British Journal of Pharmacology (1996) 119, 261-268

Pyrimidinoceptor-mediated activation of phospholipase C and phospholipase A_2 in RAW 264.7 macrophages

[1]W.W. Lin & *Y.T. Lee

Department of Pharmacology, *Center for Cardiovascular Research, College of Medicine, National Taiwan University, Taipei, Taiwan

1 As well as the presence of P_{2Z} purinoceptors previously found in macrophages, we identified pyrimidinoceptors in RAW 264.7 cells, which activate phospholipase C (PLC) and phospholipase A_2 (PLA_2).

2 The relative potency of agonists to stimulate inositol phosphate (IP) formation and arachidonic acid (AA) release was UTP = UDP >> ATP, ATPγS, 2MeSATP. For both signalling pathways, the EC_{50} values for UTP and UDP (3 μM) were significantly lower than that for ATP and all other analogues tested (>100 μM).

3 UTP and UDP displayed no additivity in terms of IP formation and AA release at maximally effective concentrations.

4 UTP-, but not ATP-, evoked AA release was 60% inhibited by pertussis toxin (PTX), while stimulation of IP formation by both agonists was unaffected. Short-term treatment with phorbol 12-myristate 13-acetate (PMA) led to a dose-dependent inhibition of IP responses to UTP and UDP, but failed to affect the AA responses. Removal of extracellular Ca^{2+} inhibited the PI response to UTP, but abolished its AA response.

5 ATP-induction of these two transmembrane signal pathways was decreased in high Mg^{2+}-containing medium but potentiated by the removal of extracellular Mg^{2+}.

6 Suramin and reactive blue displayed equal potency to inhibit the IP responses of UTP and ATP.

7 Both UTP and UDP (0.1-100 μM) induced a sustained increase in $[Ca^{2+}]_i$ which lasted for more than 10 min.

8 Taken together, these results indicate that in mouse RAW 264.7 macrophages, pyrimidinoceptors with specificity for UTP and UDP mediate the activation of PLC and cytosolic (c) PLA_2. The activation of PLC is via a PTX-insensitive G protein, whereas that of $cPLA_2$ is via a PTX-sensitive G protein-dependent pathway. The sustained Ca^{2+} influx caused by UTP contributes to the activation of $cPLA_2$. RAW 264.7 cells also possess P_{2Z} purinoceptors which mediate ATP^{4-}-induced PLC and PLA_2 activation.

Keywords: Uridine 5'-triphosphate (UTP); adenosine 5'-triphosphate (ATP); phospholipase C; phospholipase A_2; RAW 264:7 macrophages; pyrimidinoceptor

Introduction

P_2 purinoceptors are cell surface receptors and extracellular adenosine 5'-triphosphate (ATP) may serve as a mediator of cell-to-cell communication by interacting with P_2 purinoceptors. According to the selective agonists, P_2 purinoceptor subclasses include P_{2X}, P_{2Y}, P_{2U}, P_{2Z} and P_{2T} (Fredholm *et al.*, 1994; Harden *et al.*, 1995). P_{2X} purinoceptors are ion channels directly gated by ATP, and α,β-methylene ATP (α,β-MeATP) and β,γ-methylene ATP (β,γ-MeATP) are the most potent agonists. P_{2Y} purinoceptors belong to the family of guanosine 5'-triphosphate (GTP) binding protein-coupled receptors, which activate phosphoinositide (PI)-specific phospholipase C (PLC) or inhibit adenylyl cyclase (Barnard *et al.*, 1994; Lin & Chuang, 1994), the most potent agonist being 2-methylthio-ATP (2MeSATP). P_{2U} purinoceptors share many features of P_{2Y} purinoceptors and have equal affinity for ATP and UTP. P_{2Z} purinoceptors have been identified as nonspecific pores permeable to solutes of molecular weight up to 900, the most potent agonist being ATP in its fully dissociated form (ATP^{4-}). P_{2T} purinoceptors are adenosine 5'-diphosphate (ADP)-specific receptors in platelets.

Uridine 5'-triphosphate (UTP), in addition to acting on P_{2U} purinoceptors, was also proposed to exert its effects after binding to uridine nucleotide-selective G protein-linked receptors (pyrimidinoceptors) (Seifert & Schultz, 1989). Pyrimidinoceptors that are activated specifically by UTP rather than ATP have been suggested to exist in rabbit basilar and ear arteries (Von Kugelgen *et al.*, 1987; Von Kugelgen & Starke, 1990), the isolated perfused rat liver (Haussinger *et al.*, 1987), rat sympathetic ganglia (Connolly & Harrison, 1994), NG108-15 neuroblastoma (Lin, 1994; Reiser, 1995) and C_6-2B rat glioma cells (Lazarowski & Harden, 1994).

In phagocytic cells, activation of PI-PLC and phospholipase A_2 (PLA_2) signalling pathways are amongst the earliest events triggered by inflammatory stimuli, and are believed to play a role in triggering or modulating chemotaxis, secretion, phagocytosis and superoxide release (Elsbach & Weiss, 1988). It has been suggested that significant amounts of extracellular ATP, released from injured tissues and platelets, may accumulate locally at vascular sites of thrombus formation and infection/inflammation, and thus modify the function of phagocytes present at such inflammatory sites. UTP, which is also an endogenous nucleotide released from platelets (Goetz *et al.*, 1971), might be involved in the inflammatory events. In phagocytic cells, the PI turnover and Ca^{2+} mobilization caused by ATP has been observed in some cell types, such as human neutrophils, neutrophil/monocyte progenitor cells (Dubyak *et al.*, 1988; Cowen *et al.*, 1989), mouse peritoneal macrophages (Pfeilschifter *et al.*, 1989), and mouse J774 macrophages (Greenberg *et al.*,

[1]Author for correspondence.

1988). Moreover, PLA_2 activation by ATP was also demonstrated in human neutrophils (Cockcroft & Stutchfield, 1989), HL-60 cells (Xing & Matter, 1992), and mouse peritoneal macrophages (Pfeilschifter *et al.*, 1989). Although the signalling pathways of ATP have been extensively studied, not much information about the signalling and function of UTP is available.

Two distinct types of P_2 purinoceptors for extracellular ATP have been shown in BAC 1.2 F5 and J774 macrophages. One is the P_{2Z} purinoceptor and the other, capable of activating PI-PLC and inducing Ca^{2+} mobilization, is presently unknown (Greenberg *et al.*, 1988; El-Moatassim & Dubyak, 1992). The goal of the current work was to identify the P_2 purinoceptor subtypes which mediate PI turnover, and arachidonic acid (AA) release in mouse RAW 264.7 macrophages. This cell line displays many of the properties of mature macrophages (Raschke *et al.*, 1978) and possesses cytosolic PLA_2 ($cPLA_2$) (Channon & Leslie, 1990). The identification of the signalling pathways mediated by nucleotides in macrophages may provide information to delineate further their physiological and pathological roles.

Methods

Cell culture

RAW 264.7 cells, generously provided by Dr Yen-Jen Sung (Department of Anatomy, National Yang-Ming Univ. School of Medicine), were grown in 35-mm Petri dishes at 37°C in Dubecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum, 100 u ml^{-1} penicillin and 100 μg ml^{-1} streptomycin, in a humidified atmosphere of 95% air and 5% CO_2.

Measurements of PI turnover

The hydrolysis of PI was measured as the accumulation of inositol phosphates (IP) in the presence of 10 mM LiCl as described previously (Lin & Chuang, 1994). Confluent cells on 35-mm Petri dishes were labelled with [^{3}H]-*myo*-inositol (2.5 μCi per dish) in the growth medium for 24 h. The cells were then washed with physiological saline solution (PSS, composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 1.8, $MgCl_2$ 1.2, KH_2PO_4 1.2, glucose 11 and HEPES 20, pH 7.4) containing 10 mM LiCl and incubated at 37°C for 20 min. After this preincubation, the indicated drugs were added and then incubated for another 30 min. The reaction was terminated by aspiration of the reaction solution and addition of ice-cold methanol. The cells were scraped and [^{3}H]-IP was isolated by using an AG-1X8 (formate form, 100–200 mesh, Bio-Rad, Richmond, CA) column and eluted with 0.2 N ammonium formate/0.1 N formic acid. PSS was used for the measurement of PI turnover and AA release, unless otherwise indicated. In the 'Ca^{2+}-free' conditions, Ca^{2+} ions in PSS were omitted and 1 mM EGTA was supplemented. In "Mg^{2+}-free" and 'high Mg^{2+}' conditions, Mg^{2+} ions were either omitted or increased to 9 mM.

[^{3}H]-AA release

Cells in 35 mm dishes were incubated in 5% CO_2 for 24 h with 0.3 μCi ml^{-1} [^{3}H]-AA in DMEM. After being labelled, monolayers were washed three times with PSS and incubated in PSS containing 0.5% fatty acid-free bovine serum albumin. Cells were then stimulated by the agonist at 37°C for 30 min, unless otherwise indicated. When stated, pretreated drugs were added to the cells 20 min before the treatment with the agonists. At the end of the incubation, media were removed and centrifuged at 250 *g* for 5 min to remove floating cells, and the radioactivity in the supernatant was measured. Monolayers were solubilized for determination of total [^{3}H]-AA incorporation.

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

Cells grown on glass slides were loaded with 3 μM fura-II/AM and pluronic F-127 (0.02% v/v) in DMEM medium at 37°C for 45 min. Fluorescence was monitored on a PTI M-series spectrofluorometer with dual excitation wavelengths of 340 and 380 nm and emission wavelength of 510 nm. $[Ca^{2+}]_i$ was calculated by the equation described by Grynkiewiez *et al.* (1985).

Materials

Cell culture media and supplements were obtained from Gibco BRL (Grand Island, NY). [^{3}H]-*myo*-inositol (20 Ci $mmol^{-1}$) and [^{3}H]-AA (100 Ci $mmol^{-1}$) were purchased from New England Nuclear (Boston, MA). Reactive blue, 2MeSATP, α,β-MeATP and β,γ-MeATP were obtained from RBI (Natick, MA), and all other chemicals were obtained from Sigma. FPL 67156 (6-N,N-diethyl-D-β, γ-dibromomethylene ATP) was kindly provided by Dr P. Leff (Fisons plc., U.K.).

Statistical analysis

Each experiment was performed in duplicate and reproduced several times (*n* refers to the number independent experiments). Data are presented as mean ± s.e.mean values. The significance of differences between the means was evaluated by the Student's *t* test and value of $P<0.05$ was considered significant. The error bar was omitted when it was within the symbol representing the mean value. When the agonist response at maximal concentration used and in the absence of antagonists was regarded as 100%, the value at 50% of the maximal increase (EC_{50}) was estimated from the concentration-response curve. The pA_2 value, the negative logarithm of the molar concentration of antagonist in the presence of which the EC_{50} value of UTP is increased by 2 times, was calculated.

Results

Measurement of [^{3}H]-IP accumulation in the presence of LiCl was used as an index for the activation of PI turnover by various P_2 purinoceptor agonists. We found that UTP and UDP were equipotent and much more potent than other agonists tested. The EC_{50} values were 3 μM for both agonists, and higher than 100 μM for others (Figure 1a). The threshold concentration of ATP or ATPγS required to produce PI turnover was 300 μM. Similar efficacy (approximately 12–14 fold of control) was reached by UTP, UDP, ATP, ATPγS and 2MeSATP within the tested concentrations up to 1 mM. The rank order of potency based on the maximal response obtained within 1 mM was as follows: UTP = UDP >> ATP, ATPγS, 2MeSATP. The IP response of UMP (1 mM) and 3′-O-(4-benzoyl)benzoyl-ATP (BzATP) (1 mM) was 250% and 500% of control, respectively. AP_5A, AP_4A, α,β-MeATP, β,γ-MeATP and adenosine at 1 mM were completely ineffective (data not shown). This ranking is not in agreement with those proposed for P_{2U} and P_{2Y} subtypes.

The potencies of nucleotide analogues on AA release almost matched their potencies on IP formation (Figure 1b). The amount of basal [^{3}H]-AA release was 1.51 ± 0.12% of the total [^{3}H]-AA incorporated into phospholipids. UTP and UDP were much more potent (EC_{50} values of 3 μM and 10 μM, respectively) than other agonists in promoting AA release. ATP, 2MeSATP, and UMP did not increase AA release until the concentration was raised to 1 mM. The extent of AA release caused by UTP (100 μM), UDP (100 μM), ATP (1 mM), 2MeSATP (1 mM) and UMP (1 mM) were 300 ± 20% ($n=33$), 243 ± 25% ($n=4$), 389 ± 33% ($n=9$), 323 ± 10% ($n=3$) and 181 ± 6% ($n=3$) of control, respectively. B_zATP only increased the AA release at 1 mM, with 1387 ± 100% ($n=4$) of control. α,β-MeATP, $\beta\gamma$-MeATP, $A_{P5}A$, and $A_{P4}A$ were without effect at 1 mM (data not shown). As shown in Figure 2, the accumulation of IP formation (in the presence of LiCl) and AA

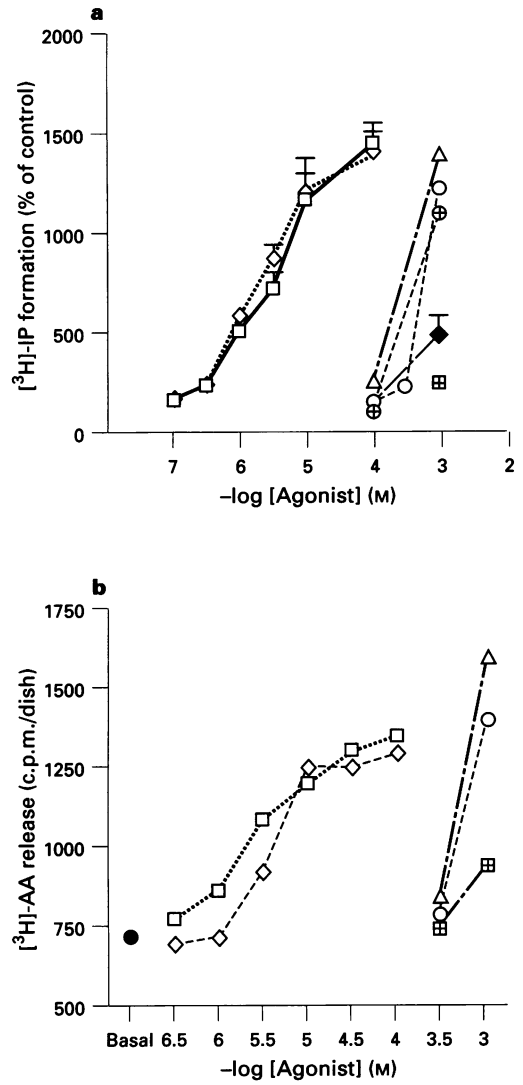

Figure 1 Concentration-response curves of agonist-induced activation of phospholipase C (PLC) and PLA_2. RAW 264.7 cells were labelled with (a) [^{3}H]-*myo*inositol or (b) [^{3}H]-arachidonic acid (AA) overnight, and the accumulation of [^{3}H]-inositol phosphate (IP) (a) or [^{3}H]-AA release (b) was measured as described in Methods. (●) Basal, (□) UTP, (◇) UDP, (○) ATP, (△) 2MeSATP, (⊞) UMP, (◆) BzATP, (⊕) ATPγS. Data represent the mean±s.e.mean (vertical lines) from at least three independent experiments. Where no error bar is represented, it is within the size of the symbol.

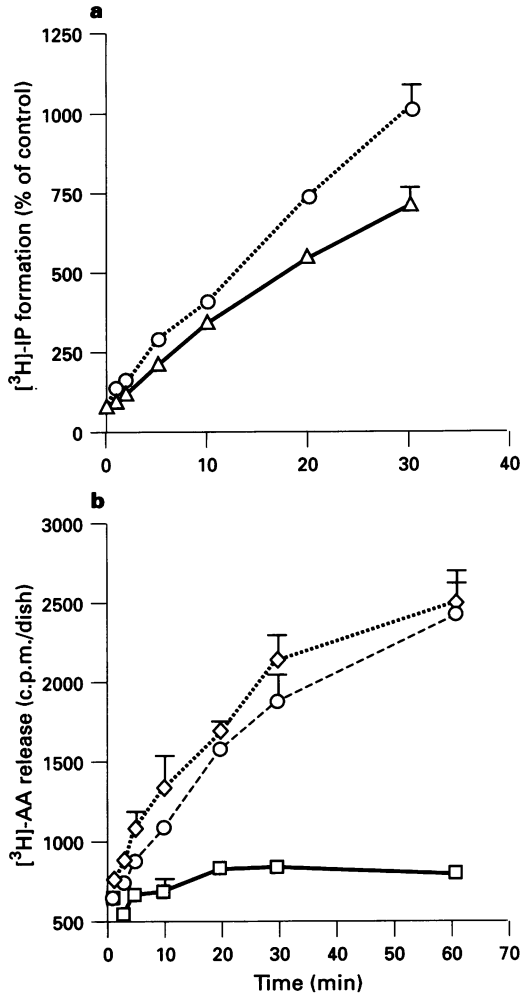

Figure 2 Time-dependent increase in [^{3}H]-inositol phosphate (IP) accumulation and [^{3}H]-arachidonic acid (AA) release caused by UTP and ATP. Cells were treated with vehicle (□), 30 (△) or 100 (◇) μM UTP or 1 mM ATP (○) for various periods, and [^{3}H]-IP accumulation (a) or [^{3}H]-AA release (b) was measured. The data shown represent the mean±s.e.mean (vertical lines) from three independent experiments.

release caused by UTP and ATP were time-dependent and were observed as rapidly as 1 min. Both the time courses of IP formation and AA release were in parallel.

In order to assess the signal pathways mediated by UTP, UDP and ATP via the same type of purinoceptor, the additivity of the responses caused by these three agonists was determined. For PI turnover, the responses caused by UTP (10 μM) in combination with ATP (1 mM) or UDP (10 μM) were non-additive (Figure 3a). For AA release, the response to UTP (100 μM) was non-additive to UDP (100 μM), but was additive to ATP (1 mM).

In order to rule out the possible modulation of PI turnover and AA release by the ATP metabolite, adenosine, we performed the additive experiment between UTP and adenosine. We found that adenosine (100 μM) did not affect the dose-response curves of UTP-induced IP formation and AA release (data not shown).

To investigate the participation of G proteins in these two signalling pathways of UTP and ATP, cells were pretreated with pertussis toxin (PTX) for 24 h. In contrast to the ineffectiveness of PTX (500 ng ml^{-1}) on the PI response to UTP, the AA release caused by UTP was reduced by approximately 60% (Figure 4a,b). On the other hand, ATP (1 mM)-induced IP accumulation and AA release were unaffected by the pretreatment with PTX (Figure 4a,b). The AA response to BzATP (1 mM) was unaffected by PTX (data not shown).

To assess the dependence of IP formation and AA release on extracellular Ca^{2+}, the responses to agonists were studied in the absence of extracellular Ca^{2+}. The AA responses to UTP (100 μM), ATP (1 mM) (Figure 4b) as well as BzATP (1 mM, data not shown) were abolished by the Ca^{2+}-free medium. With respect to PI turnover, the UTP-induced IP formation was markedly reduced in the absence of extracellular Ca^{2+} (Figure 4c), while that of ATP was abolished (data not shown).

The negative regulatory role of protein kinase C (PKC) on PLC activation was determined by short-term pretreatment with the PKC activator, phorbol 12-myristate 13-acetate (PMA). As shown in Figure 5, UTP- and UDP-induced IP formation were inhibited by PMA with the same susceptibility. The IC_{50} value of PMA was 20 nM, and the responses were about 90% inhibited by 1 μM PMA. Inactive phorbol ester, 4-α-PMA, in contrast, did not show a negative regulation of PI turnover at concentrations up to 1 μM (data not shown). The AA release by UTP and ATP, however, were unaffected by 10 min pretreatment with PMA (data not shown).

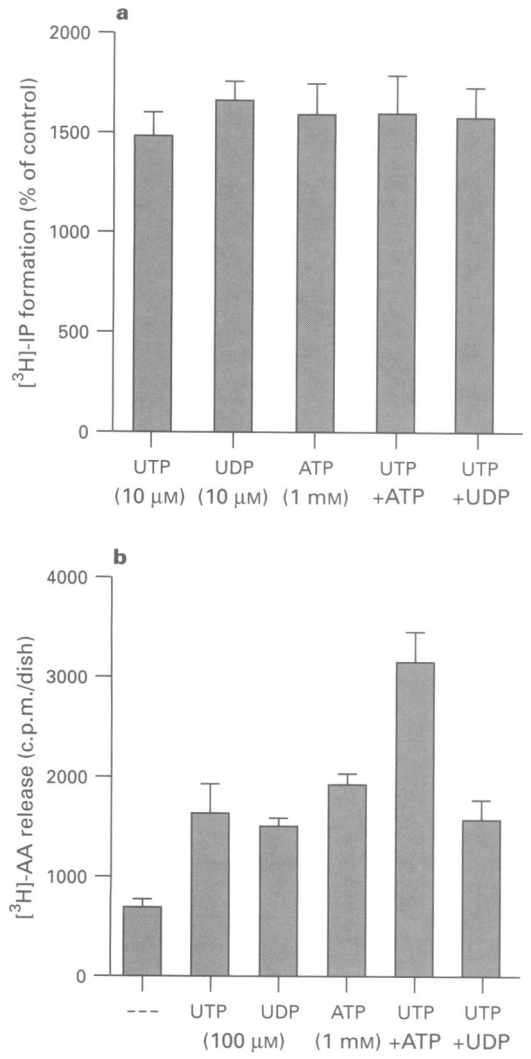

Figure 3 Additivity between the agonist-induced inositol phosphate (IP) accumulation and arachidonic acid (AA) release. Cells were treated with each agonist or in combination at the indicated concentration for 30 min, then [^{3}H]-IP accumulation (a) or [^{3}H]-AA release (b) was measured. The data shown represent the mean ± s.e.mean (vertical lines) from three independent experiments.

Figure 6 shows the effects of two competitive antagonists of P_2 purinoceptors (suramin and reactive blue) on UTP- and ATP-mediated PI turnover. Suramin and reactive blue at concentrations which did not affect the basal IP formation caused a concentration-dependent rightward shift in the dose-response curves of UTP with pA_2 values of 4.85 and 5.75, respectively. The PI response caused by ATP (1 mM) was equipotently inhibited by suramin and reactive blue as compared to UTP (10 μM) (Figure 6c).

Figure 7 shows the effects of extracellular Mg^{2+} on agonist responses. UTP-induced IP formation was unaffected either by Mg^{2+}-free or by high Mg^{2+} PSS, while the PI response caused by 1 mM ATP was potentiated by the removal of extracellular Mg^{2+} and reduced by 9 mM Mg^{2+} (Figure 7a,b). The manipulation of extracellular Mg^{2+} only affected the efficacy of the ATP-induced PI response, whereas its threshold concentration (300 μM) was unaffected (data not shown). For AA release, the response to UTP (100 μM) was not affected by the removal of extracellular Mg^{2+}, but was partially suppressed (about 30%) by high Mg^{2+}. On the other hand, the response to ATP was potentiated in the absence of Mg^{2+} and markedly reduced by high Mg^{2+} (Figure 7c).

UTP and UDP rapidly increased $[Ca^{2+}]_i$ and the time course of the response was concentration-dependent. More

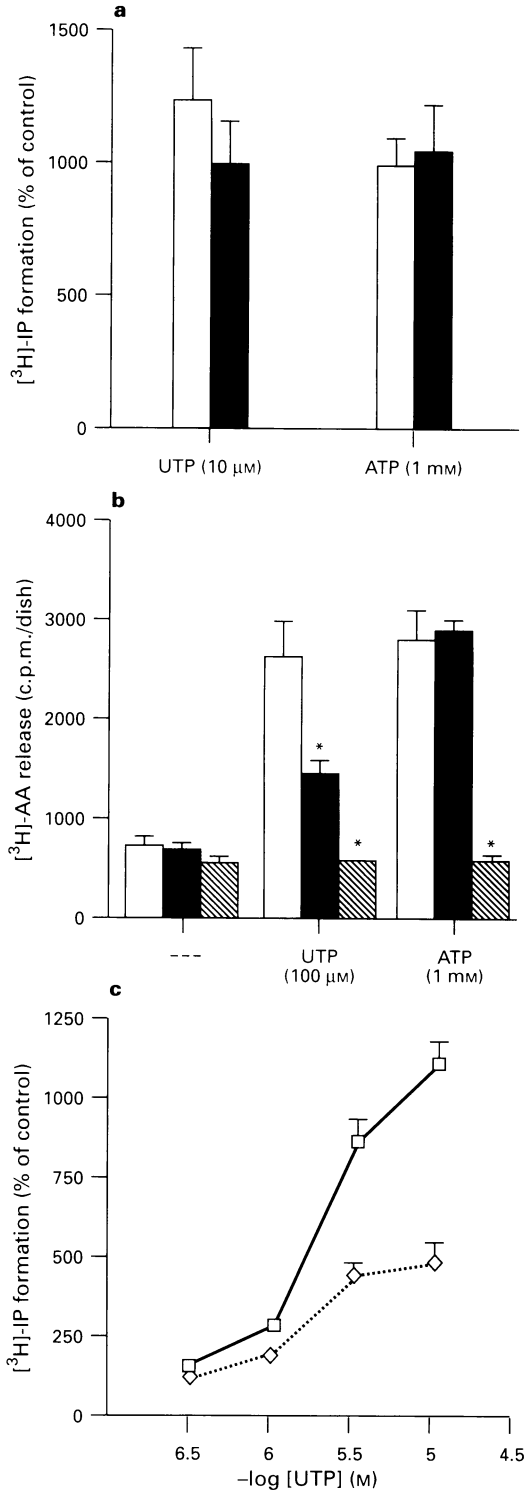

Figure 4 Effects of pertussis toxin (PTX) and the removal of extracellular Ca^{2+} on UTP- and ATP-induced [^{3}H]-inositol phosphate (IP) accumulation and [^{3}H]-arachidonic acid (AA) release. Cells were either pretreated with vehicle (open columns) or (□) in (c), pertussis toxin (500 ng ml^{-1}) for 24 h in DMEM (solid columns) or with Ca^{2+}-free PSS containing 1 mM EGTA (hatched columns in b; (◇) in c) for 10 min, then UTP at indicated concentrations or ATP (1 mM) was added, and [^{3}H]-IP accumulation (a) and (c) or [^{3}H]-AA release (b) was measured. The data shown represent the mean ± s.e.mean (vertical lines) from three independent experiments. *$P<0.05$ compared to the agonist response in normal PSS without (PTX) pretreatment.

interestingly, $[Ca^{2+}]_i$ was increased and sustained for at least 10 min within the concentration tested (Figure 8a,b). In the absence of extracellular Ca^{2+}, the plateau increase evoked by

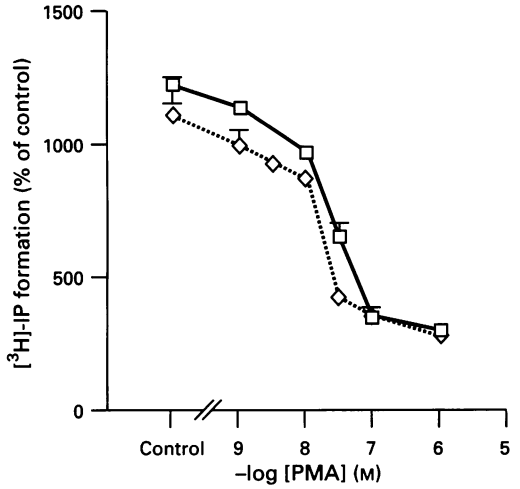

Figure 5 Effects of short-term treatment with phorbol 12-myristate 13-acetate (PMA) on UTP- and UDP-induced inositol phosphate (IP) formation. Cells labelled with [^{3}H]-*myo*inositol were pretreated with vehicle or PMA at the indicated concentrations 10 min before the addition of 10 μM UTP (□) or UDP (◇). Thirty minutes after stimulation, [^{3}H]-IP accumulation was measured. The data shown represent the mean ± s.e.mean (vertical lines) from a typical experiment.

UTP rapidly fell to base-line (Figure 8c). The $[Ca^{2+}]_i$ responses of UTP and UDP were unaffected by the extracellular Mg^{2+} (data not shown).

Discussion

The purpose of the present study was to characterize the receptor subtype involved in the activation of PLC and PLA_2 by nucleotide analogues in the mouse macrophage RAW 264.7 cell line. We found that receptors with high sensitivity to UTP and UDP are expressed in RAW 264.7 cells and their activation results in the stimulation of PLC and PLA_2. The rank orders of potency for increasing PI turnover and AA release (UTP = UDP >> ATP, ATPγS, 2MeSATP > α,β-MeATP β,γ-MeATP) are not in agreement with those classically described for P_{2U}, P_{2Y} and P_{2X} purinoceptor subtypes. 2MeSATP, the head of the P_{2Y} series, is much less potent for both signalling pathways. α,β-MeATP and β,γ-MeATP, two selective agonists for P_{2X} purinoceptors, were virtually inactive. For classical P_{2U} subtypes, UTP is equipotent to ATP in various cells (O'Connor *et al.*, 1991), and this proposal has been supported by data from the transfection with mouse and human P_{2U} purinoceptors (Lustig *et al.*, 1993; Parr *et al.*, 1994). In RAW 264.7 cells, however, the receptors responsible for UTP- and UDP-induced activation of PLC and PLA_2 appear not to be the classical P_{2U} subtype because of the much higher potency of UTP and UDP than ATP. This finding suggests that pyrimidinoceptors are expressed in mouse macrophage RAW 264.7 cells and they are coupled to the activation of phospholipases.

The hypothesis that ATP and UTP can activate distinct receptors, i.e. P_2 purinoceptors and pyrimidinoceptors respectively, has also been proposed (Seifert & Schultz, 1989). Pyrimidinoceptors have been suggested to exist in rabbit basilar and ear arteries (Von Kugelgen *et al.*, 1987; Von Kugelgen & Starke, 1990), the perfused isolated liver of the rat (Haussinger *et al.*, 1987), rat superior cervical ganglion (Connolly & Harrison, 1994), mouse neuroblastoma NG108-15 cells (Lin, 1994; Reiser, 1995), rat C_6-2B glioma cells (Lazarowski & Harden, 1994), HL-60 and neutrophils (Seifert & Schultz, 1989). Similar to the PI and AA responses in RAW 264.7 cells, with the PI and guanosine 3′:5′-cyclic monophosphate (cyclicGMP) responses (induced by UTP and UDP) in NG108-15

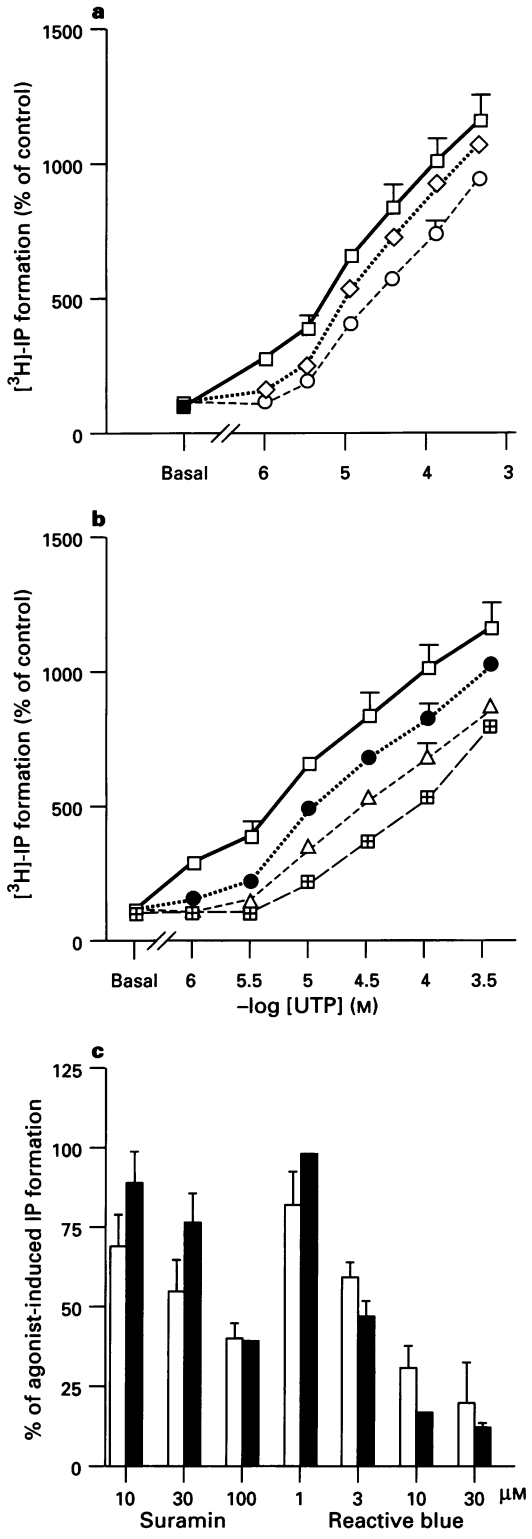

Figure 6 Effects of suramin and reactive blue on UTP- and ATP-induced [^{3}H]-inositol phosphate (IP) accumulation. Cells were pretreated with vehicle (□), 10 μM (◇) or 30 μM (○) suramin (a), 3 μM (●), 10 μM (△) or 30 μM ( ) reactive blue (b), 20 min before the addition of various concentrations of UTP, and then [^{3}H]-IP formation was measured. In (c), the inhibitory effects of suramin and reactive blue on the PI response to 10 μM UTP (open columns) and 1 mM ATP (solid columns) are summarized. The data shown represent the mean ± s.e.mean (vertical lines) from three independent experiments.

cells (Lin, 1994; Reiser, 1995) and the PI and AA responses (to UDP) in C_6-2B glioma cells (Lazarowski & Harden, 1994) UTP and UDP are much more potent than other nucleotide

analogues. All three cell lines respond to submicromolar concentrations of UTP and UDP, and thus display a higher sensitivity to uridine nucleotide. The biochemical and molecular features of these pyrimidinoceptors need further investigation.

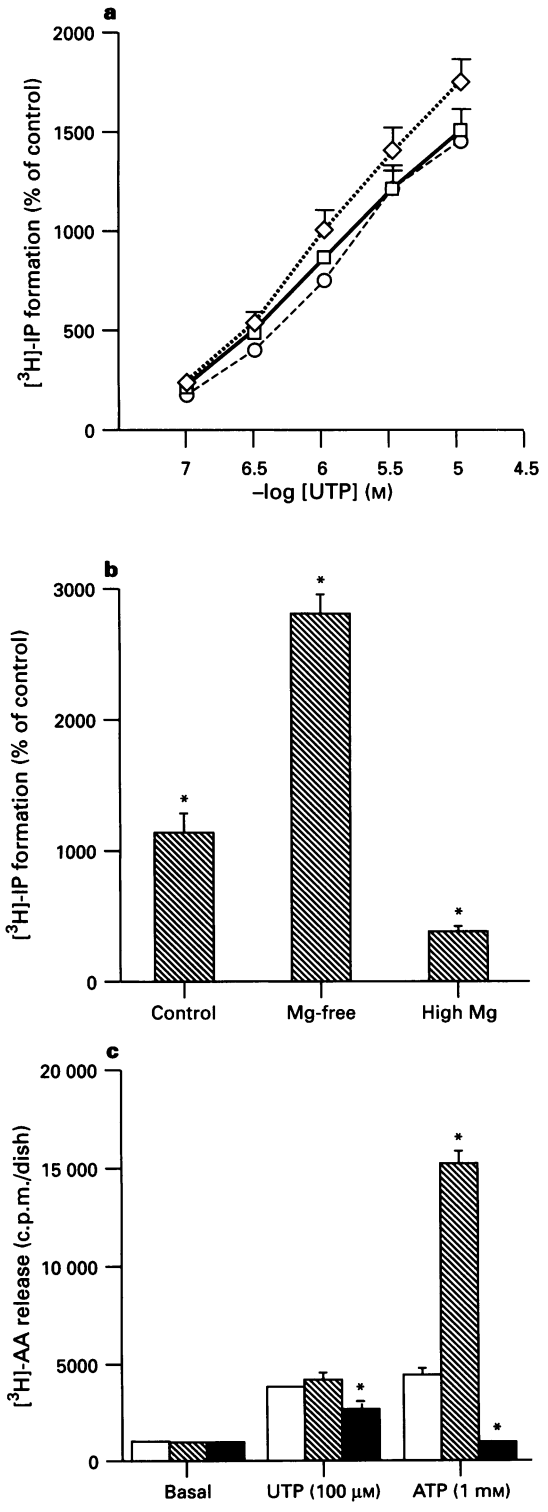

Figure 7 Effects of extracellular Mg^{2+} on UTP- and ATP-induced inositol phosphate (IP) formation and [^{3}H]-arachidonic acid (AA) release. Cells were treated with the indicated concentrations of UTP (a,c) or 1 mM ATP (b,c) in normal PSS ((□) in (a) and open columns in (c)), Mg^{2+}-free PSS [◇ in (a) and hatched columns in (c)] or high Mg^{2+} (9 mM) PSS ((○) in (a) and solid columns in (c)) and [^{3}H]-IP formation (a,b) or [^{3}H]-AA release (c) was measured. The data shown represent the mean ± s.e.mean (vertical lines) from three independent experiments. $*P<0.05$ compared to the agonist response in normal PSS.

The lower potency of ATP for activation of these two transmembrane signalling pathways in RAW 264.7 cells does not appear to be due to either the regulation by its metabolite, adenosine, or the rapid breakdown by ecto-ATPase. The former possibility can be ruled out by the findings that adenosine at 100 μM did not affect either basal and UTP-induced PI turnover or basal and prostaglandin (PGE_1)-induced cyclic-cAMP formation in this cell line (data not shown). The latter possibility was tested by studying the effects of FPL 67156, which is a potent and selective inhibitor of ecto-ATPase (Crack *et al.*, 1995). We found that FPL 67156 at 100 μM did not significantly affect the dose-response curve of ATP-induced IP formation (data not shown). In addition, ATPγS, which is resistant to degradation by ecto-ATPase, displayed a similar potency to ATP. All these observations further strengthen the suggestion that a unique pyrimidinoceptor with specificity for UTP and UDP, is expressed in RAW 264.7 cells.

The present results suggest that P_{2Z} purinoceptors, which possess pore-forming activity, possibly mediate the signalling effects of ATP. This suggestion relies on the finding that BzATP, a selective agonist on the P_{2Z} purinoceptors of macrophages (Erb *et al.*, 1990; El-Moatassim & Dubyak, 1992) and lymphocytes (Wiley *et al.*, 1994), can also induce PI turnover and AA release at concentrations higher than 300 μM in RAW 264.7 cells. Both the AA responses to ATP and BzATP are nonadditive (data not shown), and are resistent to PTX. The latter effect is not the case for UTP and UDP (see below). Moreover, the lower efficacy for ATP-induced PI and AA responses in the presence of high Mg^{2+} compared with that found in the absence of extracellular Mg^{2+}, indicates that the tetrabasic form of ATP (ATP^{4-}) mediates the signalling of ATP. This observation is consistent with the definition of P_{2Z} purinoceptors in mast cells, macrophages, parotid acinar cells and a variety of transformed cells, where P_{2Z} purinoceptors are activated by ATP in its fully dissociated form (ATP^{4-}) and couple to plasma membrane channels for monovalent ions and normally impermeant metabolites. It has been shown that UTP is unable to act on P_{2Z} purinoceptors of macrophages to induce permeabilization and depolarization (Greenberg *et al.*, 1988; El-Moatassim & Dubyak, 1992). Thus, we conclude that different receptors and mechanisms (see below) contribute to the signalling cascades of UTP and ATP in macrophages.

Although selective and potent antagonists for P_2 purinoceptors and pyrimidinoceptors are still lacking, the antagonistic profiles of two putative P_2 purinoceptor antagonists

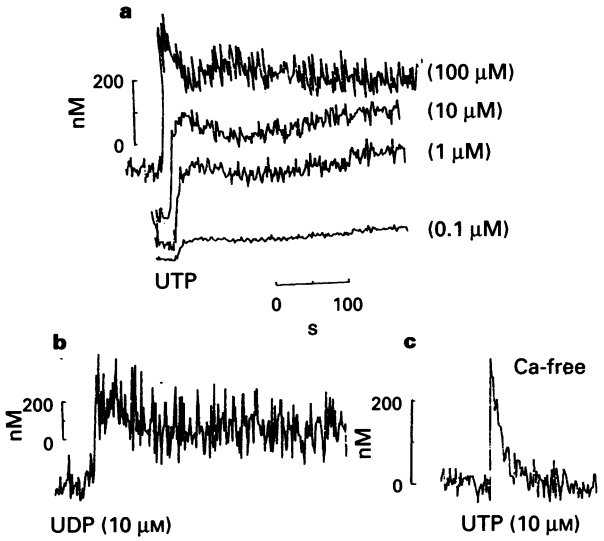

Figure 8 Traces showing the effects of UTP and UDP on $[Ca^{2+}]_i$ in RAW 264.7 cells. Each trace was obtained from cells grown on different coverslides and treated with UTP (a) or UDP (b) at the indicated concentrations. (c) The response to UTP was performed in Ca^{2+}-free PSS.

were used in this study to characterize pyrimidinoceptors. Suramin has been shown to be a competitive antagonist of P_2 purinoceptors, but it does not appear to distinguish between the subtypes (Fredholm *et al.*, 1994). Reactive blue is shown to possess selectivity for P_{2Y} purinoceptors, although nonspecific effects have also been described (Fredholm *et al.*, 1994). In this study, we found that suramin, 10–100 μM, and reactive blue, 1–30 μM, can equipotently inhibit UTP- and ATP-induced PLC activation in a competitive manner, further demonstrating their nonselective effect on pyrimidinoceptors and P_{2Z} purinoceptors.

In this study, the data indicate that the mechanisms responsible for UTP-induced PI turnover and AA release are correlated, although they are not exactly identical. To date, the mechanism involved in the regulation of $cPLA_2$ is incompletely understood. The activation of $cPLA_2$ has been proposed to occur via a number of mechanisms, including $[Ca^{2+}]_i$ increase, protein phosphorylation and G protein activation (Mayer & Marshall, 1993). All these observations led us to investigate the role of Ca^{2+}, PKC activation and G-protein on UTP- and ATP-induced AA release in RAW 264.7 cells.

Increased $[Ca^{2+}]_i$ levels, which result in the translocation of $cPLA_2$ from the cytosol to the membrane (Clark *et al.*, 1991), is generally assumed to be a primary regulator of $cPLA_2$ activity. As indicated by the results observed with the fura-II method, UTP in RAW 264.7 cells induced a significant increase in $[Ca^{2+}]_i$ preceding AA release and the Ca^{2+} response to UTP (0.1–100 μM) was sustained (even within 10 min). When the extracellular Ca^{2+} was removed, only a transient Ca^{2+} peak was observed for UTP, indicating that the transient Ca^{2+} spike results from the release of intracellular Ca^{2+} from its store, and the sustained phase relies on Ca^{2+} influx. The mechanisms involved in UTP-activated sustained Ca^{2+} influx are currently unknown. Under Ca^{2+}-free conditions, UTP- and ATP-induced AA release were completely abolished, consistent with the previous finding that receptor-stimulated PLA_2 activation is coupled to the influx of external Ca^{2+}, and not to the mobilization of intracellular Ca^{2+} from its store (Brooks *et al.*, 1989). Therefore, in RAW 264.7 cells as well as in a variety of other cell types, the influx of extracellular Ca^{2+} is tightly coupled to the agonist-promoted $cPLA_2$ activation.

Regulation of AA release by mitogen-activated protein kinase (MAPK) and PKC has been shown in various cells. With respect to the mechanism, MAPK- and PKC-dependent phosphorylation of $cPLA_2$ appears to act synergistically with Ca^{2+} for full activation (Chahraborti *et al.*, 1992; Lin *et al.*, 1993; Paglin *et al.*, 1993). In RAW 264.7 cells, although short-term pretreatment of cells with PMA inhibited UTP- and UDP-stimulated IP formation, no effect was observed for UTP-induced AA release. The underlying mechanisms responsible for this discrepancy are presently under investigation.

In this study, PTX inhibited UTP-evoked AA release, but not UTP-induced PI hydrolysis, suggesting that PTX-sensitive G protein is involved in the agonist-induced $cPLA_2$ activation. A PTX-sensitive G protein involved in the coupling between P_2 purinoceptors and $cPLA_2$ has been observed in some cell types (Mayer & Marshall, 1993), such as the P_{2U} purinoceptors in neutrophil-like HL-60 granulocytes (Xing & Matter, 1992), P_{2Y} purinoceptors in astrocytes (Bruner & Murphy, 1993), P_2 purinoceptors in endothelial cells (Gerritsen & Mannix, 1990), and airway epithelium (Lazarowski *et al.*, 1994). More direct evidence has shown that in Chinese hamster ovary cells, PTX catalyzed ADP-ribosylation of the Gi2 subunit and mutant Gi2α subunit could inhibit $cPLA_2$-mediated AA release in response to thrombin and ATP, independent of Ca^{2+} mobilization and MAPK regulation (Winitz *et al.*, 1994). At present, the physical direct coupling of $cPLA_2$ with Gi protein has not been demonstrated and the network interaction between the Giα subunit and/or $\beta\gamma$ complexes for regulating $cPLA_2$ activity is unclear. The possible anchoring of $cPLA_2$ to the membrane during its translocation via the $\beta\gamma$ subunits of G-proteins has been suggested (Jelsema & Axelrod, 1987; Kim *et al.*, 1989). Winitz *et al.* (1994) proposed that the Gi2α subunit can regulate an effector other than $cPLA_2$, and that this event is involved in the control of protein kinase networks that regulate $cPLA_2$ activity and AA release. While the nature of the PTX-sensitive pathway remains obscure, it is likely to be dependent on the Ca^{2+} increase in RAW 264.7 cells, because when cells were incubated in Ca^{2+}-free medium, AA release was completely inhibited.

In conclusion, the pyrimidinoceptors with UTP and UDP specificity in RAW 264.7 macrophages are linked to PLC by a PTX-insensitive G protein. The activation of cPLA2 by pyrimidinoceptors is primarily due to the sustained Ca^{2+} influx caused by UTP and UDP, and partially results from the activation of PTX-sensitive G protein. P_{2Z} purinoceptors are responsible for the ATP-induced PLC and $cPLA_2$ activation.

This research was supported by a research grant (DOH83-HR-301) from the National Institute of Health, Center for Cardiovascular Research.

References

BARNARD, E.A., BURNSTOCK, G. & WEBB, T.E. (1994). G Protein-coupled receptors for ATP and other nucleotides: a new receptor family. *Trends Pharmacol. Sci.*, **15**, 67–70.

BROOKS, R.C., MCCARTHY, K.D., LAPETINA, E.G. & MORELL, P. (1989). Receptor-stimulated phospholipase A_2 activation is coupled to influx of external calcium and not to mobilization of intracellular calcium in $C_6$2B glioma cells. *J. Biol. Chem.*, **264**, 20147–20153.

BRUNER, G. & MURPHY, S. (1993). Purinergic P_{2Y} receptor on astrocytes are directly coupled to phospholipase A_2. *Glia*, **7**, 219–224.

CHAHRABORTI, S., MICHAEL, J.R. & SANYAL, T. (1992). Defining the role of protein kinase C in calcium-ionophore-(A23187)-mediated activation of phospholipase A_2 in pulmonary endothelium. *Eur. J. Biochem.*, **206**, 965–972.

CHANNON, J.Y. & LESLIE, C.C. (1990). A calcium-dependent mechanism for associating a soluble arachidonyl-hydrolyzing phospholipase A_2 with membrane in the macrophage cell line RAW 264.7. *J. Biol. Chem.*, **265**, 5409–5413.

CLARK, J.D., LIN, L.-L., KRIZ, R.W., RAMESHA, C.S., SULTZMAN, L.A., LIN, A.Y., MILONA, N. & KNOPF, J.L. (1991). A novel arachidonic acid-selective cytosolic PLA_2 contains a Ca^{2+}-dependent translocation domain with homology to PKC and GAP. *Cell*, **65**, 1043–1051.

COCKCROFT, S. & STUTCHFIELD, J. (1989). The receptors for ATP and fMetLeuPhe are independently coupled to phospholipases C and A_2 via G-protein(s). *Biochem. J.*, **263**, 715–723.

CONNOLLY, G.P. & HARRISON, P.J. (1994). Reactive blue 2 discriminates between responses mediated by UTP and those evoked by ATP or α,β-methylene-ATP on rat sympathetic ganglia. *Eur. J. Pharmacol.*, **259**, 95–99.

COWEN, D.S., LAZARUS, H.M., SHURIN, S.B., STOLL, S.E. & DUBYAK, G.R. (1989). Extracellular adenosine triphosphate activates calcium mobilization in human phagocytic leukocytes and neutrophil/monocyte progenitor cells. *J. Clin. Invest.*, **83**, 1651–1660.

CRACK, B.E., POLLARD, C.E., BEUKERS, M.W., ROBERTS, S.M., HUNT, S.F., INGALL, A.H., MCKECHNIE, K.C.W., IJZERMAN, A.P. & LEFF, P. (1995). Pharmacological and biochemical analysis of FPL 67156, a novel, selective inhibitor of ecto-ATPase. *Br. J. Pharmacol.*, **114**, 475–481.

DUBYAK, G.R., COWEN, D.S. & MUELLER, L.M. (1988). Activation of inositol phospholipid breakdown in HL60 cells by P_2-purinergic receptors: evidence for mediation by both pertussis toxin-sensitive and -insensitive mechanisms. *J. Biol. Chem.*, **263**, 18107–18117.

EL-MOATASSIM, C. & DUBYAK, G.R. (1992). A novel pathway for the activation of phospholipase D by P_{2Z} purinergic receptors in BAC1.2F5 macrophages. *J. Biol. Chem.*, **267**, 23664–23673.

ELSBACH, P. & WEISS, J. (1988). Phagocytosis of bacteria and phospholipid degradation. *Biochim. Biophys. Acta*, **947**, 29–52.

ERB, L., LUSTIG, K.D., AHMED, A.H., GONZALEZ, F.A. & WEISMAN, G.A. (1990). Covalent incorporation of 3′-O-(4-benzoyl)benzoyl-ATP into a P_2 purinoceptor in transformed mouse fibroblasts. *J. Biol. Chem.*, **265**, 7424–7431.

FREDHOLM, B.B., ABBRACCHIO, M.P., BURNSTOCK, G., DALY, J.W., HARDEN, K., JACOBSON, K.A., LEFF, P. & WILLIAMS, M. (1994). Nomenclature and classification of purinoceptors. *Pharmacol. Rev.*, **46**, 143–156.

GERRITSEN, M.E. & MANNIX, R.J. (1990). G-Proteins and phospholipase activation in endothelial cells. *Adv. Exp. Med. Biol.*, **275**, 115–124.

GOETZ, U., DAPRADA, M. & PLETSCHER, A. (1971). Adenine, guanine, and uridine-5′-phosphonucleotides in blood platelets and storage organelles of various species. *J. Pharmacol. Exp. Ther.*, **178**, 210–215.

GREENBERG, S., VIRGILIO, F.D., STEINBERG, T.H. & SILVERSTEIN, S.C. (1988). Extracellular nucleotides mediate Ca^{2+} fluxes in J774 macrophages by two distinct mechanisms. *J. Biol. Chem.*, **263**, 10337–10343.

GRYNKIEWIEZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260**, 3440–3450.

HARDEN, T.K., BOYER, J.L. & NICHOLAS, R.A. (1995). P_2-Purinergic receptors: subtype-associated signaling responses and structure. *Ann. Rev. Pharmacol. Toxicol.*, **35**, 541–579.

HAUSSINGER, D., STEHLE, T. & GEROK, W. (1987). Actions of extracellular UTP and ATP in perfused rat liver. *Eur. J. Pharmacol.*, **167**, 65–71.

JELSEMA, C.L. & AXELROD, J. (1987). Stimulation of phospholipase A_2 activity in bovine rod outer segments by the β,γ subunits of transducin and its inhibition by the α subunit. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 3623–3627.

KIM, D., LEWIS, D.L., GRAZIADEI, L., NEER, E.J., BAR-SAGI, D. & CLAPHAM, D.E. (1989). G-protein $\beta\gamma$-subunits activate the cardiac muscarinic K^+-channel via phospholipase A_2. *Nature*, **337**, 557–560.

LAZAROWSKI, E.R., BOUCHER, R.C. & HARDEN, T.K. (1994). Calcium-dependent release of arachidonic acid in response to purinergic receptor activation in airway epithelium. *Am. J. Physiol.*, **266**, C406–415.

LAZAROWSKI, E.R. & HARDEN, T.K. (1994). Identification of a uridine nucleotide-selective G-protein-linked receptor that activates phospholipase C. *J. Biol. Chem.*, **269**, 11830–11836.

LIN, L.L., WARTMANN, M., LIN, A.Y., KNOPF, J.L., SETH, A. & DAVIS, R.J. (1993). $cPLA_2$ is phosphorylated and activated by MAP kinase. *Cell*, **72**, 269–278.

LIN, W.W. (1994). Heterogeneity of nucleotide receptors in NG 108-15 neuroblastoma and C_6 glioma cells for mediating phosphoinositide turnover. *J. Neurochem.*, **62**, 536–542.

LIN, W.W. & CHUANG, D.-M. (1994). Different signal transduction pathways are coupled to the nucleotide receptor and the P_{2Y} receptor in C_6 glioma cells. *J. Pharmacol. Exp. Ther.*, **269**, 926–931.

LUSITG, K.D., SHIAU, A.K., BRAKE, A.J. & JULIUS, D. (1993). Expression cloning of an ATP receptor from mouse neuroblastoma cells. *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 5113–5117.

MAYER, R.J. & MARSHALL, L.A. (1993). New insights on mammalian phospholipase A_2(s): comparison of arachidonyl-selective and -nonselective enzymes. *FASEB J.*, **7**, 339–348.

O'CONNOR, S.E., DAINTY, I.A. & LEFF, P. (1991). Further subclassification of ATP receptors based on agonist studies. *Trends Pharmacol. Sci.*, **12**, 137–141.

PAGLIN, S., ROY, R. & POLGAR, P. (1993). Characterization of hormonally regulated and particulate-associated phospholipase A_2 from bovine endothelial cells. *J. Biol. Chem.*, **268**, 11697–11702.

PARR, C.E., SULLIVAN, D.M., PARADISO, A.M., LAZAROWSKI, E.R., BURCH, L.H., OLSEN, J.C., ERB, L., WEISMAN, G.A., BOUCHER, R.C. & TURNER, J.T. (1994). Cloning and expression of a human P_{2U} nucleotide receptor, a target for cystic fibrosis pharmacotherapy. *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 3275–3279.

PFEILSCHIFTER, J., THURING, B. & FESTA, F. (1989). Extracellular ATP stimulates poly- (inositol phospholipid) hydrolysis and eicosanoid synthesis in mouse peritoneal macrophages in culture. *Eur. J. Biochem.*, **186**, 509–513.

RASCHKE, W.C., BAIRD, S., NAKOINZ, I. & RALPH, P. (1978). Functional macrophage cell lines transformed by Ableson leukemia virus. *Cell*, **15**, 261–267.

REISER, G. (1995). Ca^{2+}- and nitric oxide-dependent stimulation of cyclic GMP synthesis in neuronal cell line induced by P_2-purinergic/pyrimidinergic receptor. *J. Neurochem.*, **64**, 61–68.

SEIFERT, R. & SCHULTZ, G. (1989). Involvement of pyrimidinoceptors in the regulation of cell functions by urine and by uracil nucleotides. *Trends Pharmacol. Sci.*, **10**, 365–369.

VON KUGELGEN, I., HAUSSINGER, D. & STARKE, K. (1987). Evidence for a vasoconstriction-mediating receptor for UTP, distinct from the P_2-purinoceptor, in rabbit ear artery. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **336**, 556–560.

VON KUGELGEN, I. & STARKE, K. (1990). Evidence for two separate vasoconstriction-mediating nucleotide receptors, both distinct from the P_{2X}-receptor, in rabbit basilar artery: a receptor for pyrimidine nucleotides and a receptor for purine nucleotides. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **341**, 538–546.

WILEY, J.S., CHEN, J.R., SNOOK, M.B. & JAMIESON, G.P. (1994). The P_{2Z}-purinoceptor of human lymphocytes: actions of nucleotide agonists and irreversible inhibition by oxidized ATP *Br. J. Pharmacol.*, **112**, 946 – 950.

WINITZ, S., GUPTA, S.K., QIAN, N.-X., HEASLEY, L.E., NEMENOFF, R.A. & JOHNSON, G.L. (1994). Expression of a mutant Gi2α subunit inhibits ATP and thrombin stimulation of cytoplasmic phospholipase A_2-mediated arachidonic acid release independent of Ca^{2+} and mitogen-activated protein kinase regulation. *J. Biol. Chem.*, **269**, 1889–1895.

XING, M. & MATTER, R. (1992). Phosphorylation-dependent regulation of phospholipase A_2 by G-proteins and Ca^{2+} in HL60 granulocytes. *J. Biol. Chem.*, **267**, 25966–25975.

(Received November 20, 1995
Revised April 30, 1996
Accepted June 13, 1996)