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Adenine Nucleotides Inhibit Cytokine Generation by Human Mast Cells through a Gs-Coupled Receptor

Chunli Feng,2‡ Amin G. Mery,2∗‡ Elizabeth M. Beller,‡ Christa Favot,‡ and Joshua A. Boyce3∗†‡§

ATP and ADP activate functionally distinct G protein-coupled purinergic (P2Y) receptors. We determined the expression and function of adenine nucleotide-specific P2Y receptors on cord blood-derived human mast cells (hMCS). Human MCs expressed mRNA encoding the ADP-specific P2Y1, P2Y12, and P2Y13 receptors; the ATP/UTP-specific P2Y2 receptor; and the ATP-selective P2Y11 receptor. ADP (0.05–50 μM) induced calcium flux that was completely blocked by a P2Y1 receptor-selective antagonist and was not cross-desensitized by ATP. Low doses of ADP induced strong phosphorylation of ERK and p38 MAPKs; higher doses stimulated eicosanoid production and exocytosis. Although MAPK phosphorylation was blocked by a combination of P2Y1- and P2Y12-selective antagonists, neither interfered with secretion responses. Unexpectedly, both ADP and ATP inhibited the generation of TNF-α in response to the TLR2 ligand, peptidoglycan, and blocked the production of TNF-α, IL-8, and MIP-1β in response to leukotriene D4. These effects were mimicked by two ATP analogues, adenosine 5′-O-(3-thiotriphosphate) and 2′,3′-O-(4-benzoyl-benzoyl) adenosine 5′-triphosphate (BzATP), but not by adenosine. ADP, ATP, adenosine 5′-O-(3-thiotriphosphate), and 2′,3′-O-(4-benzoyl-benzoyl) adenosine 5′-triphosphate each induced cAMP accumulation, stimulated the phosphorylation of CREB, and up-regulated the expression of inducible cAMP early repressor, a CREB-dependent inhibitor of cytokine transcription. Human MCs thus express several ADP-selective P2Y receptors and at least one Gs-coupled ADP/ATP receptor. Nucleotides could therefore contribute to MC-dependent microvascular leakage in atherosclerosis, tissue injury, and innate immunity while simultaneously limiting the extent of subsequent inflammation by attenuating the generation of inducible cytokines by MCs. The Journal of Immunology, 2004, 173: 7539–7547.

Nucleotides, the substrates for nucleic acid synthesis, are also ubiquitous extracellular mediators. ATP is stored in high (micromolar) concentrations in the secretory granules of platelets, neurons, and some leukocytes (reviewed in Ref. 1) and is released in a regulated fashion upon activation of these cells. In addition, high concentrations of cytosolic nucleotides are released into the extracellular space in response to cell death, hypoxia, trauma, and infection. Extracellular nucleotides initiate or modulate cellular responses in multiple organ systems by binding and activating specific cell surface receptors, collectively termed purinergic (P2)4 receptors (reviewed in Ref. 2). Nucleotides are released by endothelial cells, and P2 receptors are essential for platelet aggregation and play a role in vascular disease. Two distinct families of P2 receptors exist: P2X receptors are subunits of multimeric, ligand-gated ion channels that bind ATP (3). P2Y receptors are seven-transmembrane-spanning, G protein-coupled receptors (GPCRs). Both P2 receptor families are expressed on smooth muscle, neuronal tissues, and hemopoietic cells (4). P2Y receptors exhibit clear-cut differential specificity for different nucleotides. The P2Y2 receptor binds both ATP and UTP, and the human P2Y4 receptor prefers UTP, whereas the P2Y11 receptor preferentially binds ATP. P2Y1, P2Y12, and P2Y13 (5) receptors all preferentially bind ADP, whereas the P2Y6 receptor exhibits a binding preference for UDP (reviewed in Refs. 1 and 2). The P2Y14 receptor is specific for UDP-glucose (6). Not only is the number of known P2 receptors large, but single-cell types commonly express more than one receptor specific for the same nucleotide (7, 8), suggesting nonredundant, complementary functions for these receptors. For example, coexpression of ADP-specific P2Y1 and P2Y12 receptors by platelets is essential for normal aggregation responses to both exogenous and endogenous ADP in vitro and in vivo (9–13). The expression of several P2Y receptors by leukocytes (4) suggests likely functions in immunity and inflammation.

Among immune effector cells, mast cells (MCs) are uniquely situated around blood vessels and nerves throughout the body, especially at interfaces with the external environment. Whereas their effector role in IgE-dependent allergic and immune responses is well understood, accumulating evidence supports their involvement in a broad range of nonallergic inflammatory diseases and indicates a key role for these cells in innate antimicrobial responses (14, 15). MCs abound in atherosclerotic plaques and show morphological evidence of activation in such lesions (16). Moreover, cardiovascular events such as myocardial ischemia are associated with
with elevations in plasma levels of histamine (17) and tryptase (18), two products of MC secretory granules that are released on cell activation. Activated MCs are also abundant sources of leukotrienes (LTs), which are products of the 5-lipoxygenase pathway. These smooth muscle-active mediators have an accepted role in asthma and have recently been implicated in the pathophysiology of atherosclerosis (19). Although the mechanisms responsible for MC activation in atherosclerosis are not known, MCs express a range of activating receptors for small soluble mediators as well as Ig and microbial constituents (20–24). Human MCs (hMCs) from a variety of sources express several mRNAs that encode P2X receptor subtypes (25). Rat peritoneal MCs release histamine and generate PGD2 when stimulated with ATP (26); this response is blocked by antagonists of both P2X and P2Y receptors. ATP and UTP (1–100 μM) each enhance exocytosis of histamine by human lung MCs stimulated by cross-linkage of FcεRI (27), an effect attributed to the P2Y2 receptor. ATP is also stored in MC granules, and its release by a rat MC line activated by FcεRI cross-linkage resulted in propagation and radial spread of calcium fluxes in adjacent cells, which were blocked by a global P2 receptor antagonist, suramin (28). Taken together, these studies support the concept that MCs express more than one P2 receptor type and that ATP and/or its metabolites modulate MC activation through both autocrine and paracrine pathways. These possibilities have potential relevance for multiple disease processes.

Extracellular ATP in vivo is probably maintained in equilibrium with ADP through ectonucleotidases and nucleoside diphosphokinas (29). The capacity for ATP to modulate MC activation, and the existence of P2Y receptor subtypes with distinct preferences for ATP or ADP, prompted us to study whether cultured cord blood-derived hMCs express ADP-selective P2Y receptors and whether these receptors modulate the activation of hMCs. The results of our studies indicate that hMCs express mRNAs for the ADP-preferring P2Y1, P2Y12, and P2Y13 receptors as well as the P2X1 and P2X4 receptors and demonstrate functionality of the corresponding proteins by characteristic biochemical signatures. The P2Y1 receptor mediates calcium flux by hMCs in response to ADP (but not ATP) and synergizes with P2X2 receptors to mediate phosphorylation of ERK and p38 MAPKs. Despite these signatures, ADP and ATP both fail to induce cytokine generation de novo, even at high (100 μM) doses. Instead, ADP, ATP, and a panel of ATP analogues (but not adenosine) interfere with TNF-α production by hMCs activated by peptidoglycan (PGN), a component of Staphylococcus aureus cell walls that is an agonist of TLR2, and block LTD4-induced production of a panel of cytokines, ADP, ATP, and its analogues all induce accumulation of cAMP in hMCs and up-regulate the expression of inducible cAMP early repressor (ICER), a repressor of NF-AT and AP-1-mediated transcription (30). The presence on MCs of excitatory receptors for adenosine nucleotides may facilitate the acute tissue swelling response to nucleotides in atherosclerosis, tissue injury, and innate immunity, whereas the presence of at least one Gs-coupled inhibitory receptor for ATP and ADP may limit consequent inflammation in such circumstances by interfering with inducible cytokine gene expression.

Materials and Methods
Nucleotides, analogues, and antagonists

The following reagents were purchased from Sigma-Aldrich (St. Louis, MO); ATP, ADP, UTP, and UDP; and the ATP analogues adenosine 5′-O-(3-thiotriphosphate) (ATPβS), 2′,3′-O-(4-benzoyl-benzoyl) adenosine 5′-triphosphate (BzATP), and dATP; the P2Y1-selective antagonist adenosine 3′-phosphate 5′-phosphate (A3P5P); the P2Y12-selective antagonist 2-methylthioadenosine 5′-monophosphate (2-MesAMP); and the nonselective P2 receptor antagonist pyridoxalphosphate-6-azophenyl-2′,4′-disulfonl acid.

Derivation of hMCs

Human MCs were derived from cord blood mononuclear cells cultured in the presence of stem cell factor (SCF; 100 ng/ml), IL-6 (50 ng/ml), and IL-10 (10 ng/ml; all from R&D Systems, Minneapolis, MN), as previously described (31). Cells were harvested for studies when 95% stained metachromatically with toluidine blue (typically 6–9 wk). To test the size and conditions for FcεRI-dependent exocytosis, cytokine generation, and eicosanoid production, hMCs were transferred to new medium containing SCF and IL-4 (10 ng/ml; R&D Systems) for 5 days at 37°C and 5% CO2 before activation (32, 33).

RT-PCR

To determine the steady state expression by cultured hMCs of mRNAs encoding P2Y receptors, total RNA was extracted from hMCs at 6–8 wk of culture with Tri-Reagent (Molecular Research, Cincinnati, OH). For RT-PCR, RNA samples were primed with oligo(dT) and reverse transcribed according to the manufacturer’s protocol in an RT kit (BD Clontech, Palo Alto, CA). The following primers were designed to amplify coding sequences of the indicated receptors: P2Y1 sense strand, 5′-AT GACGCAAGGTGCTTGGCC-3′; P2Y12 sense strand, 5′-AGAATG GAGATACAAGGCCTCTGA-3′; P2Y12 sense strand, 5′-CCCG CACGAC AATTCACC-3′; P2Y12 sense strand, 5′-GCCGAGATGACAC GAG-3′; P2Y13 sense strand, 5′-GGTTTTTGTTCATACCTCCAG-3′; P2Y13 sense strand, 5′-CTTAAAGGAAGACATCTTTTAC-3′; P2Y2 sense strand, 5′-ATGGCAGCAGACCTGGGCCCCTGG-3′; P2Y2 sense strand, 5′-CTCACGCGGTAATGTATGTTTC-3′; P2Y11 sense strand, 5′-CTCGGGTGCAAGTCTGTCGCC-3′; P2Y11 antisense strand, 5′-CAGCATGTTGGTAGAC-3′; P2X1 sense strand, 5′-CCG GACACAGATCAGTTTCCACG-3′; P2X1 sense strand, 5′- GCTTTGTACGTTTGAAGCTGAAAA-3′; P2X4 sense strand, 5′-GGTTA CACAGATGTTTTCTTAC-3′; P2X5 sense strand, 5′-GTTACCAA GACGTGCGACACCT-3′; P2X5 antisense strand, 5′-CCTGCTGGCAAG TTCTTC-3′; P2X7 sense strand, 5′-AGGOGAAGCTTCTTCTCTG TAT-3′; and P2X7 antisense strand, 5′-CAATCCGTTATTCGCCGCTTG-3′.

All these primers readily amplified bands from genomic DNA that were consistent with the sizes of the respective target sequences. PCR was performed with 0.4 U of Taq polymerase (PerkinElmer, Foster City, CA) for 35 cycles in a PerkinElmer Thermal Cycler with the following parameters: 94°C for 1 min, 94°C for 30 s, 50°C (or, in the case of the P2X primers, 55°C) for 2 min, and 72°C for 4 min. Specific primers for human GAPDH (BD Clontech) were run as positive controls. The PCR products were resolved on ethidium bromide-stained gels containing 0.5% agarose/0.5% Synergel (Diversified Biotech, Boston, MA).

Calcium mobilization

To measure changes in the cytosolic concentration of free calcium, hMCs (0.5–1 × 106 cells/sample) were washed and resuspended in HBSS containing 1 mM CaCl2, 1 mM MgCl2, and 0.1% BSA. The cells were labeled with fura 2-AM (Molecular Probes, Eugene, OR) for 30 min at 37°C, washed, and resuspended in the same HBSS buffer. Some cell samples were pretreated with A3P5P (0.5–100 μM), 2-MesAMP (0.5–100 μM), or 6-N,N-diethyl-thiobenzyl-b-d-glycine methylester (5′-triphosphate (ARL67156; 50 μM; Sigma-Aldrich), an inhibitor of ectonucleotidase activity (34), before the agonists were added. Other cell samples were stimulated after incubation for 4 h with pertussis toxin (PTX; 100 ng/ml; Sigma-Aldrich) to interfere with Gαi, protease signaling. The hMCs were stimulated with various concentrations of ATP, ADP, UTP, adenosine, or the analogues of ATP (35). The changes in intracellular calcium were measured using excitation at 340 and 380 nm in a fluorescence spectrophotometer (F-4500; Hitachi, Hialeah, FL). The relative ratios of fluorescence emitted at 510 nm were recorded and displayed as a reflection of intracellular concentrations of calcium.

Cell activation for secretion

Human MCs primed with IL-4 were passively sensitized with human myeloma IgE (Chemicon International, Temecula, CA; 2 μg/ml) overnight on the fourth day of priming to saturate FcεRI. On the following day, the cells were washed and resuspended in fresh medium at a concentration of 1 × 106/ml, except in the case of samples used for analysis of exocytosis, which were suspended at a 10-fold higher cell density. For exocytosis, samples of 2 × 106 hMCs were challenged with ADP or ATP at the concentrations listed in Table I (100 μM each). hMCs were stimulated with a rabbit anti-human IgE polyclonal Ab (Calbiochem, San Diego, CA; 1 μg/ml) or with staphylococcal PGN (10 μg/ml; Sigma-Aldrich) with or without the addition of ADP, ATP, or ATP analogues.

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Throughout the activation process, SCF was maintained at a constant concentration of 100 ng/ml to ensure optimal cell viability. Activation was stopped by incubating the samples on ice, and the supernatants were separated from the cell pellets by centrifugation at 200 × g in an Eppendorf microcentrifuge at 4°C. After lysis of the cell pellet by resuspension in medium and sonication, the content of β- hexosaminidase (β-hex) was measured by spectrophotometric analysis of the hydrolysis of p-nitrophenyl- β-2-acetamido-2-deoxyglucopyranoside as described previously (36). The percent release values were calculated according to the formula %R = (R/P) × 100, where S and P refer to the absorbance detected in equal volumes of supernatant and pellet, respectively, after subtraction of the background absorbance of medium alone. Nonspecific release from replicate samples treated with medium alone was subtracted, and the data were expressed as the mean net release from duplicate preparations.

MAPK phosphorylation

Samples of 2 × 10^6 hMCs were stimulated in triplicate with various doses of ADP and ATP (0.05–50 μM), with and without anti-IgE (1 μg/ml), in the wells of 96-well, flat-bottom plates. Supernatants were collected at 30 min and stored at −20°C until further analysis. Specific ELISAs were used to detect LTC4, LTD4, LTE4 (Cayman Chemical, Ann Arbor, MI), and PGD2 (Amersham Biosciences, Arlington Heights, IL), respectively. For measurements of cytokine generation, triplicate samples of 1 × 10^6 cells were stimulated with PGF (10 μg/ml; Sigma-Aldrich), anti-IgE (1 μg/ml), LTB4 (100 μM; Cayman), or medium alone in the presence or the absence of ADP, ATP, ATPYs, BzATP, or adenosine at various concentrations in the wells of 96-well, flat-bottom plates. The agonists were optimal for cytokine generation by IL-4-primed hMCs and were chosen on the basis of preliminary dose-response experiments as well as previously published data (32, 37). In some experiments the cells were pretreated for 30 min with H89 (10 μM; Sigma-Aldrich), a selective inhibitor of protein kinase A (PKA). At 6 h, supernatants were harvested and frozen at −70°C until further analysis. Specific ELISAs were used to measure the concentrations of TNF-α, IL-8, and IL-5 (all from eBiosciences, San Diego, CA) and MIP-1β (Endogen, Woburn, MA), according to the directions provided by the manufacturers. The data for each experiment were tabulated as the mean values from triplicate samples. The cytokine quantities in the samples stimulated with medium without agonists were subtracted from the corresponding quantities in the samples stimulated with agonists to obtain the net quantity produced for each cytokine.

cAMP measurements

Primed hMCs were stimulated with various nucleotides for 10 min in triplicate samples of 2 × 10^6 cells. The stimulations were performed in medium, except for experiments in which the effect of extracellular calcium was examined. Indomethacin (1 μM) was added to some experiments with 2-MesAMP (5 μM) as required. The reactions were stopped by incubating the samples on ice. Cells were lysed with a lysis buffer provided in a commercial Biotrak cAMP ELISA kit (Amersham Biosciences), which was used according to the manufacturer’s protocol. The bound radioactivity was determined. In these experiments, cells were stimulated in HBSS either with or without calcium and magnesium. In some experiments cells were pretreated with indomethacin (1 μM), a dose that completely inhibited PGD2 production, to rule out a contribution from endogenous prostanooids to cAMP accumulation. The reactions were stopped by incubating the samples on ice. Cells were lysed with a lysis buffer provided in a commercial Biotrak cAMP ELISA kit (Amersham Biosciences), which was used according to the manufacturer’s protocol. The bound radioactivity was determined. In these experiments, cells were pretreated for 4–24 h with PTX, or A3P5P was added immediately before stimulation. The cAMP values for each condition were normalized to the values for the unstimulated cells and are expressed as a percentage of the control value.

MAPK phosphorylation

Samples of 2 × 10^6 hMCs were stimulated for various periods with ADP (5 μM) in the presence or the absence of A3P5P, 2-MesAMP, or both (50 μM and 5 μM, respectively). The reactions were stopped by incubating the samples on ice at 5 min (the time at which maximal phosphorylation of ERK was observed), and the cells were lysed in a buffer containing 1% SDS, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, 5 μg/ml leupeptin, and 1 μg/ml pepstatin in 10 mM Tris (pH 8.0). The samples were loaded with an equal volume of Tris-glycine-SDS loading buffer (NOVEX, San Diego, CA) onto precast 12% Tris-glycine-SDS gels, and the proteins were separated by electrophoresis at 30 mA and 150 mV. After overnight transfer to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA), the blots were blocked for 1 h in 1 × TBS with 0.5% Tween 20 (Bio-Rad; TTBS) containing 3% nonfat dry milk and 0.25% normal goat serum and were incubated in the same buffer containing 1/2000 dilutions of anti-Active ERK, c-Jun, and p38 MAPK Abs (Promega, Madison, WI) for 2 h at room temperature. After three washing steps in TTBS, the blots were incubated again for 1 h in blocking buffer with a 1/5000 dilution of a goat anti-rabbit HRP-conjugated secondary Ab (Bio-Rad). The blots were washed again in TTBS, and bands were detected with ECL. The same strips were stripped and probed again with rabbit polyclonal Abs that detect total ERK, p38, and JNK at dilutions of 1/2000 (Cell Signaling Technologies, Beverly, MA). Maximal phosphorylation of ERK was observed at 5 min; thus, this period was chosen as the harvest time for subsequent experiments.

Immunoblotting for phosphorylation of CREB and induced expression of ICER

Primed hMCs were stimulated for 30 min to 3 h with various concentrations of nucleotides and were lysed as described above. In some experiments the cells were pretreated with H89 (10 μM) for 1 h. Samples of 3 × 10^6 cells were loaded onto precast gels and subjected to electrophoresis and blotting. The blots were incubated with rabbit polyclonal Abs specific for total CREB and for CREB phosphorylated at serine 133 (both from Cell Signaling Technologies) at dilutions of 1/500 and with a rabbit polyclonal antiserum that recognizes all forms of cAMP response element modulator (CREM), including ICER (provided by Dr. C. Molina, University of New Jersey Medical School, Newark, NJ) at a concentration of 1/1000 (30). The 20-kDa band corresponding to ICER was visualized with ECL.

Real-time PCR

Expression of TNF-α and MIP-1α mRNA was determined using real-time PCR performed on ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Samples of 5–10 × 10^6 primed, sensitized hMCs were stimulated for 2 h with anti-IgE, PGN, or medium alone in the presence of SCF at a constant concentration of 100 ng/ml. RNA was prepared using an RNeasy Mini kit (Qiagen, Valencia, CA) and was treated with RNase-free DNase (Qiagen, Valencia, CA) according to the manufacturer’s protocols. RT was performed on samples of 2 μg of DNase-treated RNA using TaqMan RT reagents (Applied Biosystems). Primers and minor groove binder-labeled probes for the amplification of TNF-α and human β2-microglobulin and the corresponding VIC dye were purchased from Applied Biosystems.

Results

Identification and pharmacologic characterization of ADP and ATP-binding P2Y receptors on hMCs

To determine whether hMCs expressed functional P2Y receptors for ADP and ATP, fura 2-loaded hMCs were stimulated with various concentrations of these nucleotides and monitored for changes in absorbance at 380 and 510 nm. ADP strongly induced dose-dependent increases in intracellular calcium, with maximal responses at concentrations between 0.5 and 5 μM in most experiments (n = 4; as shown for a single donor; Fig. 1A), nearing a plateau at 5 μM. ATP (5 μM; Fig. 1B) and UTP (not shown) also induced a calcium flux of a consistently smaller magnitude than that observed in response to the same dose of ADP. BzATP, dATP, ATPyS, adenosine, and AMP failed to cause calcium fluxes at concentrations up to 100 μM (n = 2; not shown). Priming of the hMCs for 5 days with IL-4 (10 ng/ml) in the continued presence of SCF did not alter the magnitude of the ADP- or ATP-induced calcium fluxes and did not change the dose-response characteristics (not shown). Neither the magnitude nor the duration of the ADP-induced calcium fluxes were altered by the addition of ARL67156 or by PTX pretreatment (n = 2 for each; as shown for individual experiments; Fig. 1B). ATP and ADP (5 μM each) did not cross-desensitize each other’s calcium fluxes (Fig. 1C). ADP-induced (but not ATP-induced) calcium flux was blocked completely by the P2Y1-selective antagonist, A3P5P, at 50 μM and was unaltered by 2-MesAMP (Fig. 1B). RT-PCR revealed the presence of transcripts encoding the ADP-selective P2Y1, P2Y12, and P2Y13 receptors as well as the ATP-preferring P2Y2 and P2Y11 receptors (n = 3; as shown for one experiment; Fig. 1D). Transcripts encoding the P2X1 and P2X4 receptors were also readily detected (n = 3; as shown for one experiment; Fig. 1D), whereas those encoding P2X5 and P2X7 receptors were either only weakly detectable or undetectable in the same samples. The profile of detected transcripts was not altered by DNase treatment of the RNA before reverse transcription (n = 2; data not shown).
Neither ADP nor ATP directly induced exocytosis at 0.05–5 μM (Fig. 2). A3P5P and 2-MesAMP induced the phosphorylation of ERK MAPK and more weakly elicited p38 MAPK phosphorylation (Fig. 2). C, Cross-desensitization experiment between ATP and ADP, added in both forward and reverse orders. D, Ethidium bromide-stained agarose gel of DNA fragments amplified by 35 cycles of PCR from DNase-treated, reverse transcribed RNA from hMCs showing bands that correspond to the steady state mRNA encoding the indicated P2Y and P2X receptors. The m.w. markers are indicated on the left. Data depicted in A–D are from single experiments representative of at least two performed.

Stimulation of hMCs with ADP (5 μM) for 5 min strongly induced the phosphorylation of ERK MAPK and more weakly elicited p38 MAPK phosphorylation (Fig. 2). A3P5P and 2-MesAMP (100 μM each) each attenuated ADP-induced ERK phosphorylation when used alone, and the combination of the two inhibitors was additive, while also decreasing p38 phosphorylation (n = 2; as shown for one experiment; Fig. 2). JNK phosphorylation was not detected under these conditions (n = 2; not shown).

Secretion responses to stimulation with ADP and ATP

IL-4-primed hMCs were stimulated with various concentrations of ADP or ATP, both with and without FceRI cross-linkage. The content of β-hex was measured in both the supernatants and pellets, and these values were used to calculate the percent release. Supernatants were also assayed for the presence of cysteinyl LTs (cys-LTs) and PGD2 as indices of arachidonic acid metabolism. Neither ADP nor ATP directly induced exocytosis at 0.05–5 μM, whereas both weakly induced exocytosis at 50 μM (10 ± 4 and 7 ± 4% net β-hex release (n = 4) vs 19 ± 2% net release in response to FceRI cross-linkage; not shown). Spontaneous release was low (generally <5%). Both ADP and ATP amplified FceRI-induced net β-hex release by an additional ~5% (~25% increase over anti-IgE alone) when added as a second stimulus (n = 3; not shown). At 50 μM ADP or ATP, primed hMCs generated small quantities of PGD2 (4.6 ± 4 and 9 ± 2 ng/106 hMCs in response to ADP and ATP vs 59 ± 41 ng/106 hMCs in response to FceRI cross-linkage; n = 3; data not shown) and cys-LTs (466 ± 172 and 334 ± 150 pg/106 hMCs in response to ADP and ATP vs 18 ± 4 ng/106 hMCs in response to FceRI cross-linkage; n = 3; data not shown). Stimulation with ATPyS, BzATP, and dATP (50 μM each) also elicited small amounts of PGD2 and cys-LT generation, which approached 25–70% of that induced by ADP (n = 2; data not shown). PPADs (100 μM) decreased cys-LT generation in response to both ADP (98 ± 1% inhibition) and ATP (70 ± 11%), whereas A3P5P and 2-MesAMP had no effect (data not shown).

Effect of nucleotides on cytokine generation

To determine whether adenine nucleotides would induce the generation of cytokines, primed hMCs were stimulated with various concentrations of ADP or ATP in the presence of SCF to maintain optimal viability. As positive control stimuli, other cell samples were activated with FceRI cross-linkage, staphylococcal PGN (10 μg/ml), or LTD4 (100 nM) in some experiments. Some of these samples were treated concomitantly with various concentrations of ADP or ATP. Supernatants were harvested at 6 h for measurement of cytokines. Neither ATP nor ADP used alone elicited TNF-α, IL-8, or MIP-1β generation at doses up to 100 μM (n = 6; data not shown). FceRI cross-linkage, PGN, and LTD4 each induced the generation of these cytokines by IL-4-primed hMCs. When added as a concomitant stimulus, neither ADP nor ATP significantly altered FceRI-induced generation of TNF-α (Fig. 3B), IL-8, or MIP-1β (not shown), although there was a trend toward inhibition at higher nucleotide doses. However, both nucleotides significantly decreased the production of TNF-α in response to PGN (Fig. 3A) and LTD4 (B) in a dose-dependent fashion, decreased IL-5 production in a similar dose-dependent fashion (n = 2; not shown), and at 5 μM also inhibited the LTD4-mediated generation of IL-8 and MIP-1β (Fig. 3D). The inhibitory effects of ADP and ATP on...
FIGURE 3. Effect of adenine nucleotides on cytokine generation. IL-4-primed, sensitized hMCs were stimulated with PGN (10 μg/ml; A), anti-IgE (1 μg/ml; B), or a buffer control in the presence of the indicated nucleotide concentrations. Supernatants were harvested 6 h after stimulation, and the presence of TNF-α was assessed by ELISA. Data for PGN are the mean ± SEM from four experiments, whereas the data for anti-IgE are the mean ± SEM from three experiments. * Significant inhibition (p ≤ 0.05). Adenosine at 0.05–50 μM had no effect (n = 2; not shown). C. Effects of various concentrations of ADP on TNF-α production by hMCs in response to LTD4 (100 nM). Data are the mean ± half-range from two experiments. D. Effect of ADP (5 μM) on the production of TNF-α, IL-8, and MIP-1β by primed hMCs in response to LTD4. Data displayed are the mean ± SEM from three experiments.

PGN-induced TNF-α generation were mimicked by both ATPγS and BzATP at the same dosing ranges (64 ± 25 and 56 ± 6% inhibition, respectively, at 50 μM; mean ± half-range; n = 2). Real-time PCR analysis revealed that both ADP and ATPγS at 50 μM significantly attenuated the steady state expression of TNF-α mRNA induced by PGN (n = 3; Fig. 4A) and also decreased LTD4-induced TNF-α mRNA expression by >50% (n = 2; Fig. 4B). In the cases of all agonists used, the inhibitory effects of ADP and ATP were not reversed by the presence of H89 and were not blocked by PPADs, A3P5P, or 2-MesAMP, alone or in combination. Adenosine did not affect cytokine generation at concentrations up to 100 μM (n = 2; data not shown).

Effect of adenine nucleotides on adenylyl cyclase-dependent signaling pathways

To determine whether adenine nucleotides exerted their inhibitory effects through receptors that stimulate adenylyl cyclase, cAMP was measured in hMCs stimulated for 10 min with various concentrations of these nucleotides. ADP and ATP (0.5–100 μM) were equipotent for increasing intracellular levels of cAMP in a dose-dependent fashion (n = 4; Fig. 5A). This effect was slightly potentiated by overnight pretreatment of the cells with PTX (n = 2; data not shown). A3P5P did not affect ADP-induced cAMP accumulation, whereas 2-MesAMP cross-reacted with AMP in the detection assay and could not be studied (not shown). BzATP, ATPγS, and dATP also strongly stimulated cAMP accumulation, each being more potent than ADP and ATP at equivalent concentrations (n = 2; Fig. 5A). UDP did not affect cAMP accumulation (n = 1; data not shown). Neither pretreatment with indomethacin nor depletion of extracellular calcium affected cAMP accumulation in response to ADP, ATP, ATPγS, and BzATP (50 μM each; n = 2; data not shown). Stimulation with ATPγS transiently induced the phosphorylation of CREB and up-regulated the expression of ICER in a time- and dose-dependent fashion (n = 3; as shown for one experiment; Fig. 5B). These effects were mimicked by ADP (n = 3; as shown for one experiment; Fig. 5B) and by ATP and BzATP at a concentration of 50 μM each (n = 1 for each; data not shown). Pretreatment of the hMCs with H89 attenuated CREB phosphorylation at 30 min and the up-regulation of ICER at 30 min and 2 h, although it did not prevent substantial up-regulation of ICER at 3 h (n = 3; shown for a single experiment in Fig. 5B). Preincubation of hMCs with ADP or ATPγS (50 μM) for various time periods to up-regulate ICER expression before activation did not further inhibit PGN-induced TNF-α generation, but tended to enhance inhibition of FceRI-dependent TNF-α generation relative to the addition of nucleotides concomitantly with anti-IgE (n = 2; Fig. 5C). Preincubation with nucleotides did not inhibit FceRI-dependent exocytosis (n = 2; not shown).

FIGURE 4. Real-time PCR analysis showing the effects of ADP and ATPγS on the steady state levels of TNF-α mRNA expressed by hMCs stimulated with PGN (A; 10 μg/ml) and LTD4 (B; 100 nM). IL-4-primed hMCs were stimulated for 2 h in the absence or the presence of each nucleotide at the indicated concentrations. The level of TNF-α mRNA is normalized to the internal control gene, β2-microglobulin, and is expressed as a percentage of the expression by the cells not treated with nucleotides. The results for PGN-stimulated samples are the mean ± SEM from three experiments, whereas those for LTD4 are the mean ± half-range from two experiments. * Statistical significance (p < 0.05).
Discussion

Nucleotides are probably among the first and most abundant soluble mediators to be released in tissue injury, infection, hypoxia, and shea stress (38–40). Their release by cell lysis, exocytosis of nucleotide-containing granules, and efflux through membrane transport proteins causes their concentrations in extracellular fluids to increase into micromolar ranges, and higher concentrations are probably achieved at cell-cell synapses (1). The key role for ADP and its receptors in vascular biology and platelet aggregation in vivo is well established by studies with P2Y1 and P2Y12 receptor-null mice (12, 13) and is validated in humans by the clinical efficacy of P2Y1 receptor antagonists as antiplatelet agents (41, 42). The constitutive perivascular location of MCs throughout the body and their abundance in atherosclerotic plaques probably permit their exposure to endothelial- and platelet-derived nucleotides in vivo (43). Reports that ATP activates rodent MCs or hMCs suggest that these cells express functionally significant P2X and/or P2Y receptors (26, 27). The fact that other hemopoietic cells express multiple members of this family (44, 45) prompted the current study, in which we sought to define the profile of P2Y receptors for ICER expression are depicted below. Data are from single experiments, representative of three performed. Similar data were obtained with BzATP and ATP as stimuli (n = 2; not shown). C, Time-dependent effects of nucleotides on suppression of TNF-α generation by hMCs. Cells were preincubated for the indicated times with ADP or ATP (50 μM) to up-regulate ICER expression before subsequent stimulation for 6 h with FcεR1 cross-linkage or PGN (10 μM). Data are the mean ± half-range for two experiments and are expressed as a percentage of the control value (amount of TNF-α generated by samples not treated with nucleotides).

receptors that are expressed by rodent MCs (23) and an hMC line, HMC-1 (24). The complete blockade of ADP-mediated calcium flux by A3P5P (Fig. 1B) indicates that this response requires the P2Y1 receptor, the mRNA for which was readily detected by RT-PCR (Fig. 1C). Although heterologously expressed P2Y1 receptors can also bind and respond to ATP (with 30-fold lower affinity than to ADP) (46), the lack of cross-desensitization between ADP and ATP for calcium flux on hMCs (Fig. 1C) and the lack of blockade of the ATP-induced calcium flux by A3P5P are consistent with the additional presence of a response through one or more of the ATP-prefering receptors. Although we detected transcripts encoding the ATP-prefering P2Y11 receptor in the cells of two of four donors tested (as shown for one donor; Fig. 1), the lack of calcium fluxes in response to BzATP, ATP-γS, and dATP (each of which is a potent agonist for P2Y11 receptors) (47, 48) mitigates against a substantial P2Y11 contribution to the ATP-induced calcium response. The P2Y2 receptor (also detected by RT-PCR at low levels) prefers ATP and UTP over ADP (49). Thus, the fact that UTP and ATP both caused weak calcium fluxes is consistent with the function of the P2Y2 receptor in hMCs and with the results of earlier studies in lung hMCs (27), as is the modest enhancement of exocytosis by ATP (and, in our study, ADP). Transcripts encoding the P2X1 and P2X4 receptors were also detected, a profile consistent with that previously reported by others using cord blood-derived hMCs or lung MCs (25). Although additional contributions from these P2X receptors to the ATP-dependent calcium flux is possible, their ability to serve as sodium channels may ablate the ability to detect their calcium fluxes in sodium-containing buffer solutions (50) such as that used in our study.

Unlike the G_{protein}-coupled P2Y1 receptor, the ADP-selective P2Y12 and P2Y13 receptors both use G_{protein} proteins to inhibit adenylate cyclase (51–53). Stimulation of platelets with ADP through

FIGURE 5. Adenyl cyclase-related signaling by adenine nucleotides and temporal effects on inhibition of TNF-α production. A, Accumulation of cAMP in primed hMCs stimulated for 10 min with the indicated doses of ADP, ATP, BzATP, and ATP-γS. The data are expressed as a percentage of the control value (taken as the average cAMP content of unstimulated hMCs in each experiment) and are the mean ± SEM for three experiments for ADP and ATP. Data for BzATP and ATP-γS are the mean ± half-range from two experiments. B, Up-regulation of ICER expression and phosphorylation of CREB in hMCs stimulated for various periods with ATPγS (50 μM) in the presence or the absence of H89 (10 μM). Dose-dependent effects of ADP and ATP-γS on ICER expression are depicted below. Data are from single experiments, representative of three performed. Similar data were obtained with BzATP and ATP as stimuli (n = 2; not shown). C, Time-dependent effects of nucleotides on suppression of TNF-α generation by hMCs. Cells were preincubated for the indicated times with ADP or ATPγS (50 μM) to up-regulate ICER expression before subsequent stimulation for 6 h with FcεR1 cross-linkage or PGN (10 μM). Data are the mean ± half-range for two experiments and are expressed as a percentage of the control value (amount of TNF-α generated by samples not treated with nucleotides).
the P2Y12 receptor provides a calcium-independent signal that activates the small GTPase RAP-1B (51, 52) and is complementary to the calcium-dependent signal from P2Y1 for MAPK activation and aggregation in response to exogenous or endogenous ADP (9, 11). Although ADP-mediated calcium fluxes in hMCs were P2Y1-dependent in our study, the combined inhibitory effects of A3P5P and 2-MesAMP on ADP-induced phosphorylation of ERK and p38 MAPKs suggests contributions from both P2Y1 and P2Y12 receptors to this response (Fig. 2), similar to their behavior in platelets. Although calcium flux and ERK phosphorylation are prerequisites to the activation of cytosolic phospholipase A2, the initial step in the synthesis of both cys-LTs and PGD2 by MCs and other cell types (54, 55), only small amounts of PGD2 and cys-LTs were secreted in response to ADP or ATP, and their secretion was only evident at higher doses of nucleotides. Furthermore, P2Y1 and P2Y12 blockade did not prevent this response, possibly reflecting the inability of these antagonists to inhibit at higher agonist concentrations. Alternatively, 50 μM doses of nucleotides may elicit contributions from P2Y13, P2X, or even adenosine receptors. Indeed, the capacity of P2Y1 and P2Y12 receptors to mediate strong calcium fluxes in response to ATP, ATP[S, and BzATP all suppress cytokine secretion by hMCs, consistent with prior observations made using rat peritoneal MCs (22).

Calcium flux and MAPK phosphorylation are both early and essential events in de novo generation of cytokines by MCs activated through other GPCRs (56). In our previous studies, exogenous LTC4, LTD4, and the nucleotide UDP each induced cytokine generation by IL-4-primed hMCs through a pathway involving the complementary functions of cys LT1 and cys LT2 receptors, which are structural homologues of the P2Y receptors (21, 37). Given the evident signaling parallels between the cys-LT receptors and the P2Y receptors, it was initially surprising that adenine nucleotides failed to elicit cytokine generation by primed hMCs, even at high (100 μM) doses. Indeed, ADP, ATP (Fig. 3), and ATP analogues significantly suppressed TNF-α production by cells stimulated with PGN, a ligand for the TLR2/TLR6 heterodimer than mediates innate immune responses to Gram-positive organisms (15). The inhibitory effect was probably mediated, at least in part, at the level of transcription or transcript stability, based on decrements in steady state TNF-α mRNA (Fig. 4). ADP also strikingly suppressed the generation of TNF-α, IL-8, and MIP-1β by cells stimulated with LTD4 (Fig. 3). The fact that adenosine did not interfere (or augment) cytokine generation implies a lack of involvement of P1 receptors. Moreover, the inability of A3P5P, 2-MesAMP, and pyridoxalphosphate-6-azophenyl-2’,4’-disulfonic acid to reverse the suppressive effect of nucleotides on cytokine production suggests that this action may be independent of a known member of the P2 receptor family.

GPCRs that interfere with activation of immune cells commonly couple to G proteins, stimulating adenylyl cyclase and initiating cAMP-dependent inhibitory signaling pathways. Examples include the receptors for PGE2 (EP2 and EP4 receptors) (57, 58) and the β2-adrenergic receptor (59). To date, the P2Y11 receptor is the only cloned ATP-binding P2Y family member known to stimulate G proteins. When expressed heterologously in transfected cells, P2Y11 receptors also mediate strong calcium fluxes in response to ATP and its analogues (60). Recently, Duhan et al. (61) reported that ATP, ATP[S, and BzATP all suppress cytokine secretion by polyclonally stimulated human T lymphocytes through a putative, unidentified P2 receptor that stimulates the accumulation of cAMP. ADP was not examined. Because the ability of both ADP and ATP to interfere with cytokine production in our study suggested the potential presence of a Gαi-coupled P2Y family member, we measured cAMP accumulation in hMCs stimulated by these nucleotides. ADP and ATP both induced modest cAMP accumulation (Fig. 5A), being equipotent and effective at the same dose range at which they interfered with TNF-α generation (Fig. 3). Moreover, ATP[S, BzATP, and dATP more potently stimulated cAMP accumulation despite their inability to elicit calcium fluxes. The accumulation of cAMP did not require extracellular calcium, implying a lack of involvement P2X receptor-mediated ion flux in this function, whereas the lack of effect of indomethacin indicates that endogenous generation of PGs (which may also stimulate Gαi) is not required. This pharmacologic profile is thus similar to that reported by Duhant and suggests the existence of a dominantly Gαi-coupled receptor for adenine nucleotides that is expressed by both hMCs and lymphocytes. Although it is possible that this activity reflects the P2Y11 receptor, the lack of calcium flux in response to ATP and its analogues argues against this idea. Alternatively, the responsible receptor may be among several orphan GPCRs that have sequence homology to the P2Y family (62).

ICER is a product of an alternate promoter in the CREM gene that autoregulates CREM-dependent transcription. Stimulation that elevates cAMP levels transiently induces ICER expression in human thymocytes. In these cells, ICER blocks binding of NF-AT and AP-1 transcription factors to their target sites in the promoters of TNF-α and other cytokines and suppresses their expression (63). Consistent with these same actions in hMCs, ADP, ATP, ATP[S, and BzATP all strongly up-regulated the expression of ICER (Fig. 5B). Pretreatment of hMCs with ADP or ATP[S before activation tended to inhibit FceRI-dependent TNF-α generation to a greater extent than did concomitant nucleotide treatment. Phosphorylation of CREB at serine 133, a prerequisite for induced ICER expression, was observed at 30 min in ATP[S-stimulated hMCs and was decreased by pretreatment with the PKA inhibitor H89. Nonetheless, there was residual CREB phosphorylation, and ICER expression was still strongly up-regulated at 3 h even in cells treated with H89. Moreover, H89 failed to reverse the inhibitory effect of ADP or ATP on cytokine generation, a finding reminiscent of previous studies examining the inhibitory effect of exogenous PGE2 and cAMP on cytokine generation by human T lymphocytes (64) and macrophages (58). Our data (Fig. 5) thus suggest a component of ICER induction that is H89 resistant and that may recapitulate inhibitory pathways initiated by other Gαi-coupled receptors. This component may reflect the capacity of such receptor systems to activate kinases other than PKA that can target CREB in immune cells (65). Based on our previous studies (37), the particular sensitivity of cys-LT-mediated cytokine generation to inhibition by nucleotides may reflect an absolute requirement for NF-AT, a target of ICER.

This study reveals that hMCs express complementary receptors for ADP in addition to ATP receptors. Moreover, the results implicate at least one Gαi-coupled ADP/ATP receptor in a nucleotide-initiated signaling pathway that is inhibitory for cytokine production, and adenosine may prevent nucleotides themselves from initiating this process while attenuating cytokine generation initiated by other receptor systems. Adenine nucleotide-mediated stimulation of MCs through activating P2 receptors could relate to MC activation during coronary events, tissue injury, and infections. Although such activation could facilitate vascular permeability responses by permitting the rapid release of smooth muscle-active mediators, the simultaneous stimulation of inhibitory pathways could prevent overexuberant secretion of potentially harmful cytokines into the tissue microenvironment, thus limiting the extent of tissue pathology. Identification of the responsible receptor(s) could present a novel therapeutic target for asthma, arthritis, and other disorders with a prominent contribution from MCs and their products.
References


