammatory cytokine milieu.

Our results concerning the ionic basis of P2X$_7$R-induced cathepsin release provide new insight into mechanisms by which P2X$_7$R signals to the dual events of cathepsin and IL-1$\beta$ release and may partially resolve a long-standing controversy regarding how IL-1$\beta$ is released from activated macrophages. IL-1$\beta$ is a leaderless protein unable to follow classical secretory pathways (3–5), and, despite much work to delineate molecular and cellular mechanisms of its release process, remains an unresolved issue (12, 17, 28, 59). Lysosomal activation in response to ATP stimulation of PAMP-primed macrophages has previously been suggested to be one of the means whereby IL-1$\beta$ is transported to the outside of the cell via exocytosis of IL-1$\beta$–containing lysosomes (17). However, several more recent studies have clearly dissociated the presence of caspase-1 and IL-1$\beta$ from lysosomes (12, 43, 59). In the current study, we found that P2X$_7$-induced lysosomal activation and cathepsin release from unprimed macrophages were only partially dependent on calcium and, most significantly, were unaltered in the presence of high extracellular potassium ions. This is in striking contrast to P2X$_7$-mediated caspase-1 activation and IL-1$\beta$ release, which is highly calcium sensitive and is abolished in the presence of high potassium solutions (39, 47). Lysosomal secretion can be triggered by the P2X$_7$-mediated calcium increase in the cells (12) and therefore be responsible for the partial calcium dependence on cathepsin release that we found in this study. This is supported by the fact that, in BMDM, intracellular calcium increase triggered by the calcium ionophore ionomycin also induced the release of lysosomal contents to the extracellular space. Nigericin, a proton/potassium ionophore known to induce IL-1$\beta$ release from primed cells by triggering potassium efflux in a similar way to P2X$_7$ (39), also induced cathepsin release. This is not due to

**Discussion**

The novel and most therapeutically significant finding of the current study is that P2X$_7$R stimulation evokes rapid release of lysosomal cathepsins from murine and human macrophages independent from PAMP signaling in quantities sufficient to induce in vitro degradation of extracellular matrix. Previous studies have demonstrated ATP-induced release of cathepsins from PAMP-activated macrophages (12, 17) even when IL-1$\beta$ secretion is impaired (12). However, the possibility that this may occur in unprimed macrophages in which proinflammatory cytokines are not present, being completely unrelated to IL-1$\beta$/IL-18 release, and that these cathepsins may have relevant biological activity once released has not been considered. The ATP-induced release of cathepsins was shown to be solely due to P2X$_7$R stimulation without contribution from other purine receptors because ATP concentration-response profiles (EC$_{50}$ = 0.360 mM) were characteristic of P2X$_7$R. ATP evoked no release from P2X$_7$-gene-deleted mouse BMDMs, and highly selective P2X$_7$R antagonists completely blocked ATP-evoked cathepsin release from human lung alveolar macrophages. This P2X$_7$-mediated release was independent of cell death because reduction of macrophage cytoly-sis induced by ATP activation of the receptor by the use of glycine (29) did not affect the levels of cathepsin release. We also found that P2X$_7$R-agonist-evoked activation of cathepsin B (39, 47) and membrane currents (data not shown) in resting and PAMP-primed macrophages and that blocking cathepsin activity using the pan-cathepsin inhibitor E-64 in PAMP-primed macrophages did not alter P2X$_7$-mediated IL-1$\beta$ release. Taken together, these results have important consequences in terms of potential roles for P2X$_7$R in noninflammatory joint diseases because they allow us to conclude that P2X$_7$R activation signals to two separate signaling cascades, both of which operate during inflammatory conditions in a high proinflammatory cytokine

**FIGURE 5.** Inhibition of cathepsin release by selective P2X$_7$R antagonists but not other anti-inflammatory compounds. A, ATP-induced cathepsin release inhibition by the selective P2X$_7$R antagonists AZ11648720 (circles) and AZ10573295 (squares) and the inactive analog AZ10603690 (triangles) at human lung macrophages; n = 6 for each point. B, Actions of P2X$_7$R antagonist AZ10573295, anakinra, etanercept, MAB 206 (anti–IL-6), sulfasalazine, prednisolone, diclofenac, and methotrexate on ATP-induced cathepsin release. Concentrations of these anti-inflammatory agents were 10-fold greater than their therapeutic maximums; n = 3–6 for each.

**FIGURE 6.** P2X$_7$R-induced cathepsin release degrades extracellular matrix in vitro. Supernatants from wild-type or P2X$_7$R$^{-/-}$ mouse BMDMs were assayed for their ability to degrade collagen extracellular matrix by cathespin A, C or MMP (B, D) zymography. Representative zymogram from unstimulated and ATP-stimulated (5 mM for 20 min) supernatants are shown in A and B with densitometry analysis of the bands shown in C and D; n = 3. Densitometry results are shown as band intensity in ATP-stimulated supernatants relative to unstimulated supernatants.
potassium efflux per se, as we have shown this is not needed for the process (Fig. 3C), but possibly to the lysosomal alkalization induced by nigericin because it is known that a rise in lysosomal pH triggers lysosomal secretion (49). According to this, ammonium chloride, another compound capable of increasing lysosomal pH, also induced the release of cathepsins in BMDM, but this release was not impaired in the absence of calcium. Alkalination of lysosomal compartments can precede lysosome destabilization (60), and we have shown that ATP activation of P2X7-R on BMDM induces such an effect (Fig. 2A). These data suggest a mechanism by which P2X7-R activation induces cathepsin release through lysosomal secretion by a calcium-dependent direct exocytosis and calcium-independent pH-dependent lysosome destabilization. These results not only further support recent evidence against a role for lysosomal export of IL-1β but also establish a different physiological role for P2X7-R-induced lysosomal activation, that of releasing cathepsins into the extracellular environment.

Cathepsin B was the primary active protease in supernatants from ATP-stimulated human lung macrophages as indicated by the decrease in total cathepsin activity by cathepsin B immunodepletion, by selective cathepsin B, but not cathepsin K inhibitors, and by bands corresponding to cathepsin B active chain in zymography assays. Interestingly, we did not detect an increase in MMP activity in the supernatants from these macrophages, whereas a recent study on human monocytes and B lymphocytes obtained from peripheral blood showed that ATP stimulation induced rapid release of MMP9 (16). As MMPs are the other major class of degradative extracellular enzymes, this may reflect tissue/cell-specific release patterns or physiological state of the cells. Further investigations into release patterns of degradative enzymes in response to P2X7-R stimulation in synovial cells and osteoclasts from healthy and diseased joints will be required to delineate specific cathepsins and/or MMPs that may be involved in tissue damage by this mechanism. Nevertheless, the potential role of cathepsin B in RA and OA has generated increasing interest as a therapeutic target in view of studies showing markedly increased levels of cathepsin B in synovial fluid from patients with RA in sufficient quantities to read-

Disclosures

The authors have no financial conflicts of interest.

References


