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Inhibition of Neutrophil Apoptosis by ATP Is Mediated by the $P2Y_{11}$ Receptor[1]

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Neutrophils undergo rapid constitutive apoptosis that is delayed by a range of pathogen- and host-derived inflammatory mediators. We have investigated the ability of the nucleotide ATP, to which neutrophils are exposed both in the circulation and at sites of inflammation, to modulate the lifespan of human neutrophils. We found that physiologically relevant concentrations of ATP cause a concentration-dependent delay of neutrophil apoptosis (assessed by morphology, annexin V/To-Pro3 staining, and mitochondrial membrane permeabilization). We found that even brief exposure to ATP (10 min) was sufficient to cause a long-lasting delay of apoptosis and showed that the effects were not mediated by ATP breakdown to adenosine. The P2 receptor mediating the antiapoptotic actions of ATP was identified using a combination of more selective ATP analogs, receptor expression studies, and study of downstream signaling pathways. Neutrophils were shown to express the $P2Y_{11}$ receptor and inhibition of $P2Y_{11}$ signaling using the antagonist NF157 abrogated the ATP-mediated delay of neutrophil apoptosis, as did inhibition of type I cAMP-dependent protein kinases activated downstream of $P2Y_{11}$, without effects on constitutive apoptosis. Specific targeting of $P2Y_{11}$ could retain key immune functions of neutrophils but reduce the injurious effects of increased neutrophil longevity during inflammation. *The Journal of Immunology,* **2007, 179: 8544–8553.**

Apoptosis (programmed cell death) of neutrophils is an important control point in the physiological resolution of innate immune responses. In this context, neutrophil apoptosis must be delayed until essential host functions such as pathogen clearance are completed but then proceed promptly to abrogate inflammation and avoid tissue damage (1). Neutrophils are typically short-lived cells that can engage a constitutive program of apoptosis (2), leading to down-regulation of neutrophil proinflammatory functions and clearance by phagocytes (2, 3). There is abundant evidence that the balance between neutrophil survival and death by apoptosis is exquisitely regulated by a range of extracellular factors, including host-derived cytokines and pathogen-derived molecules to which neutrophils are exposed in the circulation and in tissues (4, 5). The potential for extracellular nucleotides, particularly ATP, to modulate neutrophil survival has not been investigated.

Neutrophils are exposed to ATP in a variety of in vivo situations, and its role as a signaling molecule in pathophysiological situations is increasingly recognized (6). ATP is released into the circulation after activation of platelets and endothelial cells (7, 8), for example, in acute coronary syndromes (7), potentially exposing circulating neutrophils to high local concentrations. Within 3 min after vessel wall injury, ATP concentrations of 20 μM can be detected (9), and 1×10^7 platelets can release concentrations of >55 μM (8). ATP is also released from dying cells (10), notably in chronic inflammatory conditions such as cystic fibrosis (11, 12). The effects of ATP are mediated via P2 receptors (13), which are further divided into P2X and P2Y subfamilies (14). Both are widely expressed in tissues and implicated in diverse cellular functions. ATP has been shown to modulate neutrophil proinflammatory functions, including chemotaxis (15), NADPH oxidase-dependent superoxide anion generation (16), and secretion of granule contents (17, 18).

We hypothesized that extracellular ATP may be a critical regulator of neutrophil apoptosis. We found that even short exposures to ATP delay neutrophil apoptosis, an effect that is independent of increases in intracellular calcium ($[Ca^{2+}]_i$)[3] but dependent on type I cAMP-dependent protein kinases. Studies of receptor expression and use of P2 subtype inhibitors and agonists identified $P2Y_{11}$ as the purinergic receptor mediating the antiapoptotic effect. These studies identify a novel potential therapeutic target for the amelioration of neutrophilic inflammation in a wide range of inflammatory diseases.

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[3] Abbreviations used in this paper: $[Ca^{2+}]_i$, intracellular calcium; $\alpha\beta$MeATP, $\alpha\beta$-methylene-ATP; ATP-γ-S, AT[γ-thio]P; BzATP, 2′-3′-*O*-(4-benzoylbenzoyl)-ATP; cA-PK, cAMP-dependent protein kinases; HEK, human embryonic kidney; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide; 2Me-SATP, 2-(methylthio)-ATP; N^6-MB-cAMP, N^6-monobutyryladenosine-cAMP; PLC, phospholipase C; *Rp*-8-Br-cAMPS (*Rp* isomer), 8-bromoadenosine-3′,5′-cyclic monophosphorothioate; U73122, 1-{6-{[17β-3-methoxyestra-1,3,4(10)-trien-17-yl]amino}hexyl}-1*H*-pyrrole-2,5-dione; fluo-4-AM, fluo-4-acetoxymethyl ester; EC_{50}, half-maximal effective concentration; $[cAMP]_i$, intracellular cAMP.

Materials and Methods

Materials

All chemicals were from Sigma-Aldrich unless otherwise stated. The phospholipase C (PLC) inhibitor 1-{6-{[17β-3-methoxyestra-1,3,4(10)-trien-17-yl]amino}hexyl}-1*H*-pyrrole-2,5-dione (U73122) and its inactive analog 1-(6-{[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino}hexyl}})-2,5-pyrrolidinedione, 8-bromoadenosine-3′,5′-cyclic monophosphorothioate (*Rp*-8-Br-cAMPS), and pluronic acid were purchased from Calbiochem. Fluo-4-acetoxymethyl ester (fluo-4-AM) was obtained from Molecular Probes. The $P2Y_{11}$ and $P2X_7$ (APR-008) receptor Abs for Western blotting were from Alomone Laboratories. The $P2X_7$ Ab for flow cytometry was a gift from GlaxoSmithKline (19), and the IgG2b isotype control was from Sigma-Aldrich. The $P2Y_{11}$ antagonist NF157 was synthesized as previously described (20).

Neutrophil isolation and culture

Human neutrophils were isolated by dextran sedimentation and plasma-Percoll gradient centrifugation from whole blood of normal volunteers (21) with written informed consent and the approval of the South Sheffield Research Ethics Committee. Purity (>94%) was assessed by counting >300 cells on duplicate cytospins, with contaminating cells being mostly eosinophils. In some experiments, neutrophils were further purified by negative magnetic selection, increasing neutrophil purity to >99.9% by removal of the low level of contaminating cells, especially PBMCs, that can affect the responses of neutrophils (22). These neutrophils are referred to in text as highly purified neutrophils. Neutrophils were suspended at 2.5×10^6/ml in RPMI with 1% penicillin-streptomycin and 10% FCS (all from Invitrogen) and cultured in 96-well Falcon Flexiwell plates (BD Biosciences). Freshly isolated neutrophils were designated as time 0.

ATP and ATP analogs were added at time 0 and incubated for 5 h unless otherwise stated. Neutrophils treated with *Rp*-8-Br-cAMPS or NF157 were preincubated for 30 min before addition of ATP or ATP analogs.

Assessment of cell viability and apoptosis

Nuclear morphology was assessed on Giemsa-stained cytospins, with blinded observers counting >300 cells per slide on duplicate cytospins. Hemocytometer counts were performed at all time points, both to assess cell retrieval and for trypan blue exclusion. No significant differences in cell retrieval were noted with different treatments, and necrosis was <2% throughout. In some experiments, neutrophils were washed in PBS and stained with PE-labeled annexin V (BD Biosciences) and To-Pro3 iodide (Molecular Probes) to identify apoptotic (annexin V^+) and necrotic (To-$Pro3^+$) cells (23). Controls included EDTA treatment (to determine the annexin V population), freeze-thawed, necrotic cells (To-$Pro3^+$ cells) and constitutively aged neutrophils (annexin V^+), to set appropriate gating. To detect the loss of mitochondrial membrane potential ($\Delta\psi_m$), neutrophils were incubated with 10 μM 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; Molecular Probes) at 37°C, and loss of $\Delta\psi_m$ was assayed by gain in FL-1 fluorescence (24). Samples were analyzed by FACSCalibur flow cytometer (BD Biosciences), with 10,000 events recorded and analyzed by CellQuest software (BD Biosciences).

Measurement of neutrophil $[Ca^{2+}]_i$

Ca^{2+} transients were measured in populations of human neutrophils loaded with the Ca^{2+} indicator fluo-4-AM using the magnetically stirred Cairn Integra fluorometer (Cairn Research) and a previously described method (25). Briefly, neutrophils were incubated with 2 μM fluo-4-AM and 0.1% pluronic acid for 30 min at 37°C in buffer containing 136 mM NaCl, 1.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5 mM $NaHCO_3$, 1.2 mM $CaCl_2$, 5.5 mM glucose, 20 mM HEPES, and 5 mM EGTA. Cells were washed once in the same buffer with or without EGTA, and then 5×10^6 cells/ml were placed in a 1-ml cuvet for calcium measurements using the Cairn Integra fluorometer (Cairn Research). Cumulative agonist concentration-response curves were obtained, and results normalized to maximum fluorescence induced by digitonin (2 mM) applied at the end of the experiment. Digitonin-induced fluorescence varied by <10% in a single set of experiments (six cuvets/experiment; data not shown). Curves shown are representative of the whole cell population.

Analysis of mRNA expression

Total RNA was extracted from highly pure populations of neutrophils using TRI-reagent according to the manufacturer's instructions. To obtain cDNA, 1 μg of total RNA was primed with oligodeoxythymidylate primers and reverse transcribed with avian myeloblastosis virus reverse transcriptase (Promega). Primers for human P2X receptors were designed based on published sequence data. The primers used (forward, reverse) were as follows. $P2X_1$: TTTCATCGTGACCCCGAAGCAG, TCAAAGCGAATCCCAAACACC; $P2X_2$: ACCTGCCCCGAGAGCATAAG, AATGACCCCGATGACACCACCC; $P2X_3$: CACCTCGGTCTTTGTCATCATCAC, TGTTGAACTTGCCAGCATTCC; $P2X_4$: ACAGCAACGGAGTCTCAACAGG, CCTTCCCAAACACAATGATGTCG; $P2X_5$: TCCTGGCGTACCTGGTCGTATGG, CAGTCGCTGTCCTTGGAGCACG; $P2X_6$: GGTGACCAACTTCCTTGTGACG, CCCAGTGAACTCTGATGCCTACAG; $P2X_7$: TGCGATGGACTTCACAGATTTG, TGCCCTTCACTCTTCGGAAAC. PCR settings were 94°C for 1 min and 55°C for 2 min, followed by extension at 72°C for 3 min. Each PCR reaction was performed with 35 cycles, and the PCR products were resolved by electrophoresis on 1.5% agarose gel.

Western blotting

Highly pure populations of neutrophils were washed with ice-cold PBS and lysed with 2× SDS lysis buffer (100 mM Tris-HCl (pH 6.8), 100 mM DTT, 20% SDS, 20% glycerol, and 0.2% bromophenol blue, made up in water) containing EDTA-free protease inhibitor mixture, at 90°C for 10 min. The protein was subjected to 8% SDS-PAGE and then electrophoretically transferred from the gel onto a 0.45 μM polyvinylidene fluoride microporous membrane (Bio-Rad). The membrane was probed with a rabbit polyclonal Ab directed against the human $P2Y_{11}$ or $P2X_7$ receptor, with or without the control peptide Ag (at 1 μg of peptide per μg of Ab), overnight at 4°C. After a washing, the membrane was incubated with HRP-conjugated goat anti-rabbit Ab (DAKO) and visualized using the ECL system (Amersham Biosciences) followed by autoradiography.

Flow cytometry for $P2X_7$ expression

Highly purified neutrophils, human embryonic kidney (HEK)-293 cells (negative control), and HEK-293 cells transfected with human $P2X_7$ (positive control; Ref. 26) were used to detect the presence of $P2X_7$. Extracellular expression was detected using fixed cells and total $P2X_7$, both extracellular and intracellular, using cells that were fixed then permeabilized. Fixation and permeabilization buffers were from eBioscience. Cells were then incubated with the $P2X_7$ receptor Ab (1.25 μg/μl) or control IgG2b Ab (1.5 ng/ml) for 30 min, followed by an anti-mouse IgG FITC-conjugated secondary Ab (10 μl/100 μl; Sigma-Aldrich). Analysis of $P2X_7$ receptor expression was by flow cytometry, using a dual-laser FACSCalibur (BD Biosciences) and CellQuest software (BD Biosciences), with appropriate single-stained samples for setting of compensation. We confirmed that we were able to detect increased expression of CD11b, a protein present intracellularly, following permeabilization (data not shown).

Measurement of neutrophil intracellular cAMP ($[cAMP]_i$)

Neutrophils have low concentrations of cAMP and thus were cultured at 3×10^6/ml, as previously described (27), and preincubated with 3-isobutyl-1-methylxanthine (1 mM) for 20 min to inhibit breakdown of cAMP, before stimulation with ATP, AT[γ-thio]P (ATP-γ-S), or 2′-3′-*O*-(4-benzoylbenzoyl)-ATP (BzATP), all at 100 μM, for 8 min. Cells were lysed with 0.1 M HCl and then spun at $850 \times g$ for 10 min. Supernatants were acetylated to detect intracellular cAMP, using a direct immunoassay kit (Sigma-Aldrich; measuring sensitivity, 0.039 pmol/ml) according to the manufacturer's instructions. Data are expressed as fold increase in intracellular cAMP compared with unstimulated cells.

Statistical analysis

All data are expressed as mean ± SEM. Data were analyzed as appropriate by Student's *t* test or ANOVA with either Dunnett's or Bonferroni's (selected pairs) posttest using the Prism 4.0 program (GraphPad). Results were considered to be statistically significant where $p < 0.05$. Statistically significant differences from controls are indicated by: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$. Differences between treated populations are indicated by: #, $p < 0.05$; ##, $p < 0.01$; and ###, $p < 0.001$.

Results

ATP delays neutrophil apoptosis in a concentration-dependent manner

Incubation of neutrophils with ATP resulted in concentration-dependent reductions in neutrophil apoptosis at 5 h that were significant at ATP concentrations of 1 μM and above (Fig. 1*A*). Such concentrations are physiological and readily achieved in vivo (8, 9). This delay of apoptosis was maintained over a prolonged time course (Fig. 1*B*). Inhibition of apoptosis was assessed by light

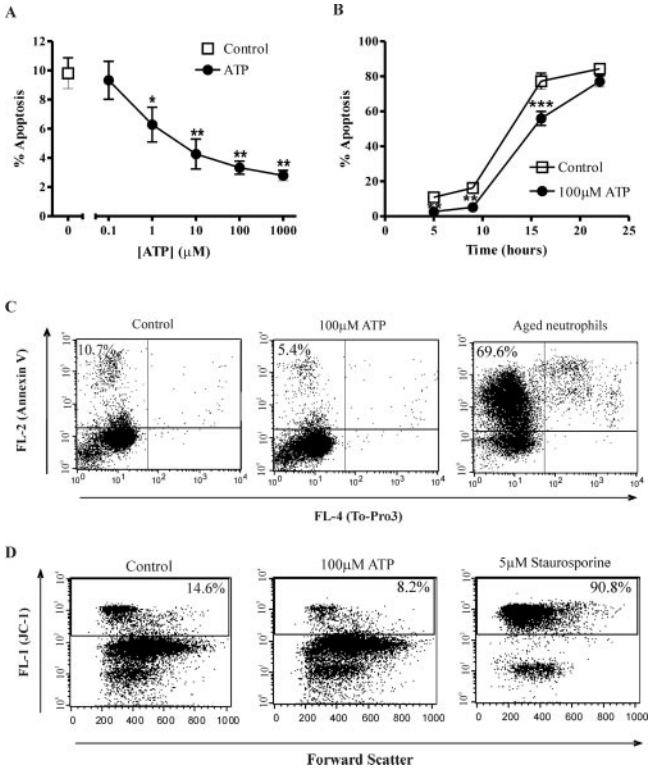

FIGURE 1. ATP inhibition of neutrophil apoptosis is concentration and time dependent. Neutrophils were cultured for varying periods of time in the presence or absence of various concentrations of ATP. Apoptosis was assessed by morphological criteria, and data are expressed as mean percent apoptosis ± SEM from at least three independent experiments, each from a separate donor. *A,* Neutrophils were cultured either alone (□) or with various concentrations of ATP (0.1–1000 μM; ●) for 5 h. *B,* Neutrophils were incubated in the presence (●) or absence (□) of ATP (100 μM) for 5, 9, 16, and 22 h before assessment of apoptosis. *C,* Neutrophils were cultured in the presence or absence of ATP (100 μM) for 5 h, and apoptosis and necrosis were assessed by staining with annexin V/To-Pro3 and flow cytometry. Representative dot plots show annexin V and To-Pro3 staining in untreated cells (negative control), following ATP treatment or constitutively aged, 24-h neutrophils (positive control). Results are representative of three independent experiments in which ATP delayed the onset of apoptosis compared with untreated cells (mean ± SEM 5.2 ± 0.8% compared with 9.1 ± 0.8% in control cells, $p < 0.05$). *D,* Neutrophils were cultured for 4 h with ATP (100 μM) or staurosporine (5 μM) and then stained with the mitochondrial dye, JC-1 and assessed by flow cytometry. Representative dot plots show JC-1 fluorescence in untreated cells (negative control) and following ATP or staurosporine (positive control) treatment. An increase in FL-1 (green) fluorescence indicates loss of mitochondrial membrane potential in neutrophils. Results are representative of three independent experiments in which ATP significantly maintained membrane potential compared with untreated cells (10.0 ± 1.1% apoptosis in ATP-treated cells compared with 21.4 ± 2.5% in control cells, $p < 0.05$).

microscopy using morphological features (2); in additional experiments, these changes were correlated with evidence that ATP also delayed cell membrane changes of apoptosis (annexin V binding; Fig. 1*C*) and loss of mitochondrial membrane potential (JC-1 staining; Fig. 1*D*). There was no evidence of necrotic cell death on trypan blue exclusion or To-Pro3 staining (data not shown), nor of differences in cell retrieval on hemocytometer counts with ATP treatment compared with controls. We have previously shown that the antiapoptotic effects of a prototypic proinflammatory mediator, LPS, are principally dependent on the small numbers of mononuclear cells present in neutrophil populations prepared by gradient centrifugation (22). We therefore compared the antiapoptotic effects of ATP on neutrophils prepared by the standard method or with an additional purification step based on immunomagnetic bead purification to achieve >98% purity (22). There were no differences in the antiapoptotic effects of ATP over a range of concentrations (Table I).

The exposure of neutrophils to significant concentrations of ATP may be of relatively short duration in vivo, because ATP is readily broken down (28), yet the survival effect of ATP treatment of neutrophils was long-lasting. We therefore determined whether prolonged neutrophil survival could be initiated by short exposures to ATP. In Fig. 2*A,* neutrophils were cultured for 5 h, and apoptosis was measured. For the first 10, 30, 60, or 180 min of this period, neutrophils were treated with ATP (or medium only as a control), after which time they were washed and returned to fresh medium. Other neutrophils were cultured with medium alone or ATP throughout the time course, without additional washing. These data show that a 10-min exposure to ATP (100 μM) at the beginning of the experiment induced similar levels of neutrophil survival as when cells were exposed to ATP throughout the time course, demonstrating rapid engagement of an extremely effective survival mechanism. We also determined whether neutrophils exposed to ATP after they had aged in culture could still have a measurable increase in survival after ATP exposure. We found that ATP had a significant survival effect on neutrophils when added up to 180 min after culture (Fig. 2*B*).

The lifespan of ATP in the tissues and circulation is tightly regulated by the actions of ectonucleotidases that hydrolyze ATP to adenosine, which can itself delay neutrophil apoptosis (29). To confirm that the effects of ATP on neutrophil apoptosis were not due to breakdown to adenosine, we used a stable ATP analog, ATP-γ-S, that is resistant to hydrolysis by ecto-ATPases or phosphatases (30). ATP-γ-S also effectively inhibited neutrophil apoptosis as shown in Fig. 2*C*.

Effect of different ATP analogs on neutrophil apoptosis

To determine the P2 receptor subtypes responsible for ATP-mediated delay of apoptosis, a variety of approaches were needed because of the lack of a comprehensive battery of specific agonists and antagonists. Initially, ATP analogs with differing preferences for P2 receptor subtypes were studied for their effect on neutrophil apoptosis (Fig. 3). BzATP, a typical P2X$_7$ agonist, delayed neutrophil apoptosis at concentrations of 1 μM and above. In contrast,

Table I. *Presence of contaminating cells does not influence inhibition of neutrophil apoptosis by ATP*[a]

	% Apoptosis	
[ATP] (μM)	Percoll purified (94.2 ± 1.6%)[b]	Highly purified (98.8 ± 0.8%)[b]
0	12.9 ± 0.8	15.2 ± 1.6
0.1	11.1 ± 0.4	10.7 ± 1.3[c]
1	7.8 ± 0.7[d]	5.8 ± 1.1[d]
10	4.6 ± 0.4[d]	5.1 ± 0.7[d]
100	4.7 ± 1.0[d]	4.1 ± 0.6[d]
1000	4.3 ± 0.5[d]	3.7 ± 0.6[d]

[a] Neutrophils were isolated by plasma-Percoll gradients and further purified by immunomagnetic selection to remove contaminating eosinophils and PBMCs. Neutrophils were treated with ATP (0.1–1000 μM) for 5 h. Apoptosis was assessed by morphological criteria, and data are expressed as mean percent apoptosis ± SEM from three independent experiments, each from a separate donor. For both isolation methods, ATP significantly inhibited apoptosis in a concentration-dependent manner. There was no significant difference between the two isolation methods ($p = 0.6614$).

[b] *Mean ± SEM neutrophil purity from each isolation method.*

[c] $p < 0.01$.

[d] $p < 0.001$.

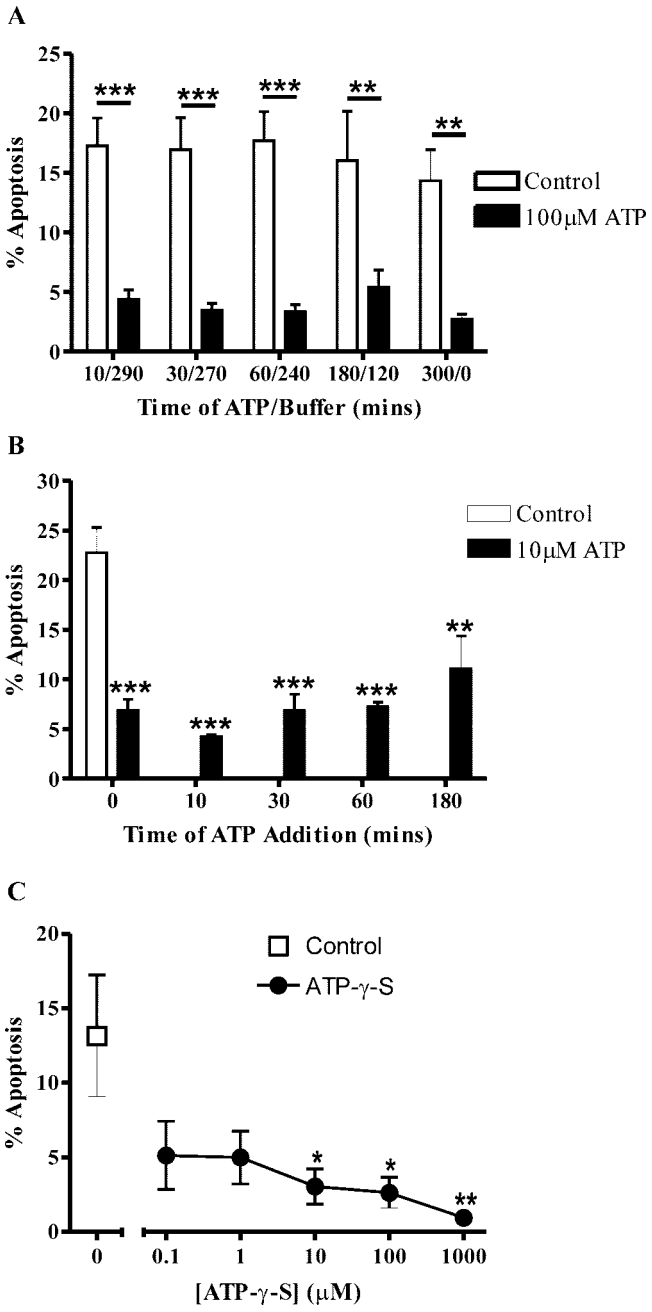

FIGURE 2. Delay of neutrophil apoptosis is mediated by short exposures to ATP. Neutrophils were cultured in the presence (■) or absence (□) of ATP. Data are expressed as mean percent apoptosis ± SEM, assessed by morphological criteria, from at least three independent experiments, each from a separate donor. *A,* Neutrophils were cultured with or without ATP (100 μM) for varying lengths of time as indicated, after which cells were washed and resuspended in fresh medium to remove any remaining extracellular ATP. Cells were cultured for a total of 5 h before assessment of apoptosis. *B,* Neutrophils were cultured with or without ATP (10 μM) for 5 h, with ATP added at the time points indicated. Apoptosis was then assessed at 5 h. ATP significantly delayed apoptosis to similar levels at each time point. *C,* Neutrophils were incubated in the presence (●) or absence (□) of varying concentrations of the stable ATP analog, ATP-γ-S, for 5 h, and apoptosis was assessed.

$\alpha\beta$-methylene-ATP ($\alpha\beta$MeATP, which preferentially activates $P2X_1$ and $P2X_3$ receptors), 2-(methylthio)-ATP (2MeSATP;a $P2Y_1$, $P2X_1$, and $P2X_5$ agonist) and UTP (a $P2Y_2$ and $P2Y_4$ agonist) did not alter the rate of neutrophil apoptosis at any tested concentration.

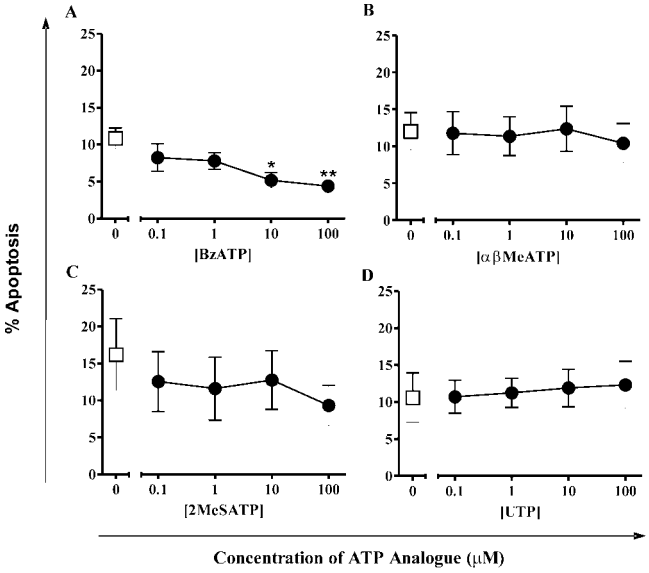

FIGURE 3. Effects of ATP analogs on neutrophil apoptosis. Neutrophils were cultured in the presence (●) or absence (□) of varying concentrations of ATP analogs for 5 h, and apoptosis was assessed by morphological criteria. Data are expressed as mean percent apoptosis ± SEM from at least three independent experiments, each from a separate donor. *A,* BzATP; *B,* $\alpha\beta$MeATP; *C,* 2MeSATP; *D,* UTP.

$P2X_7$ receptor subtype is not expressed by human neutrophils

ATP-γ-S and BzATP are the only agonists, other than ATP, that are known to activate $P2X_7$, the purine receptor most often associated with apoptosis in immune cells (31, 32). Therefore, we initially examined the possibility that $P2X_7$ receptors mediate the antiapoptotic effects of ATP on neutrophils. However, it is unclear whether human neutrophils do (16) or do not (33) express $P2X_7$ receptors, We therefore sought evidence of mRNA expression in highly purified neutrophil populations. As shown in Fig. 4*A,* only mRNAs for $P2X_1$ and $P2X_5$ were detected in these highly pure populations. If the samples were spiked with 5% PBMCs, however, transcripts for $P2X_4$, $P2X_6$ and $P2X_7$ were also intermittently detected (Fig. 4*B*). Because $P2X_4$ and $P2X_7$ are highly expressed in monocytes (34), it is possible that their detection in previous studies was because of the presence of contaminating cells. There are, however, clear precedents for human neutrophils to express proteins but for the corresponding mRNA to be undetectable. Examples include lactoferrin and myeloperoxidase, major constituents of neutrophil granules (35). Because $P2X_7$ has been thought to mediate functional effects in neutrophils in a number of recent studies, we wanted to exclude its possible expression as rigorously as possible. We found that $P2X_7$ protein could not be detected by flow cytometry either intra- or extracellularly in neutrophils, although the protein was readily detected in HEK-293 cells transfected with $hP2X_7$ (Fig. 4*C*). $P2X_7$ was also not detected by Western blotting, and LPS treatment, which up-regulates $P2X_7$ expression in other cell types (36), did not induce its expression in neutrophils (Fig. 4*D*).

ATP causes elevations of $[Ca^{2+}]_i$ levels in neutrophils independently of the delay of apoptosis

To explore further the possibility that the effects of ATP were mediated via a P2Y receptor, we measured intracellular calcium levels, because the G protein-coupled seven-transmembrane P2Y receptors are known to cause elevations of cytosolic calcium levels

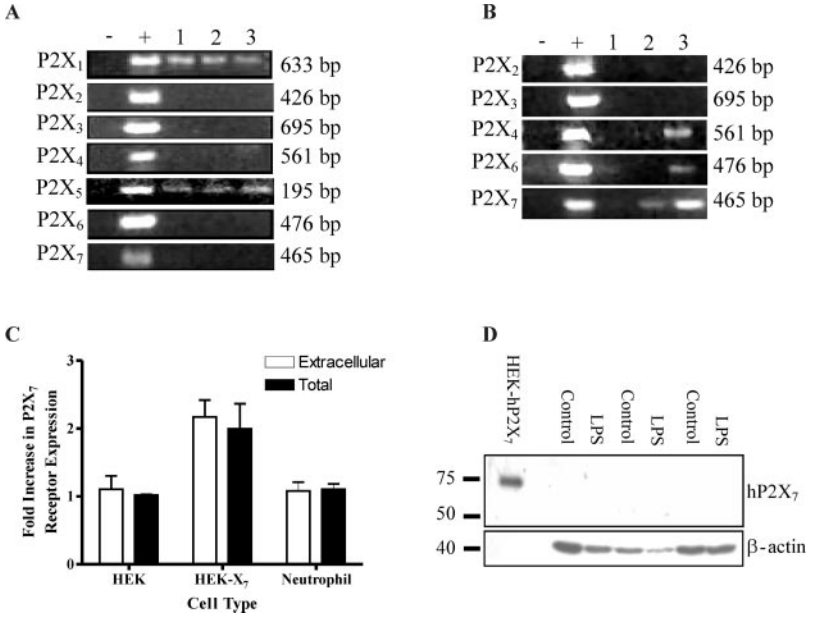

FIGURE 4. Expression of P2 receptors by human neutrophils. *A,* PCRs were performed for each of the P2X receptors according to the protocols described in *Materials and Methods*, and bands of the appropriate sizes were identified as indicated. Three samples of cDNA from highly purified human neutrophils, each from a different donor (*lanes 3*-5), were tested for the presence of each P2X receptor mRNA. *Lanes 1* and *2,* negative and positive controls, respectively. Positive controls are vectors containing the receptor subtype cDNA. *B,* The studies were repeated using samples of cDNA from highly purified human neutrophils spiked with 5% PBMCs, each from a different donor (*lanes 3–5*). *Lanes 1* and *2,* negative and positive controls, respectively. $P2X_4$, $P2X_6$, and $P2X_7$ mRNAs were detected in these populations but not in the samples from highly purified neutrophils. *C,* Cells were fixed and/or permeabilized, and $P2X_7$ receptor expression was detected by flow cytometry. Fold increase in $P2X_7$ receptor expression (mean ± SEM of three independent experiments) is plotted against cell type. HEK-X_7 cells showed significant expression of $P2X_7$ both extracellularly and intracellularly ($p < 0.01$ and $p < 0.05$, respectively) compared with both untransfected HEK cells and neutrophils. Neutrophils do not express $P2X_7$ receptors either intra- (■) or extracellularly (□). *D,* Highly purified neutrophils were cultured with or without 10 ng/ml LPS for 16 h, then Western blotted to detect $P2X_7$ receptor protein. Neither untreated nor LPS-stimulated neutrophils expressed detectable $P2X_7$ receptor protein ($n = 3$). In parallel samples, LPS inhibited apoptosis of neutrophils isolated from each donor population used for Western blotting (mean apoptosis was 74.4 ± 5.7% in control cells and 35.9 ± 4.0% in LPS-treated cells, $p < 0.01$).

via PLC activation and inositol-1,4,5-triphosphate-mediated release of calcium from intracellular stores. Moreover, we have previously shown that elevations of $[Ca^{2+}]_i$ delay neutrophil apoptosis (37) and thus speculated that this might be the mechanism of ATP-mediated delay of apoptosis. We found that ATP increased neutrophil $[Ca^{2+}]_i$ in a concentration-dependent manner, in the presence or absence of extracellular calcium, implying that the increase in $[Ca^{2+}]_i$ is mediated from intracellular stores. Addition of a PLC inhibitor, U73122, completely abolished the ATP-mediated $[Ca^{2+}]_i$ effect (Fig. 5*A*). The P2Y agonist UTP increased $[Ca^{2+}]_i$ to similar levels to ATP, again a PLC-dependent effect (Fig. 5*B*). Use of the different structural analogs of ATP showed that only the stable analog, ATP-γ-S, also mediated a calcium response (Fig. 5*C*). These data suggest the ATP-mediated inhibition of apoptosis and elevation of $[Ca^{2+}]_i$ are separate events. UTP increases $[Ca^{2+}]_i$ but has no effect on apoptosis, whereas ATP analogs that increase neutrophil survival, such as BzATP, do not increase $[Ca^{2+}]_i$. This data contrast with our previous studies linking increases in $[Ca^{2+}]_i$ with delay of neutrophil apoptosis (37), but in those studies delay of apoptosis was mediated by influx of extracellular calcium rather than release of calcium from intracellular stores mediated by ATP.

Combining the data from the effects of ATP analogs, expression data, and calcium studies allowed us to narrow the range of possible receptors mediating the delay of neutrophil apoptosis by ATP. Because two P2Y agonists (2MeSATP and UTP) did not delay neutrophil apoptosis and the delay of apoptosis was independent of $[Ca^{2+}]_i$, the anti-apoptotic effect of ATP was not typical for a P2Y receptor-mediated effect. Of the P2X receptors identified in neutrophils, $P2X_1$ was an unlikely candidate, because two recognized $P2X_1$ agonists, αβMeATP and 2MeSATP (38), did not inhibit neutrophil apoptosis. There is evidence that human $P2X_5$ may not be a functional receptor (38) and 2MeSATP, which is also an agonist at this receptor, was without effect on neutrophil apoptosis. Involvement of $P2X_7$ was effectively excluded by its lack of expression in neutrophils. We also found that a $P2X_7$ antagonist, 1-[*N,O*-bis(5-isoquinolinesulfonyl)-N-methyl-$_L$-yrosyl]-4-phenylpiperazine, did not inhibit BzATP-mediated delay of apoptosis, further evidence this effect was independent of $P2X_7$ (data not shown). BzATP is, however, also a potent agonist of the $P2Y_{11}$ receptor, which is coupled to both phosphoinositide and cyclic AMP signaling pathways (39). BzATP exerts effects upon both phosphoinositide and cAMP signaling pathways with half-maximal effective concentrations (EC_{50}) in the low micromolar range (39, 40). This is in keeping with a significant effect of BzATP on neutrophil apoptosis at a concentration of 10 μM, and it contrasts with an EC_{50} of >50 μM for BzATP at the $P2X_7$ receptor (41). Moreover, the rank order of agonist potency at the $P2Y_{11}$ receptor reveals that ATP-γ-S is at least equivalently potent to BzATP (40), again in keeping with our data and suggesting $P2Y_{11}$ as the receptor mediating anti-apoptotic signaling.

ATP-mediated delay of neutrophil apoptosis is P2Y$_{11}$ dependent

If ATP and its analogs were exerting their antiapoptotic effects via the $P2Y_{11}$ receptor, they would be predicted to cause increases in intracellular cAMP. We showed ATP, ATP-γ-S, and BzATP all increased $[cAMP]_i$ compared with untreated control cells (Fig.

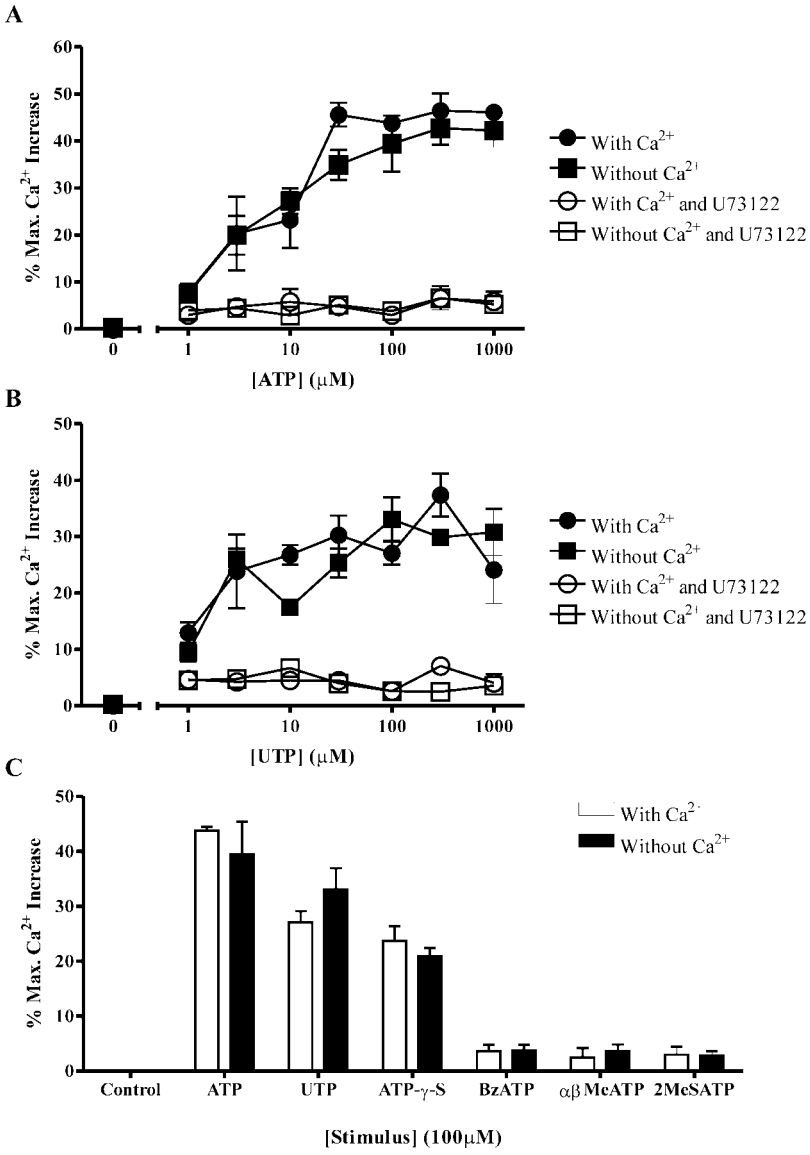

FIGURE 5. ATP and some analogs increase intracellular Ca^{2+} via activation of PLC. Neutrophils were incubated with fluo-4-AM for 30 min, then washed, and resuspended in buffer in the presence (○) or absence (□) of calcium. Cells were then treated with ATP or an ATP analog as indicated, in the absence (●, ■) or presence (○, □) of a PLC inhibitor, U73122 (10 μM). Increases in $[Ca^{2+}]_i$ were measured as a percentage of the maximum Ca^{2+} released by addition of 2 mM digitonin. Data are mean percent ± SEM maximum Ca^{2+} increase from at least three independent experiments, each from a separate donor. Neutrophils were treated with increasing concentrations of ATP (1–1000 μM; *A*) and UTP (1–1000 μM; *B*). Both ATP and UTP increase $[Ca^{2+}]_i$ from intracellular stores via a PLC-dependent mechanism. *C,* 100 μM concentrations of ATP, UTP, ATP-γ-S, BzATP, $\alpha\beta$MeATP, and 2MeSATP were added to neutrophils in Ca^{2+}-containing (□) or EGTA-containing buffer (■). Of these analogs, only ATP-γ-S caused an increase in $[Ca^{2+}]_i$ similar to the changes observed with ATP and UTP.

6*A*). A recent study showed NAD^+ exerted its proinflammatory effects on human neutrophils via the $P2Y_{11}$ receptor (42), and we found that NAD^+ also caused a concentration-dependent delay of apoptosis that was significant at concentrations of 1 μM and above (e.g., mean apoptosis ± SEM, 4.2 ± 0.5% after 1 μM NAD^+ compared with 7.9 ± 0.5% in control cells; $n = 3$; $p < 0.01$ at 5 h). During these studies, a potent antagonist at the $P2Y_{11}$ receptor, NF157, became available, which is derived from suramin, a compound with antagonist activity at most P2 receptors. NF157 was shown to have an IC_{50} of ~0.5 μM at the $P2Y_{11}$ receptor and high selectivity for $P2Y_{11}$ over all P2X and P2Y receptors tested other than $P2X_1$ (20). We found that NF157 blocked ATP-γ-S-mediated delay of neutrophil apoptosis (Fig. 6*B*) but had no effect on constitutive neutrophil apoptosis. The inhibitory effect of NF157 was overcome at higher concentrations of ATP-γ-S, confirming the competitive nature of its $P2Y_{11}$ antagonism (Fig. 6*C*). The antiapoptotic effect of ATP was also abrogated by NF157 (Fig. 6*D*). Moreover, we were able to detect $P2Y_{11}$ protein in highly purified neutrophils (Fig. 6*E*), with a band detected at 45 kDa, slightly smaller than the band detected in platelets but within the expected size range for $P2Y_{11}$ (43).

ATP-mediated delay of neutrophil apoptosis is dependent on cAMP-dependent kinases

To further confirm a role for $P2Y_{11}$ in delay of neutrophil apoptosis, we determined the effects of modulating cyclic AMP signaling pathways downstream of $P2Y_{11}$. Reagents that mediate increases in $[cAMP]_i$ delay neutrophil apoptosis (44). Krakstad et al. (45) have shown the antiapoptotic effects of cAMP on neutrophils are mediated by activation of cAMP-dependent protein kinases (cA-PK), as opposed to the recently discovered cAMP-regulated GTP exchange proteins directly activated by cAMP. ATP has also been shown to increase $[cAMP]_i$ in neutrophils (46). We confirmed the results of Krakstad et al. (45), showing the ability of a cA-PK type I agonist (N^6-monobutyryladenosine-cAMP; N^6-MB-cAMP) but not an exchange proteins directly activated by cAMP activator (8-(4-chlorophenylthio)-2′-*O*-methyladenosine-cAMP) to cause delay of neutrophil apoptosis equivalent to that seen with the stable cAMP analog, dibutyryl cAMP (data not shown). We then showed that a specific inhibitor of cA-PK type I (*Rp*-8-Br-cAMPS) was able to completely abrogate the ATP-mediated survival of neutrophils (Fig. 7*A*). Moreover, the antiapoptotic effects of all ATP analogs found to delay neutrophil apoptosis were abolished by the

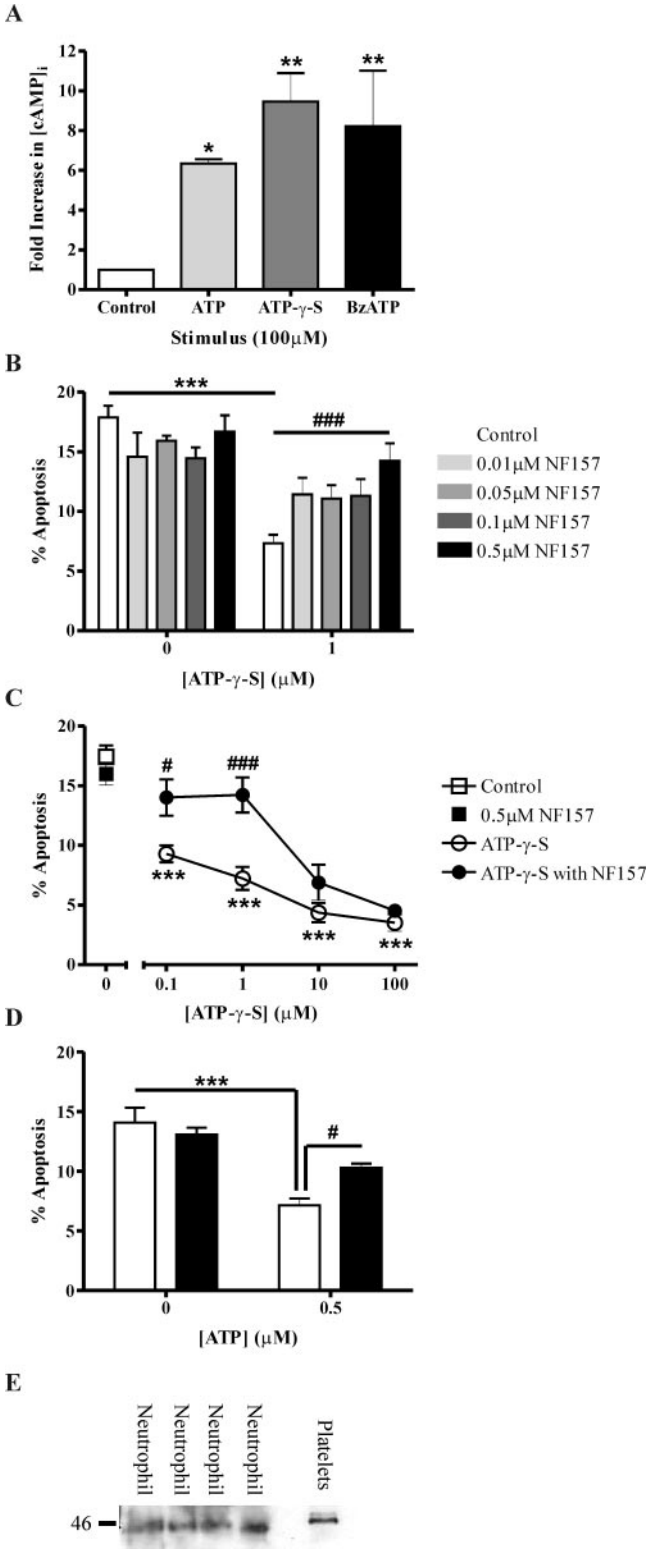

FIGURE 6. P2Y$_{11}$ receptors mediate ATP-induced delay of neutrophil apoptosis. A, Neutrophils were pretreated with 3-isobutyl-1-methylxanthine (1 mM) then with ATP, ATP-γ-S, or BzATP (100 μM) or buffer control for 8 min before lysis and detection of [cAMP]$_i$. Data are from three independent experiments, each assessed in duplicate, and is expressed as fold increase in [cAMP]$_i$ compared with buffer control. All three compounds caused a significant increase in [cAMP]$_i$. In additional experiments, neutrophils were pretreated for 30 min with the P2Y$_{11}$ receptor antagonist, NF157, at varying concentrations or buffer control, then treated with ATP-γ-S, ATP, or buffer for a total incubation time of 5 h. Apoptosis was then assessed by morphological criteria, and data are expressed as mean percent apoptosis ± SEM from a minimum of three independent experiments, each from a separate donor. *B,* Neutrophils were pretreated with NF157 (0.01–0.5 μM) before stimulation with ATP-γ-S (1 μM). The survival effect of ATP-γ-S was significantly reduced by 0.5 μM NF157 (###, $p < 0.001$). *C,* Neutrophils were pretreated with NF157 (0.5 μM) before stimulation with increasing concentrations of ATP-γ-S (0.1–100 μM). ATP-γ-S significantly inhibited neutrophil apoptosis at all concentrations compared with control cells (***, $p < 0.001$). NF157 abrogated the inhibition of apoptosis by ATP-γ-S (#, $p < 0.05$; ###, $p < 0.001$). *D,* Neutrophils were preincubated with buffer (□) or NF157 (0.5 μM) (■) followed by stimulation with ATP (0.5 μM) or buffer control. ATP reduced neutrophil apoptosis (∗), and this effect was significantly abrogated by NF157 (#). E, Detection of P2Y$_{11}$ protein expression by highly purified neutrophils, with human platelets used as a positive control. In all four donors, P2Y$_{11}$ is detected as an approximately 45-kDa protein.

inhibitor, whereas the compound had no effect on constitutive neutrophil apoptosis (Fig. 7*B*). We used a 16-h time point for these experiments, where there would be a sufficiently large inhibition of neutrophil apoptosis by ATP (Fig. 1*B*) for abrogation of these antiapoptotic effects to be clearly demonstrated. These data confirm that P2Y$_{11}$-mediated delay of apoptosis is dependent on activation of cAMP signaling pathways, an effect that is unique to P2Y$_{11}$ among P2 receptors (47).

Discussion

Extracellular ATP is a crucial mediator of the inflammatory response, in part through its nonredundant role in activation of P2X$_7$ and thus IL-1 release from macrophages (6, 48). Here, we show that ATP regulates the lifespan of the inflammatory response by a further mechanism: preservation of neutrophil survival. Together with previous work that has shown that ATP can enhance neutrophil functions including degranulation (18) and superoxide production (16), these data place ATP as a multifunctional central regulator of inflammation, the effects of which can considerably outlast the period of time for which it is present in the extracellular environment.

We showed that ATP mediates effects upon neutrophil apoptosis at concentrations readily achieved when platelets are activated, or cells are damaged (8). Significant effects were achieved at 1 μM ATP, and ATP concentrations of up to 250 μM have been detected on lytic release from cells (49). Our findings are supported by a previous study showing delay of neutrophil apoptosis by a single concentration of ATP at a single time point (50). Because ATP is rapidly metabolized by ectonucleases (CD39 and CD73) to adenosine, which is also antiapoptotic to neutrophils via actions on A$_{2A}$ receptors (29), we confirmed the effects were seen with a nonhydrolyzable ATP analog, ATP-γ-S. Neutrophils themselves have been shown to release ATP, particularly under conditions of hypoxia (51) or hypertonic stress (52), suggesting that ATP may act as an autocrine or paracrine survival factor, prolonging neutrophil lifespan at sites of inflammation.

To determine which receptor mediates the antiapoptotic effects of ATP, we used a combination of more selective ATP analogs, receptor expression studies and examination of downstream signaling pathways. Of the known P2X receptors, we found that neutrophils expressed mRNA for P2X$_1$ and P2X$_5$, but agonist studies, combined with evidence P2X$_5$ may not be functional in humans (38), again making them unlikely candidates. The combination of agonist studies and the lack of requirement for calcium signaling to delay apoptosis made classical P2Y receptors unlikely candidates to mediate the delay of apoptosis. We therefore considered the possibility that the antiapoptotic effect was mediated via P2Y$_{11}$, which is the only P2 receptor known to mediate cAMP-dependent

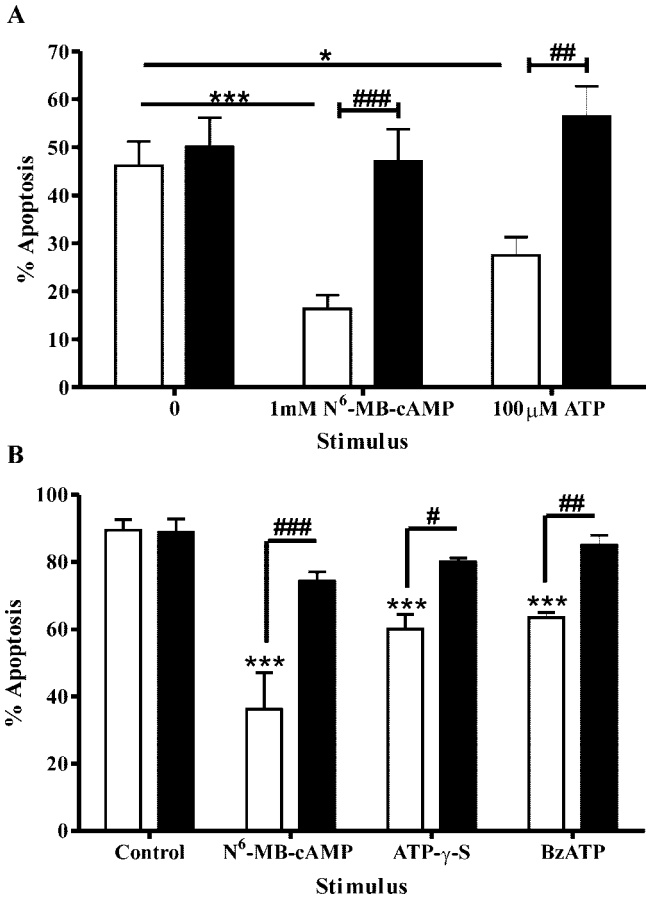

FIGURE 7. ATP inhibits neutrophil apoptosis via activation of cAMP-dependent protein kinase. *A,* Neutrophils were cultured in the presence or absence of the cA-PK type I agonist N^6-MB-cAMP (1 mM) or ATP (100 μM), alone (□) or in combination with a specific cA-PK type I inhibitor, *Rp*-8-Br-cAMPS (0.7 mM; ■), for 16 h. Apoptosis was assessed by morphological criteria and data are expressed as mean percent apoptosis ± SEM from at least three independent experiments, each from a separate donor. *Rp*-8-Br-cAMPS abrogated the enhanced neutrophil survival caused by these treatments (##, $p < 0.01$; ###, $p < 0.001$). *B,* Similar experiments were performed for two ATP analogs shown to delay neutrophil apoptosis, ATP-γ-S and BzATP (both at 100 μM), again using N^6-MB-cAMP (1 mM) as a positive control. Data are from at least three independent experiments, each from a separate donor. The ATP analogs significantly inhibited neutrophil apoptosis and these effects were blocked by *Rp*-8-Br-cAMPS.

signaling (47). We showed that ATP and other analogs causing delay of neutrophil apoptosis also caused increases in intracellular cAMP levels. Moreover, a recently synthesized selective $P2Y_{11}$ inhibitor, NF157, abrogated the antiapoptotic effect of ATP, and the effects of ATP were also abolished by inhibition of signaling via cAMP-dependent protein kinases, further implicating $P2Y_{11}$. The delay of apoptosis by ATP analogs was achieved at concentrations in keeping with the described EC_{50} values for BzATP and ATP-γ-S as agonists of cAMP production in $P2Y_{11}$-transfected cells (7.2 and 3.4 μM, respectively) in complete medium (40), although a 10-fold lower EC_{50} for BzATP has been reported in divalent free medium (53). We did not detect a significant rise in intracellular calcium after BzATP treatment of neutrophils. Previous studies have shown increases in HL-60 cells (16), but there are no reports of a calcium rise in neutrophils in response to BzATP. We were able to detect a rise in intracellular calcium in response to ATP, ATP-γ-S, and UTP, which will activate $P2Y_2$ receptors at the concentrations used but did not detect a calcium rise in response to BzATP or to 2MeSATP which, as a $P2Y_1$ agonist, would also be expected to cause an increase in $[Ca^{2+}]_i$ via activation of PLC. This suggests that the calcium response we detected was predominantly $P2Y_2$ mediated and there is evidence that BzATP is only a weak partial agonist at human $P2Y_2$ receptors (54). We have not been able to further demonstrate the role of $P2Y_{11}$ by study of $P2Y_{11}$-deficient neutrophils, because, to our knowledge, a $P2Y_{11}$-deficient mouse has not been developed and because genetic manipulation of primary human neutrophils is notoriously difficult.

$P2Y_{11}$ is unique as a P2 receptor coupling both to PLC and to adenylate cyclase (39). $P2Y_{11}$ is expressed on several leukocyte populations, including neutrophils (52), lymphocytes (55), monocytes (34), and monocyte-derived dendritic cells (56), and is associated with maturation of bone marrow precursor populations (57). More recently, $P2Y_{11}$ was found to be abundantly expressed in the endothelium (58).

In the course of our experiments, we found that neutrophils express a limited repertoire of P2X receptors and, in particular, do not express $P2X_7$ at mRNA or protein level in resting or LPS-stimulated cells, in agreement with the work of Chen et al. (15), who also failed to detect $P2X_7$ mRNA. This contrasts with previous studies showing $P2X_7$ expression in human neutrophils at the mRNA level (16, 52). In both of these studies, neutrophils were purified using dextran sedimentation and gradient centrifugation, with neutrophil purities of approximately 97%. As shown in Fig. 4*B,* neutrophil populations contaminated with PBMCs can have detectable mRNA for $P2X_7$ as well as $P2X_4$ and $P2X_6$, and this may explain the discrepant results. Gu et al. (59) detected intracellular $P2X_7$ protein expression in human neutrophils. We were unable to detect $P2X_7$ in neutrophils by flow cytometry or by Western blotting under conditions where the protein was detected in other cell types. Thus, $P2X_7$ expression may be present at low levels in neutrophils, but it is likely that contamination of neutrophils with PBMC has contributed to a confused picture in which the role of $P2X_7$ may have become overstated. Our data are supported by the finding of Labasi et al. (60) that granulocytes from $P2X_7$-deficient mice showed no lack of shape change or L-selectin shedding in response to ATP, in contrast to monocytes and lymphocytes, which the authors speculated reflected lack of surface expression of $P2X_7$ on neutrophils.

In a number of studies, functional effects of BzATP upon neutrophils have been attributed to $P2X_7$-mediated actions (16, 61). BzATP, however, is also an agonist at other receptors, notably $P2Y_{11}$ but also $P2X_1$ (38), and the potency of BzATP in delay of neutrophil apoptosis is more in keeping with effects at the $P2Y_{11}$ receptor (40). We also considered the possibility that ATP may exert its effects on neutrophil apoptosis indirectly, by activation of $P2X_7$ receptors expressed on mononuclear cells. We have previously shown using highly purified LPS, a TLR4 agonist, that the antiapoptotic effect of LPS on human neutrophils is principally mediated via indirect effects on the small numbers of monocytes present in the cell preparations, inducing release of neutrophil survival factors (22, 62). In our studies, the antiapoptotic effects of ATP were the same in gradient-purified and highly purified neutrophil populations, supporting the view that ATP and ATP analogs were mediating their antiapoptotic effects directly on neutrophils but via a receptor other than $P2X_7$.

Extracellular ATP can cause induction of cell death, either apoptotic or cytolytic, in many cell types e.g., endothelial cells (32), lymphocytes (31), and hepatocytes (63). In neutrophils, in contrast, ATP mediates a significant delay of apoptosis. Thus, an environment associated with cell damage and death mediates prolongation

of effect of an important cell of the innate immune system, potentially facilitating resolution of tissue injury. The effects of ATP on neutrophils are, however, dependent on activation of cA-PK via elevation of intracellular cAMP. cAMP also induces apoptosis in many cell types, including thymocytes (64) and B lymphocytes (65), yet is antiapoptotic to both neutrophils (44) and eosinophils (66). Krakstad et al. (45) showed that cAMP inhibited neutrophil apoptosis by activation of cA-PK type I, the dominant isoform in neutrophils (67). Elevation of cAMP stabilizes the antiapoptotic Bcl-2 protein, Mcl-1, and phosphorylates Bad, providing potential downstream mechanisms of its antiapoptotic actions (68).

$P2X_7$ has profoundly proinflammatory effects on monocyte-macrophage populations, and we now propose that enhancement of neutrophil lifespan, and thus proinflammatory functions given that these are regulated by onset of apoptosis (3), is separately regulated via $P2Y_{11}$. The lack of effect of $P2Y_{11}$ antagonism on constitutive apoptosis suggests that specific targeting of $P2Y_{11}$ could provide a mechanism that retains key immune functions of neutrophils but reduces the injurious effects of neutrophil longevity at inflamed sites. Additional experiments are under way to determine the full consequences of early $P2Y_{11}$ antagonism on neutrophil phenotypes generated by proinflammatory stimuli. Strategies to selectively drive neutrophil apoptosis using cyclin-dependent kinase inhibitors (69) have recently shown the potential of this strategy to reduce inflammation in a range of animal models. Our data suggest that encounters of the neutrophil with ATP in the microvasculature at sites of inflammation, clotting, and tissue damage may induce a prolonged survival phenotype that can be rapidly initiated and subsequently carried into tissues. These data further suggest that $P2Y_{11}$ antagonists will be most efficacious when administered systemically and may conceivably deprive the neutrophil of a mechanism important in the generation of a fully activated tissue neutrophil.

Disclosures

The authors have no financial conflict of interest.

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