Purinergic P2Y2 Receptors Induce Increased MCP-1/CCL2 Synthesis and Release from Rat Alveolar and Peritoneal Macrophages

Leanne Stokes and Annmarie Surprenant

*J Immunol* 2007; 179:6016-6023; doi: 10.4049/jimmunol.179.9.6016

http://www.jimmunol.org/content/179/9/6016

---

References

This article *cites 42 articles*, 16 of which you can access for free at:

http://www.jimmunol.org/content/179/9/6016.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
Macrophages play a key role in inflammation by synthesis and release of proinflammatory cytokines and chemokines. Extracellular nucleotides released at sites of tissue damage may be an early danger signal for immune cells, and ATP-gated P2X₇ receptors are well known to mediate the rapid release of proinflammatory IL-1β and IL-18. However, there is little direct evidence for the involvement of other purine receptor subtypes in the release of other cytokines or chemokines. We initially used protein arrays to address whether extracellular ATP can release cytokines and/or chemokines from rat NR8383 alveolar macrophage, which lack the P2X₇ receptor. ATPγS increased the release of the proinflammatory chemokine, MCP-1 (MCP-1/CCL2). Pharmacological profiling identified the receptor responsible as the P2Y₂ receptor. Brief activation (10 min) of P2Y₂ receptors increased MCP-1 mRNA levels within 30 min and increased its release at 60 min. Similar results were obtained from rat peritoneal macrophages. We investigated likely downstream signaling cascades that may be involved, specifically the canonical Gq-mediated phospholipase C (PLC) and subsequent MAP kinase pathways, and Gi/Go-mediated signaling. We could find no evidence for these pathways being involved in the P2Y₂R-induced increase in mRNA levels although inhibition of PLC blocked the UTP-induced increased release of MCP-1. Thus, the PLC-activated pathway can account for the increased release of MCP-1, but a novel signaling pathway may be involved in the increase in MCP-1 mRNA by activation of P2Y₂ receptors in alveolar and peritoneal macrophage.


E

xtracellular nucleotides (ADP, ATP and UTP) are released from secretory cells such as nerve terminals, mast cells and platelets via regulated (i.e., exocytotic) release mechanisms, as well as from nonsecretory cells such as epithelia and vascular endothelia via poorly understood constitutive release mechanisms (1–3). Nucleotide release occurs in response to both physiological stimuli (nerve activity, blood flow, digestion) and pathological stimuli (hypoxia, infection, inflammation, trauma). In particular, the ability of these released nucleotides to modulate the release of cytokines and/or chemokines from immune effector cells has generated increasing interest in regards to the development of novel anti-inflammatory therapeutics targeted to specific nucleotide receptors (4–6). Two families of plasma membrane receptors mediate the actions of extracellular nucleotides, the metabotropic G-protein coupled P2Y purinergic receptors, and the ionotropic P2X purinergic receptor ion channels (5, 7).

There are eight mammalian metabotropic P2Y receptors (P2Y₁,₂,₄,₆,₁₁,₁₂,₁₃,₁₄), most of which (P2Y₁,₂,₄,₆,₁₁) couple to Gq-protein-mediated stimulation of the phospholipase C (PLC) signaling cascade (3, 5, 8, 9) and it is this group of P2Y receptor subtypes that have been implicated in alterations in chemokine and cytokine release. In human monocyte-derived dendritic cells, activation of P2Y₁ and/or P2Y₁₁ receptors has been shown to stimulate release of the anti-inflammatory cytokine IL-10, and to inhibit the endotoxin-induced release of the proinflammatory cytokine TNF-α, and chemokines MCP-1/CCL2 and macrophage inflammatory protein-1α (MIP-1α) (10–12). Activation of these same receptors can also inhibit leukotriene D₄-induced release of TNF-α, IL-8, and MIP-1β from human mast cells (13), whereas an increase in both TNF-α and IL-8 release has been found in human eosinophils and astrocytes as a result of P2Y₆ or other undetermined P2Y receptor subtypes (14–16). The P2Y₂ receptor is highly expressed in virtually all hemopoietic cells where its activation in eosinophils and neutrophils has been found to stimulate chemotaxis and superoxide production and to induce characteristics of monococytes to macrophage differentiation in a number of monocyctic cells and cell lines (2, 9, 17, 18). However, it is not known whether activation of this receptor subtype directly alters cytokine or chemokine release in any of these cell types or what signaling pathways may be involved.

There are seven members of the P2X receptor family (P2X₁₋₇), each of which can form nucleotide-gated ion channels when expressed as homomeric or heteromeric subunit assemblies (7). mRNA for three of these, P2X₁, P2X₄, and P2X₇ receptors, coexist in every immune effector cell and cell line so far examined, although protein expression has not been as routinely observed and only P2X₇ receptors have been implicated in altered cytokine release (4, 6, 7, 19). There is unequivocal evidence from studies with P2X₇ receptor knockout mice (20, 21) and highly selective P2X₇ receptor antagonists (22, 23) that activation of P2X₇ receptors in endotoxin-primed monocytes and macrophages results in a rapid
release of bioactive IL-1β and IL-18 (19, 24). However, it is the existence of this P2X receptor subtype in immune cells that complicates studies of involvement of other purine receptors in altered cytokine or chemokine function because IL-1β itself is the “early release” cytokine classically known to evoke the subsequent release of numerous other proinflammatory molecules (25).

The NR8383 rat alveolar macrophage cell line does not appear to express functional P2X receptors, but does express functional P2Y1, P2 Y2, and P2X receptors as assayed by electrophysiologic- ical and calcium flux measurements (26) and therefore may provide a valuable model in which to examine potential roles of these purinergic receptors in cytokine and chemokine release. We used these cells initially in a cytokine/chemokine array assay to ask whether extracellular ATP acting on purinergic receptors alters release profiles. We found that constitutive release of MCP-1 from these cells, and from rat peritoneal macrophage, was greatly increased by ATP or UTP acting through P2Y2 receptors. The increased release was associated with increased levels of MCP-1 mRNA. The UTP-induced increased MCP-1 release, but not the increased mRNA levels, was blocked by inhibition of PLC. The P2Y2 receptor-induced increased MCP-1 mRNA levels also could not be attributed to involvement of MAPK, PI3K, elevated intracellular calcium levels, or G_q, signaling, which may suggest a novel signaling pathway activated by P2Y2 receptors in alveolar and peritoneal macrophages.

Materials and Methods

Cell culture

The NR8383 rat alveolar macrophage cell line (American Type Culture Collection) was maintained in F12-K medium (Invitrogen Life Technologies) supplemented with 15% bovine FCS (Invitrogen Life Technologies) at 37°C in a humidified 5% CO2 incubator. Rat peritoneal macrophages were isolated by lavage of the peritoneal cavity with 5 ml of PBS. The collected cells were centrifuged and resuspended in DMEM containing 10% FCS and 2 mM l-glutamine plus 100 µg/ml streptomycin (Invitrogen Life Technologies). Adenoviral constructs were generated for rat P2X7-EE using the BD Clontech Adenovirus Expression System-1. All protocols involving rats were approved and in accord with University of Sheffield institutional committee on animal welfare. "System-1. All protocols involving rats were approved and in accord with University of Sheffield institutional committee on animal welfare.

Reagents

ATP, UTP, 2'(3')-O-(4-Benzoylbenzoyl)adenosine 5'-triphosphate (BzATP), ADP, ATPyS, suramin, 4-[(4-Formyl-5-hydroxy-6-methyl-3-[(phospho- nooxy)methyl]-2-pyridyl]azolo]-1,3-benzenesulfonilic acid (FPADS), cyclo-heximide, LPS (0155:B5 strain), Wortmannin, [3H]yl29002, and apyrase Grade 1 were purchased from Sigma. BAPTA-AM, U73122, PD98059, and SB203580 were from Calbiochem.

Protein array

The RayBiotech rat cytokine Ab array I was purchased from Tebu-Bio and performed as per the manufacturer’s instructions. Twenty-four hours before stimulation, 5 × 10^5 cells were plated in 60 mm petri dishes in F12-K medium containing 5% FCS.

Medium was removed, cells were washed twice, and then cells were incubated with vehicle, 100 ng/ml LPS as a positive control, or 100 µM ATPyS for 4 h in a total volume of 1 ml. Cell-free supernatants were collected and added to preblocked protein array membranes overnight at 4°C. Array membranes were washed three times with wash buffer and incubated with biotin-conjugated anti-cytokine Abs for 2 h at room temperature. After washing, streptavidin HRP was added for 2 h. Protein array membranes were exposed to detection buffer for 1 min and then exposed to Kodak Bio-Max MS film (Sigma-Aldrich). The resulting membrane films were analyzed using GeneSnap/GeneTools software (Syngene) to quantify spot density.

Western blotting

Western blotting was performed as detailed previously (27) according to standard protocols and proteins were visualized using anti-Glu-Glu primary Ab and HRP-conjugated secondary Ab (both at 1/2000 dilution), followed by detection using the ECL-plus kit and Kodak Bio-Max MS film.

ELISAs and secretion experiments

A duo-set ELISA development kit for rat IL-1β was purchased from R&D Systems. Ninety-six-well ELISA coated with anti-IL-1β capture Ab at 0.8 µg/ml in PBS and the detection Ab, biotinylated goat anti-rat IL-1β was used at 350 ng/ml. Recombinant rat IL-1β was used as standard over the range of 62–4000 pg/ml. Cells were plated at 5 × 10^5 cells per well in F-12K medium containing 15% FCS. Adenoviral constructs (P2X2, or control adenovirus) were added 48 h before release studies. Culture medium was replaced and replaced with 200 µl 5% FCS medium containing LPS at 100 ng/ml or 1 µg/ml. A paired Ab set to detect MCP-1 by ELISA was purchased from BD Pharmingen; it used mouse anti-rat MCP-1 (C9) at 2 µg/ml and biotinylated mouse anti-rat MCP-1 (B4) at 1 µg/ml. Recombinant rat MCP-1 (Peprotech) was used as standard over the range of 62–4000 pg/ml. Twenty-four hours before stimulation, NR8383 cells were plated at a density of 2 × 10^5 cells/well in 24-well plates (Nunc) in F12-K medium containing 1% FCS. Media was then removed and replaced with serum-free medium containing nucleotides with or without test compounds to a final volume of 250 µl/well. At indicated times supernatants were pipetted from each well, briefly centrifuged to pellet cells and 100 µl supernatant was added to the ELISA plate. Experiments were performed in triplicate in each case.

Because control constitutive release of MCP-1 varied widely from experiment to experiment (500 pg/ml to 3000 pg/ml over a 2-h time period), but not within a single experiment, all agonists and test compounds were normalized to the control value from the same experiment. For data shown in the figures, the absolute values (mean ± SEM) of control release are given in the corresponding figure legends.

Semiquantitative RT-PCR

Four × 10^5 NR8383 cells were stimulated with vehicle, nucleotides (100 µM ATP or UTP) in presence or absence of test inhibitors for 30 min in F-12K medium containing 5% FCS. Cells were removed from petri dishes by scraping and centrifuged, and the cell pellet was used to extract total RNA using the RNAeasy kit (Qiagen). Five micrograms total RNA was reverse transcribed using Superscript III RdnaE h-reverse transcriptase (Invitrogen Life Technologies) and 2.5 µM oligo dT. Primers for rat MCP-1 were designed using accession number NM_01530 and sequences were rMCP-1 sense: 5’-GGT GCT GCT CAT ACT CAT TCA GCA AG-3’ and rMCP-1 antisense: 5’-GGT CAA GAT CAT.CAC ATT CAA AGG TGC-3‘. Primers for β-actin were sense: GCC TCT CCT CTA GCC TTC TCT CTT G and antisense: CAC AGA GCT CTT GCG CTT AGG AGG. PCR was performed with Bioline Taq polymerase, 2.5 mM MgCl2, 125 µM each dNTP, and 1 µM each primer. Thermal cycling was performed in a Hybrid PCR machine and cycling conditions were 94°C for 2 min followed by 20 cycles of 94°C for 30s, 55°C for 30s, 72°C for 1 min, and a final elongation at 72°C for 5 min. The expected PCR product sizes were 260 bp (MCP-1) and 1.1 kb (β-actin). Preliminary experiments were performed to ensure that the number of PCR cycles performed (20) were nonsaturating. MCP-1 and β-actin PCR products were run on the same 1% agarose gel containing ethidium bromide and quantitated by densitometry using GeneSnap/GeneTools software (Syngene). For each sample, the relative index (RI) was calculated as RI = densitometry of MCP-1 PCR/densitometry of β-actin PCR.

Electrophysiology

Conventional whole-cell patch clamp recordings were obtained using HEKA EPC9 patch clamp amplifier as detailed previously (22, 26). Recordings were made using the following intracellular to extracellular solutions (mM): 145 NaCl, 10 HEPES, 10 EGTA: 145 NaCl, 2 CaCl2, 1 MgCl2, 2 KCl, 10 HEPES, 10 glucose (pH 7.3). Agonists were applied via fast perfusion system (RSC delivery system, BioLogic). Recordings were performed 24–48 h post adenoviral infection.

Results

NR8383 alveolar macrophages lack P2X2 receptors but not IL-1β signaling cascade

An important assumption in the present study was that NR8383 macrophages lack P2X2-Rs and therefore any nucleotide action on other cytokines or chemokines that may occur could not be due to P2X2-R-induced release of IL-1β. We made this assumption because we had previously found no evidence for their functional coupling to either ionic currents or calcium influx (26). However, low levels of P2X2-R mRNA can be detected in these cells (26) and therefore we addressed the validity of this assumption, first by
Western blot assay for protein expression of P2X₇-R. No P2X₇-R protein could be observed in contrast to the very high protein levels of P2X₄-R present in these cells (Fig. 1A). Second, whole-cell patch clamp experiments showed that the ATP analog, BzATP (30–300 μM) evoked an inward current of <50% of maximum ATP response in control cells (Fig. 1B), which is in keeping with its action as a weak partial agonist at rat P2X₄Rs (6, 7). After adenoviral infection with rP2X₇Rs, BzATP-evoked currents (100 μM BzATP) were 10–15-fold greater than currents evoked by 100 μM ATP (Fig. 1B), and were reduced by >95% by 100 nM Brilliant Blue G (data not shown); these BzATP-evoked responses are typical of rP2X₄-R-activated currents (6, 7, 22, 23). Third, we measured IL-1β levels in the supernatant from LPS-primed NR8383 macrophage after ATP stimulation but found none (Fig. 1C). The more potent P2X₇-R agonist, BzATP (100 μM) or ATPγS (100 μM) also did not evoke any IL-1β release from LPS-primed NR8383 macrophages (n = 2, data not shown). However, when we introduced the P2X₇-R protein into these cells by use of adenoviral constructs, ATP now induced very large amounts of IL-1β release from LPS-primed NR8383 macrophage (Fig. 1C). These results solidly support our initial assumption and also show that it is only the absence of P2X₄-R protein, and not any defect in IL-1β processing or release, that accounts for the lack of effect of ATP on IL-1β release from these cells.

**ATP increases the release of MCP-1 from macrophage**

We used a protein microarray to examine the constitutive release of a panel of 18 cytokines and chemokines from NR8383 cells and in response to ATP or the non-hydrolysable ATP analog, ATPγS (Fig. 2). We used ATPγS in the first instance to prevent the possible breakdown of nucleotides by ectonucleotidases (8–10). In the absence of stimulation only one of these compounds, MCP-1, was observed to be released constitutively (Fig. 2A). The addition of ATPγS resulted in a significant increase in the release of this chemokine without any significant increase in levels of the other cytokines or chemokines examined (Fig. 2B). We used LPS as a positive control in these experiments because LPS treatment is well known to increase the synthesis and release of MCP-1 (28) as well as TNF-α. Consistent with this, LPS significantly and selectively increased the release of both MCP-1 and TNF-α from NR8383 macrophage (Fig. 2, A and C). Fig. 2C plots the relative amounts of constitutively released MCP-1 and the fold increase induced by either ATPγS alone or LPS alone; ATPγS and LPS resulted in an ~2-fold and 3.5-fold increase in MCP-1 release respectively.
ATP-induced increase in MCP-1 release is due to the activation of P2Y2 receptors

We first examined whether P2X receptors are involved in the ATP-evoked increased release of MCP-1. Of the seven P2X receptors, only P2X1 and P2X4 are expressed in these cells (Ref. 26 and Fig. 1A). P2XRs are potently activated by the ATP analog, αβmeATP (7), but this P2X,R agonist (1–100 μM) had no effect on levels of MCP-1 (control vs αβmeATP: 1318 pg/ml vs 1350 pg/ml n = 3). Rat P2X4Rs are insensitive to inhibition by the P2X/P2Y receptor antagonist, suramin (29), but suramin (10, 30 μM) completely blocked the actions of ATP to increase the release of MCP-1 (control constitutive release from these cells was 2212 ± 73 pg/ml, n = 3). ATP and UTP (100 μM) also increased MCP-1 released into the supernatant from primary cultures of peritoneal macrophage in a suramin-sensitive manner; n = 3–6 experiments in each case.

FIGURE 3. P2Y2 receptors mediate increased MCP-1 release from NR8383 alveolar macrophage and rat peritoneal macrophage. A, Representative data obtained from ELISAs for MCP-1 released into the medium after 2 h incubation with each of the agonists (100 μM) as indicated; all data obtained from a single experiment and is representative of four to six similar experiments. B, Agonist concentration-response curve for ATP (○), UTP (□), and BzATP (△) obtained from all experiments as indicated in A where MCP-1 release is expressed as percent maximum from individual experiments; n = 2–5 for each point. C, Suramin (30 μM) but not PPADS (30 μM) blocks ATP (30 μM and UTP (100 μM)-induced increase in MCP-1; control constitutive release from this set of experiments was 212 ± 73 pg/ml, n = 3. D, ATP and UTP (100 μM) also increased MCP-1 released into the supernatant from primary cultures of peritoneal macrophage in a suramin-sensitive manner; n = 3–6 experiments in each case.

UTP were all equally effective at increasing MCP-1 release, with half-maximal concentrations (EC50 values) of 4.5 ± 0.6 μM, 2.5 ± 0.6 μM, and 3.6 ± 0.3 μM respectively (Fig. 3B). Similar EC50 values for these agonists have been reported for heterologously expressed P2Y2 receptors (30).

We obtained similar results from acutely isolated rat peritoneal macrophage. We found that MCP-1 was constitutively released from these cells, that ATP (10, 100 μM) and UTP (10, 100 μM) both resulted in an increased release of MCP-1, and that suramin but not PPADS abolished the UTP-induced increase in MCP-1 release (Fig. 3D). This concentration of agonist (10 μM) evoked maximum MCP-1 release as higher concentrations of ATP and UTP (100 μM) did not evoke significantly greater MCP-1 release, and suramin (30 μM) also blocked MCP-1 release evoked by these higher concentrations (n = 3). Whereas maximum responses to ATP and UTP were the same in NR8383 alveolar macrophage (Fig. 3, A and B), the maximum response to UTP was approximately twice that of ATP in primary cultures of rat peritoneal macrophages (Fig. 3D).

Constitutive release of MCP-1 does not involve P2Y2 receptors

We examined the possibility that the constitutive release of MCP-1 may be due to low-level activation of P2Y2 receptors by constitutively released ATP by preincubating cells in apyrase to hydrolyze extracellular nucleotides, if present. Apyrase alone did not alter the constitutive release of MCP-1, whereas it abolished the increased release of MCP-1 incuded by either ATP or UTP (Fig. 4A). We also examined the effects of preincubation with suramin and
PPADS on constitutive MCP-1 release and found that suramin or PPADS at concentrations ≤30 μM had no significant effect on constitutive release (Fig. 3B) but that higher concentrations (100–300 μM) virtually abolished all MCP-1 release (data not shown). These actions are most likely due to several well-documented nonspecific actions of these compounds (7, 8).

Kinetics of P2Y2R-induced increase in MCP-1 release
We examined the kinetics of MCP-1 release in response to the continued application of ATP or UTP; the earliest time point at which MCP-1 was significantly increased over control was 60 min after addition of either agonist and levels reached maximum at 120 min after agonist addition (Fig. 4B). We then asked whether a brief pulse of agonist would also lead to subsequent increased release of MCP-1 by applying UTP for 10 min and assaying for MCP-1 release 120 min later. We found a 10 min pulse of UTP was as effective to increase levels of MCP-1 release as was the continued presence of agonist for the entire duration (Fig. 4C).

P2Y2R activation increases MCP-1 mRNA levels via PLC-independent pathway
The delayed increased release of MCP-1 in response to P2Y2R activation suggested a signaling cascade involving increased synthesis. Therefore, we used a semiquantitative RT-PCR method to...
examine levels of MCP-1 mRNA relative to β-actin and found significantly increased levels within 30 min after stimulation with ATP or UTP (Fig. 5A). P2Y2R coupling through Gs protein activation can lead to increase transcription through at least three pathways: activation of PLC leading to Ras/Raf/MAP Kinase/ERK1/2 signaling, activation of PLC leading to phosphorylation of CREB, or activation of NF-κB via Gβγ protein coupling to PI3K (8, 9, 31–33). However, the PLC inhibitor, U73122 (5 or 10 μM), did not alter the UTP-induced increase in MCP-1 mRNA nor did chelation of intracellular calcium by incubating cells with BAPTA-AM (Fig. 5, B–D).

**P2Y2R activation increases MCP-1 release via the PLC-dependent pathway**

In contrast to the lack of effect of PLC blockade on MCP-1 mRNA levels, U73122 at a concentration of 10 μM (but not at 5 μM) completely prevented the increased release of MCP-1 by UTP (Fig. 6). Both concentrations of U73122 decreased basal release of MCP-1 by ~50% and the absolute amounts of MCP-1 induced by UTP were concomitantly reduced in the presence of either concentration of U73122 (Fig. 6A). By calculating the ratio of MCP-1 release (i.e., release in UTP/basal release vs release in UTP + U73122/release in U73122 alone) it can be seen that only the higher concentration of U73122 produced a significant inhibition of the UTP-evoked increased release in MCP-1 (Fig. 6B).

Similarly, preincubation of the cells with BAPTA-AM inhibited basal MCP-1 release by ~55% (Fig. 6A) and also prevented the UTP-induced increase in release, as evidenced by the calculated MCP-1 release ratio (Fig. 6B). We examined the actions of a number of other inhibitors of pathways linked to P2Y2 receptor activation on basal and UTP-evoked MCP-1 release. Blockade of Gs/Gq, coupling by preincubation with pertussis toxin, or blockade of PI3K (and ERK1/2) with wortmannin or LY294002, did not alter the UTP-induced increase in release. ATP, UTP, and BzATP all showed similar effects on MCP-1 release (Fig. 7A). PD98059 and SB203580, which block MEK1 and p38 MAPK respectively, markedly reduced the basal release of MCP-1 (by >85%) but did not alter the increase in release by UTP as evidenced by the MCP-1 release ratio (Fig. 7, A and B).

**Discussion**

This study has shown that brief activation of metabotropic P2Y2 purinergic receptors induced increased levels of MCP-1 mRNA and a delayed release of this chemokine from rat NR8383 alveolar macrophage cell lines and from primary cultures of rat peritoneal macrophage. MCP-1 (CCL2) is the prototypic, and most studied, proinflammatory chemokine that targets monocytes and T-lymphocytes expressing the CC chemokine receptor CCR2 to recruit these cells from the bloodstream to damaged tissue (34). In addition to providing chemotactic signals, MCP-1 signals to monocyte activation and induction of the respiratory burst (35, 36). Indeed, MCP-1 has been linked to numerous roles in both adaptive and innate immunity as well as in inflammatory diseases, and its synthesis and/or release is well documented to be increased in vascular endothelial and smooth muscle cells in atherosclerotic plaques, in macrophages and in glomerular epithelial cells at sites of acute and chronic inflammation (34–36). Much less information exists as to the initial physiological and pathophysiological stimuli that induce MCP-1 synthesis and/or release. Results from the present study implicate extracellular nucleotides acting at the P2Y2 receptor as a potentially important initial stimulus for MCP-1 release.

The multiplicity of purine receptors expressed on macrophage, together with a paucity of subtype-selective receptor antagonists, presents considerable obstacles to identifying which receptor subtype may be directly responsible for modulation of chemokine or cytokine release. This is particularly true for immune cells expressing P2X7 receptors because P2X7-Rs unequivocally couple to the rapid release of IL-1β from activated macrophages (usually via LPS priming) (4, 19–21); IL-1β can then lead to the release of numerous other proinflammatory molecules including MCP-1 (25). For this reason, we made use of the rat NR8383 alveolar macrophage cell line which the present (Fig. 1) and previous (26) study reveal do not express functional P2X7 receptors and so do not release IL-1β in response to extracellular ATP. Because these cells possess all other aspects of the IL-1β cascade, as evidenced by their capacity to release fully processed IL-1β in response to ATP after ectopic expression of P2X7-R, they should prove highly useful in further studies of chemokine/cytokine modulation by other stimuli which may directly or indirectly lead to release of ATP. Moreover, use of rat NR8383 macrophage additionally simplifies investigations of purinergic receptor actions because they express a more limited repertoire of receptors than other immune cells and immune cell lines, expressing mRNA for only P2Y1, P2Y2, P2Y12, and P2X4 receptors, each of which can be observed to be functional in electrophysiological assays (26). We used a supramaximal concentration of the nonhydrolysable ATP analog, ATPγS, to initially screen a 17-member cytokine/chemokine protein array and found only one, MCP-1, to be significantly increased by this agonist. It should be noted that this protocol would be expected to bias against P2Y, R actions because P2Y, Rs are ADP-sensitive and, thus, unlikely to be activated by ATPγS (8); however, use of ADPβS (an nonhydrolysable ADP analog) in this type of cytokine/chemokine array may well identify a P2Y, R-specific pathway.

Agonist concentration curves and antagonist profiles clearly identified P2Y2, R as the sole receptor underlying nucleotide-mediated MCP-1 release. ATP, UTP, and BzATP all showed similar maximum responses and EC50 values of 4–6 μM, values which are very similar to those obtained in heterologous expression assays of rat P2Y2, R (30). These agonist profiles rule out P2Y1, R (ATP, UTP, and BzATP-insensitive), P2Y12, R (UTP and BzATP-insensitive), and P2X7, R (UTP-insensitive) (8, 30). Additionally, we found that brief stimulation (~10 min) with UTP was as effective to increase MCP-1 mRNA levels and secretion as was prolonged receptor stimulation (2 h). These kinetics are in keeping with the well known rapid and pronounced desensitization of P2Y2-Rs (31). Results we obtained with the nonselective antagonists, suramin and PPADS, also provide limited, but supportive, data. We found that suramin at concentrations ≤30 μM abrogated the ATP or UTP-evoked increases in MCP-1 without exerting any effect on MCP-1 release when applied in the absence of ATP or UTP, whereas PPADS at ≤30 μM had no effect on either constitutive nor ATP/UTP-evoked MCP-1 release. These results provide further support for selective activation of P2Y2, R (suramin-sensitive/PPADS-insensitive) by ATP and UTP (8). However, higher concentrations of either antagonist inhibited both constitutive and agonist-induced release by >85%, thus emphasizing the nonspecific actions of these compounds and the need for caution in interpretations made based on their actions, especially in view of much data showing that concentrations >100 μM will inhibit virtually all P2X and P2Y receptors so far examined (7, 8). For example, in a recent study, ATPγS (10 and 100 μM) was found to increase the release of a number of inflammatory mediators, including MCP-1, from the human HMEC1 epithelial cell line. In that study, suramin and PPADS were used at concentrations of 100 and 1000 μM and found to block both constitutive and agonist-induced release of all compounds measured (37); thus, providing no information as to the receptors involved.
We also found that primary cultures of rat peritoneal macrophage exhibited constitutive release of MCP-1 as well as ATP and UTP-evoked increases in MCP-1 release and the agonist-evoked increased release was prevented by suramin (10 μM) but not PPADS (30 μM). In these macrophage the EC50 values for ATP and UTP were ~10 μM. These results are consistent with P2Y2R activation, but the maximum UTP-evoked response was 2-fold greater than the ATP-evoked response, a result that is most likely due to activation of more than a single receptor type by ATP.

In common with several studies of MCP-1 release in other cells (10, 37–39), we found substantial basal release of MCP-1 from both NR8383 alveolar macrophage and peritoneal macrophage. This was not due to presence of extracellular nucleotides because neither apyrase nor suramin altered the basal MCP-1 release. High levels of basal MCP-1 release generally have been ascribed to known or unknown conditions resulting in an activated (inflammatory) phenotype (35, 37, 39). However, we did not observe significant constitutive release of any of the other proinflammatory chemokines or cytokines assayed in our protein array, thus suggesting the high basal levels of MCP-1 in these apparently unactivated macrophages may have a functional role unrelated to inflammatory conditions.

Because MCP-1 follows the classical endoplasmic reticulum-Golgi pathway of constitutive secretion, it is reasonable to expect that the P2Y2R-induced increase in MCP-1 release is a straightforward consequence of increased protein synthesis. In keeping with this expectation, we observed increased levels of MCP-1 mRNA within 15–30 min of agonist application, and a delayed release detected only 60 min after receptor stimulation. However, inhibition of PLC by U73122, or prevention of PLC-induced increases in intracellular calcium concentrations by chelation with BAPTA-AM, effectively abolished UTP-induced increased release of MCP-1 without significantly altering the increased mRNA levels. It is difficult to explain these results based on activation of a single transduction pathway (i.e., the PLC cascade) by P2Y2 receptors although we found no evidence for the involvement of either of the other two signaling cascades that are less prominently associated with P2Y2R activation: Gαi-coupled P3K pathways and Gαq/11-coupled signaling (8, 9, 31–33). An interesting possibility is constitutive release of MCP-1 but completely prevented UTP- or ATP-evoked increases. Inhibition of Ras/Raf/ERK1/2 signaling with wortmannin or LY294002 altered neither basal nor UTP-induced MCP-1 release. These results suggest that specific, constitutively active, MAPK pathways (MEK and/or p38 MAPK) are primarily responsible for basal (but not stimulated) MCP-1 release and highlight the importance of measuring release ratios, rather than absolute levels, when assaying changes in constitutively released proteins.

ATP, ADP, and/or UTP have been known for the past decade to act indirectly as chemoattractant signals in several immune and hemopoietic cells, including neutrophils, dendritic cells, monocytes, and recently, hemopoietic stem cells (2–4, 17, 42, 43), although the purinergic receptor subtypes involved and their underlying signaling mechanism(s) have been investigated only quite recently (17, 42). In human endothelial cells, glia (17, 43) and hemopoietic stem cells (42), P2Y1Rs signal through pertussis toxin-sensitive Gαi/Gq/11 paths to engage other chemoattractant or adhesion molecules, such as integrins (17) and CXCR1/2 (42), via p-GTPase-dependent signaling. The present study provides the first demonstration that P2Y2R activation and its canonical PLC signaling cascade may also link to chemotaxis by inducing the release of MCP-1 from macrophage.

**Acknowledgments**

We thank Elizabeth Martin and Weihong Ma for valuable technical assistance.

**Disclosures**

The authors have no financial conflict of interest.

**References**


