The receptors for ATP and fMetLeuPhe are independently coupled to phospholipases C and A₂ via G-protein(s)

Relationship between phospholipase C and A₂ activation and exocytosis in HL60 cells and human neutrophils

Shamshad COCKCROFT and Jane STUTCHFIELD

Department of Experimental Pathology, School of Medicine, University College London, University Street, London WC1E 6JJ, U.K.

The relationship between phospholipase A₂ and C activation and secretion was investigated in intact human neutrophils and differentiated HL60 cells. Activation by either ATP or fMetLeuPhe leads to [³H]arachidonic acid release into the external medium from prelabelled cells. This response was inhibited when the cells were pretreated with pertussis toxin. When the [³H]arachidonic acid-labelled cells were stimulated with fMetLeuPhe, ATP or Ca²⁺ ionophore A23187, and the lipids analysed by t.l.c., the increase in free fatty acid was accompanied by decreases in label from phosphatidylinositol and phosphatidylcholine. Moreover, incorporation of label into triacylglycerol and to a lesser extent phosphatidylethanolamine was evident. Activation of secretion was evident with ATP and fMetLeuPhe but not with A23187. The pharmacological specificity of the ATP receptor in HL60 cells was investigated by measuring secretion of β -glucuronidase, formation of inositol phosphatases and release of [³H]arachidonic acid. External addition of ATP, UTP, ITP, adenosine 5'-[γ -thio]triphosphate (ATP[S]), adenosine 5'-[β -imido]triphosphate (App[NH]p), XTP, CTP, GTP, 8-bromo-ATP and guanosine 5'-[γ -thio]triphosphate (GTP[S]) to intact HL60 cells stimulated inositol phosphate production, but only the first five nucleotides were effective at stimulating secretion or [³H]arachidonic acid release. In human neutrophils, addition of ATP, ITP, UTP and ATP[S] also stimulated secretion from specific and azurophilic granules, and this was accompanied by increases in cytosolic Ca²⁺ and in [³H]arachidonic acid release. The addition of phorbol 12-myristate 13-acetate (PMA; 1 nM) prior to the addition of either fMetLeuPhe or ATP led to inhibition of phospholipase C activity. In contrast, this had no effect on phospholipase A₂ activation, whilst secretion was potentiated. Phospholipase A₂ activation by either agonist was dependent on an intact cell metabolism, as was secretion. It is concluded that (1) activation of phospholipase C does not always lead to activation of phospholipase A₂, (2) phospholipase A₂ is coupled to the receptor independently of phospholipase C via a pertussis-toxin-sensitive G-protein and (3) for secretion to take place, the receptor has to activate both phospholipases C and A₂.

INTRODUCTION

The presence of a receptor for ATP has recently been described in human neutrophils and in HL60 cells. This receptor is coupled to the phosphatidylinositol bisphosphate-specific phospholipase C via a pertussis toxin-sensitive guanine-nucleotide-binding regulatory protein, G_p [1,2]. Occupation of the receptor by ATP stimulates an increase in cytosolic Ca²⁺ which is similar in magnitude to that produced by fMetLeuPhe [2]. However, the extent of exocytotic secretion caused by fMetLeuPhe is greater than that observed with ATP [2] suggesting that other signalling pathways may be initiated that modulate the extent of secretion.

The suggestion for a second signalling pathway controlling exocytosis also comes from studies in permeabilized cell preparations including neutrophils and HL60 cells [3–5]. Here it has been demonstrated that guanine

nucleotides can promote secretion by a mechanism not involving phospholipase C activation. This effect of guanine nucleotides is attributed to the involvement of a putative G-protein called G_e, whose affinity for guanine nucleotides is regulated by Ca²⁺- and protein kinase C-mediated phosphorylation [4–6].

fMetLeuPhe, apart from activating phospholipase C, also stimulates both phospholipase A₂ [7–9] and phospholipase D activity [10–12]. Phospholipase A₂ activation, in many cell types including neutrophils, has been suggested to be regulated by a distinct G-protein termed G_s [13–17]. In contrast, there are many suggestions that activation of phospholipase A₂ is a consequence of phospholipase C activation (i.e. the intracellular messengers Ca²⁺ and diacylglycerol are responsible for triggering phospholipase A₂ activity). The main evidence to support this is the observation that Ca²⁺ ionophores and phorbol esters, individually or in combination, can activate phospho-

Abbreviations used: G-protein, guanine-nucleotide-binding regulatory protein; PMA, phorbol 12-myristate 13-acetate; PI, phosphatidylinositol; PC, phosphatidylcholine; GPI, glycerophosphoinositol; PE, phosphatidylethanolamine; GTP[S], guanosine 5'-[γ -thio]triphosphate; ATP[S], adenosine 5'-[γ -thio]triphosphate; Gpp[NH]p, guanosine 5'-[β -imido]triphosphate; App[NH]p, adenosine 5'-[β -imido]triphosphate; Gpp[CH₂]p, guanosine 5'-[β -methylene]triphosphate; App[CH₂]p, adenosine 5'-[β -methylene]triphosphate.

lipase A₂ [18–22]. In contrast, a synergism between ionophore and PMA was not observed when the rise in cytosolic Ca²⁺ was restricted to 800 nM [23].

In this paper, we have explored the relationship between phospholipase C and A₂ activation and exocytosis from HL60 cells and human neutrophils stimulated either by the ATP receptor or by the fMetLeuPhe receptor. From the pharmacological specificity of the ATP receptor in stimulating phospholipase C and A₂ activation and secretion, a correlation between the ability of a given nucleotide to stimulate secretion and phospholipase A₂ activation, but not phospholipase C activation, was observed. Moreover, phospholipase C activation stimulated by the ATP or fMetLeuPhe receptors could be inhibited by PMA without causing inhibition of phospholipase A₂ activity or secretion. We therefore conclude that activation of phospholipase A₂ by the receptor occurs independently of phospholipase C activation, and for secretion to take place, the receptor has to activate both phospholipase C and A₂.

MATERIALS AND METHODS

Materials

[5,6,8,9,11,12,14,15-³H]Arachidonic acid (201.5 Ci/mmol) was obtained from Amersham. All nucleotides were obtained from Boehringer-Mannheim, except XTP which was obtained from Sigma. The purity of all the nucleotides was assessed by h.p.l.c. as described previously [24]. All nucleotides were at least 98 % pure, except ATP[S] and GTP[S] which were only 70 % pure, the major contaminants being ADP and GDP respectively. The sources of all other materials were as described previously [2,5,25].

Preparation of human neutrophils and HL60 cells

Human neutrophils were prepared as described previously [10]. HL60 cells were grown in suspension culture and differentiated with dibutyryl cyclic AMP as described previously [5,25].

Measurement of [³H]arachidonate release and secretion

Cells (2–5 ml; 10⁷ cells·ml⁻¹) were prelabelled with 1 μCi of [³H]arachidonic acid·ml⁻¹ for 1 h in a buffered salt solution (pH 7.2) comprising 20 mM-Hepes, 137 mM-NaCl, 2.7 mM-KCl, 1 mM-MgCl₂, 1 mM-CaCl₂, 1 mg of fatty acid-free bovine albumin·ml⁻¹ and 5.6 mM-glucose. The cells were washed three times with buffer and incubated for a further 20 min. After addition of cytochalasin B (5 μg·ml⁻¹ final), aliquots of cells (100 μl) were transferred to tubes containing an equal volume of nucleotides (or as indicated) and the tubes were incubated for 10 min at 37 °C. At the end of the incubation, the cells were quenched with 0.5 ml of ice-cold 0.9 % NaCl and the cells were sedimented by centrifugation at 600 g. The supernatant was monitored for release of labelled arachidonate (and its metabolites) and secretion. For human neutrophils both β-glucuronidase (marker for azurophilic granules) and lysozyme (marker for specific granules) were measured, whereas for HL60 cells only β-glucuronidase was assayed. Enzyme assays were done as described previously [5,26]. Pretreatment with pertussis toxin (500 ng·ml⁻¹) was carried out for 2 h at 37 °C where indicated.

Lipid analysis

[³H]Arachidonate-labelled cells, stimulated with fMetLeuPhe, ATP or ionophore A23187, were quenched with chloroform/methanol (1:2, v/v) and the lipids extracted into the organic phase by further addition of water and chloroform as described previously [27]. Lipid samples were run in parallel on t.l.c. plates to separate the neutral lipids and phospholipids. Neutral lipids were separated in a solvent consisting of hexane/diethyl ether/acetic acid (80:20:1, by vol.) and phospholipids were separated in a solvent consisting of chloroform/methanol/acetic acid/water (75:45:3:1, by vol.) as described previously [27]. For localization, carrier lipids were added or autoradiographed. The spots containing the individual lipids were excised and counted by scintillation counting.

Metabolic inhibition of cells

In those experiments where the cells were metabolically inhibited, glucose was omitted from the buffer and the cells were pretreated with metabolic inhibitors (5 μM-antimycin A plus 6 mM-deoxyglucose) for 5 min.

Measurement of glycerophosphoinositol and inositol phosphates

Inositol phosphates were measured (in the presence of 10 mM-LiCl) in cells prelabelled with [³H]inositol as described previously [4,25]. In brief, 100 μl of cells was added to tubes containing an equal volume of buffer supplemented with the indicated agonists and incubated at 37 °C. At the end of the 10 min incubation, the cells were sedimented by centrifugation at 600 g at 4 °C and the supernatant was sampled for β-glucuronidase secretion. The cells were then quenched with chloroform/methanol and the aqueous phase separated from the organic phase by further addition of chloroform and water. The aqueous phase was loaded on to Dowex columns and the inositol-labelled metabolites were eluted sequentially. After washing the columns with water to remove free inositol, the columns were then eluted with 5 mM-sodium tetraborate and 60 mM-sodium formate to obtain glycerophosphoinositol (GPI). Total inositol phosphates were then eluted with 1 M-ammonium formate. The identity of GPI was confirmed by h.p.l.c. on a Partisil SAX column using a linear gradient of 0–1.7 M-ammonium formate. Over 90 % of the increase in the total inositol phosphates fraction consisted of an increase in the inositol monophosphates (inositol 1-phosphate and inositol 4-phosphate).

Measurement of cytosol Ca²⁺

Human neutrophils (5 × 10⁷ cells·ml⁻¹) were loaded at room temperature with fura 2-AM (10 μM). After 20 min the suspension was diluted 5-fold and left to recover for a further 40 min at room temperature. After centrifugation, the cells were resuspended at 10⁷ cells·ml⁻¹ and kept at room temperature until use. Fluorescence was measured using a Perkin-Elmer LS5 fluorimeter with excitation at 340 nm and emission at 510 nm. The cuvette was continuously stirred and maintained at 37 °C during measurements. Calibration of the fluorescence signals was carried out as described previously [28].

Expression of data

All determinations were carried out in duplicate and

experiments were repeated on at least three occasions. Secretion was routinely monitored in the same cell preparation used for the measurements of [³H]arachidonate release or [³H]inositol phosphate formation. Release of [³H]arachidonate is expressed as recovered c.p.m. in the supernatant (less the zero time value) as a percentage of the total c.p.m. incorporated in the cell (except in the experiment presented in Fig. 2 where the zero time value has not been deducted). Typical levels of incorporated labelled arachidonate in the cells used per incubation ranged from 100 000–500 000 c.p.m. Formation of inositol phosphates is expressed as c.p.m. obtained in the total inositol phosphates as a percentage of the total c.p.m. incorporated into the inositol-containing lipids.

RESULTS

Release of labelled arachidonate stimulated by ATP or fMetLeuPhe

Addition of external ATP or fMetLeuPhe to [³H]arachidonate-prelabelled HL60 cells or human neutrophils leads to a concentration-dependent release of [³H]arachidonate into the extracellular medium (Figs. 1*a–d*). To ensure the release of arachidonate into the extracellular medium, the buffer contained fatty acid-free albumin which acts as a trap. The magnitude of the response in both cell types was similar with either ATP or fMetLeuPhe, although the maximal response observed with ATP was much less than that observed with fMetLeuPhe. When the cells were pretreated with pertussis toxin for 2 h, the release of arachidonate by both agonists was inhibited (Figs. 1*a–d*).

The method of measuring [³H]arachidonate released into the external medium was compared with extraction of the released fatty acid with an organic solvent and separation by t.l.c. Figs. 2(*a*) and 2(*b*) illustrate the results from a parallel experiment where either (1) incub-

ations were terminated by addition of ice-cold saline and samples used for measurement of released [³H]arachidonate in the medium (and also secretion), or (2) samples were quenched with chloroform/methanol and the lipids extracted and analysed by t.l.c. The radioactivity obtained in the medium was similar to the radioactivity recovered in the position of free fatty acid by t.l.c., confirming that measurement of released [³H]arachidonate in the medium is an adequate method. In all subsequent experiments, release into the medium was used as the method.

In order to identify the source of [³H]arachidonate release, the radioactivity in the individual lipids before and after stimulation with ATP, fMetLeuPhe or the Ca²⁺ ionophore A23187 was determined in HL60 cells (Fig. 2). A decrease in the label in phosphatidylcholine (PC) and phosphatidylinositol (PI) was observed with all stimuli. This decrease was greater than the increase in free fatty acid. The remainder of the decrease could be accounted for by an increase in label in triacylglycerol and to a minor extent, phosphatidylethanolamine (PE) and diacylglycerol. Thus the major source of [³H]arachidonate is PI and PC, and the measurement of released [³H]arachidonate is an underestimate of the changes since a proportion of the mobilized [³H]arachidonate is re-incorporated mainly into triacylglycerol. Compared with ATP, fMetLeuPhe-induced changes were greater, as was the increase in secretion (Fig. 2*h*). However, A23187, although a good activator for [³H]arachidonate release, is a poor activator of enzyme secretion.

To establish that the route of arachidonate release from PI was via phospholipase A₂, the formation of GPI was monitored. GPI is derived from lyso-PI, the immediate product of the action of phospholipase A₂ on PI. GPI was measured in HL60 cells prelabelled with [³H]inositol. The concentration of labelled GPI in unstimulated cells is 4–5 times higher than the level of inositol monophosphates in unstimulated cells (see Figs.

Fig. 1. Arachidonate release stimulated by ATP or fMetLeuPhe in neutrophils and HL60 cells: effect of pertussis toxin pretreatment

Cells were labelled with [³H]arachidonic acid for 1 h, washed and incubated with the indicated concentrations of either ATP (*a, b*) or fMetLeuPhe (*c, d*). HL60 cells (*a, c*) and human neutrophils (*b, d*) were used. ●, Control cells; ○, cells pretreated with pertussis toxin.

Fig. 2. Changes in the lipid profile after stimulation with ATP, fMetLeuPhe or A23187

[³H]Arachidonate-labelled HL60 cells were incubated with ATP (1 mM), fMetLeuPhe (1 μ M) or A23187 (1 μ M) for 10 min. Either the cells were extracted with chloroform/methanol for lipid analysis by t.l.c. (b–g) or the extracellular medium was recovered after centrifugation and analysed for secretion (h) and released [³H]arachidonate (a) in the external medium. The actual c.p.m. that was present in free fatty acid measured either as released into the medium (a) or by t.l.c. (b) are indicated above the bars. The total c.p.m. applied to t.l.c. was $42\,300 \pm 1300$ ($n = 16$). Abbreviations: AA, arachidonic acid; FFA, free fatty acid; DG, diacylglycerol; TG, triacylglycerol.

5a and 5b). On stimulation with 1 μ M-fMetLeuPhe, an increase in GPI by 80–90 % occurs concurrently with the increase in inositol phosphates (see Figs. 5a and 5b). A 20–30 % increase was also observed with 1 mM-ATP (results not shown).

Specificity of the ATP receptor

We have examined the specificity of the ATP receptor for stimulation of inositol phosphate formation (Fig. 3a), β -glucuronidase secretion (Fig. 3b) and arachidonate release from HL60 cells (Fig. 3c). ATP, UTP, ITP, ATP[S], App[NH]p, XTP, CTP, GTP, 8-bromo-ATP and GTP[S] all stimulate inositol phosphate production in a concentration-dependent manner (Fig. 3a). The same agonists were also effective in stimulating a rise in cytosolic Ca^{2+} (results not shown). Of these agonists, only ATP, UTP, ITP, ATP[S] and App[NH]p were effective in stimulating secretion and arachidonate release. Other compounds that were tested (highest concentration tested was 100 μ M) and that gave no stimu-

lation of secretion, inositol phosphate production or [³H]arachidonate release, were adenosine, AMP, ADP, App[CH₂]p, Gpp[NH]p and Gpp[CH₂]p, PP_i, PPP_i and PPPP_i.

Fig. 4 compares the effects of ATP, ITP, UTP, ATP[S] and fMetLeuPhe on human neutrophils in stimulating (1) secretion from specific (lysozyme as marker) and azurophil (β -glucuronidase as marker) granules, (2) arachidonate release and (3) a rise in cytosol Ca^{2+} . Whilst the relative potency of all the agonists in stimulating secretion (from either granule type) and arachidonate release is similar, it does not correspond with the ability of the individual agonist to stimulate a rise in cytosolic Ca^{2+} . For example, ATP[S], which is a good agonist for secretion and release of [³H]arachidonate, results in a slightly smaller rise in cytosolic Ca^{2+} than does ITP, and yet ITP is a poor agonist for secretion or [³H]arachidonate release. XTP, CTP, GTP, 8-bromo-ATP and GTP[S] were unable to stimulate secretion or [³H]arachidonate release although they were effective at mobilizing intracellular Ca^{2+} (results not shown).

Fig. 3. Effect of different nucleotides on (a) inositol phosphate production, (b) β -glucuronidase secretion and (c) arachidonate release from HL60 cells

HL60 cells were incubated with the indicated nucleotides for 10 min. For the determination of inositol phosphates, the cells were prelabelled with [3 H]inositol, and for arachidonate release the cells were prelabelled with [3 H]arachidonate. The bars which represent incubations in the presence of nucleotides are: open bars, 1 μ M; hatched bars, 10 μ M; filled bars, 100 μ M.

Effect of PMA

Both the ATP receptor and the fMetLeuPhe receptor can be uncoupled from phospholipase C by PMA, an activator of protein kinase C (Figs. 5a and 6a). HL60 cells were treated with 1 nM-PMA for 5 min and then stimulated with ATP or fMetLeuPhe. Under these conditions, PMA pretreatment does not interfere with the activation of phospholipase C stimulated either directly

by Ca²⁺ or via the G-protein [29]. Thus the inhibition of inositol phosphate production by the agonist is due to the uncoupling of the receptor from the phospholipase C (Figs. 5a and 6a). In contrast, PMA has no inhibitory effect on agonist-induced phospholipase A₂ activity. This was monitored either by release of arachidonate (Figs. 5d and 6b) or by measuring an increase in GPI (Fig. 5b), a by-product of phospholipase A₂-mediated hydrolysis of PI. Secretion was also monitored, and the effect of PMA was to potentiate secretion (Figs. 5c and 6c).

Effect of metabolic inhibition

We have previously demonstrated that if HL60 cells are metabolically inhibited then phospholipase C activation is inhibited by over 95% [5]. This effect of ATP is due to depletion of the substrate, phosphatidylinositol 4,5-bisphosphate, for phospholipase C. The substrates for phospholipase A₂ are PC and PI, which are not dependent on the presence of cellular ATP. It was therefore of interest to see whether phospholipase A₂ activation was still evident after metabolic inhibition. Figs. 7(a)–7(d) illustrate that metabolic inhibition of cells leads to inhibition of stimulation of both secretion and phospholipase A₂ activation by either fMetLeuPhe or ATP.

DISCUSSION

We have recently shown that human neutrophils and HL60 cells possess a receptor for ATP which is coupled to phospholipase C via a pertussis toxin-sensitive G-protein [2]. In this paper, we demonstrate that this receptor can also stimulate phospholipase A₂ activation in HL60 cells and human neutrophils. The requirement for activation of both phospholipases for secretion to occur is also shown.

Regulation of phospholipase A₂

The most efficient way of releasing arachidonate is by direct removal of the fatty acid from the lipid by phospholipase A₂ action. However, it has been suggested that arachidonate release can also occur from diacylglycerol formed by phospholipase C action on inositol lipids [30], although this view has been questioned [31]. From the results presented here, the phospholipase A₂ route is the likely route since phospholipase C activation can be clearly dissociated from arachidonate release (see below). Moreover, an increase in GPI, which is a product of lyso-PI, can only arise by phospholipase A₂ action. The demonstration of phospholipase A₂ in the plasma membrane of neutrophils [32–34] supports this pathway as a major route for arachidonate release.

Since the receptors for ATP and fMetLeuPhe stimulate both phospholipases, it is important to establish whether activation of phospholipase A₂ is dependent on a prior activation of phospholipase C. The results presented here clearly support the view that activation of the two phospholipases by the receptor are two independent events. The evidence can be summarized as follows. (1) Stimulation of phospholipase C (e.g. by XTP or GTP) is not accompanied by activation of phospholipase A₂. (2) Phospholipase C activation via the receptor can be uncoupled by PMA, but phospholipase A₂ activation by the receptor is unimpaired. Such a dissociation of phospholipase C activation from phospholipase A₂ activation has been observed in other cell types including

Fig. 4. Comparison of effects of fMetLeuPhe with those of ATP, ITP, UTP and ATP[S] on stimulation of (a) arachidonate release, (b) changes in cytosolic Ca²⁺ and secretion from (c) azurophilic and (d) specific granules in human neutrophils

Human neutrophils were stimulated with nucleotides or fMetLeuPhe and the following parameters were monitored: (a) arachidonate release, (b) intracellular levels of Ca²⁺, (c) β-glucuronidase secretion from azurophilic granules and (d) lysozyme secretion from specific granules. For (a), (c) and (d), the nucleotide concentrations were 1 μM (open bars), 10 μM (hatched bars) and 100 μM (filled bars). For (b), the concentration of nucleotides was at 100 μM and the error bars indicate S.E.M. from four separate observations. The concentration of fMetLeuPhe was 0.1 μM.

Fig. 5. Effect of PMA on phospholipase C and A₂ activation and secretion stimulated by fMetLeuPhe in HL60 cells

HL60 cells were preincubated with 1 nM-PMA for 5 min and transferred into tubes containing the indicated concentrations of fMetLeuPhe. The formation of total inositol phosphates and GPI was measured from cells prelabeled with [³H]inositol. Arachidonate release was monitored from [³H]arachidonate-labelled cells, as was secretion. (a) Inositol phosphates (IPs), (b) GPI, (c) secretion, and (d) arachidonate (AA) release were measured. ○, Incubations in the absence of PMA; ●, incubations in the presence of 1 nM-PMA.

FRTL-5 cells [13], MDCK cells [35,36] and Swiss 3T3 cells [14], supporting the view that the receptor can activate the two phospholipases independently.

The observation that phospholipase A₂ activation is susceptible to inhibition by pertussis toxin argues in favour of the phospholipase A₂ being regulated by the receptor via a G-protein. Metabolic inhibition also leads to inhibition of phospholipase A₂ activation (Figs. 7a and 7c) and phospholipase C activation (results not shown). Whilst the inhibition of phospholipase C is expected due to substrate depletion, such arguments do not apply to phospholipase A₂ activation. The inhibition of phospholipase A₂ could be attributed to GTP depletion, since metabolic inhibition will lead to a decrease not only in ATP levels but also in GTP levels. In addition, we have observed that addition of GTP[S] to permeabilized HL60 cells leads to an activation of phospholipase A₂ (S. Cockcroft & J. Stutchfield, unpublished work), supporting the notion that phospholipase A₂ activity is regulated by a G-protein.

In principle, it could be argued that one G-protein could be responsible for stimulating both phospholipases. Thus the α -subunit would be responsible for

Fig. 6. Effect of PMA on phospholipase C and A₂ activation and secretion stimulated by ATP in HL60 cells

HL60 cells were preincubated with 1 nM-PMA for 5 min and transferred into tubes containing the indicated concentrations of ATP. Formation of total inositol phosphates was monitored in cells prelabelled with $[^3H]$ inositol and arachidonate release was monitored in $[^3H]$ arachidonate-labelled cells as was secretion. (a) Inositol phosphates (IPs), (b) arachidonate (AA) release and (c) secretion. ○, Incubations in the absence of PMA; ●, incubations in the presence of 1 nM-PMA.

Fig. 7. Effect of metabolic inhibition on arachidonate release and secretion stimulated by fMetLeuPhe or ATP from HL60 cells

HL60 cells were treated with antimycin A in the presence of deoxyglucose for 5 min and then transferred to tubes containing fMetLeuPhe (a, b) or ATP (c, d). Arachidonate (AA) release (a, c) and secretion (b, d) were monitored in control cells (●) and in metabolically inhibited cells (○).

phospholipase C activation and the $\beta\gamma$ -subunit would be responsible for phospholipase A_2 activation, since it has been demonstrated in experiments *in vitro* that $\beta\gamma$ units can stimulate phospholipase A_2 [37,38]. However, the possibility that distinct G-proteins may be involved in activating phospholipases C and A_2 cannot be totally ruled out, because pertussis toxin pretreatment can have differential effects on phospholipase C and A_2 activation depending on cell type.

G-protein-mediated regulation of phospholipase A_2 has been suggested in FRTL-5 cells [13], Swiss 3T3 fibroblasts [14], rod outer segments [16], neutrophils [9,15], mast cells [17] and *Aplysia* sensory neurons [39], and the putative G-protein has been designated G_a [13]. In *Aplysia* sensory neurons, the agonist known to stimulate phospholipase A_2 is known not to stimulate phospholipase C, adding further credence to the idea that phospholipase A_2 is controlled by a separate G-protein. Unlike G_p , which is a substrate for pertussis toxin in some cell types only [40,41], G_a appears to be a substrate for pertussis toxin in all cell types that have been investigated. These include neutrophils [8,9], HL60 cells (this paper), FRTL-5 cells [13], *Aplysia* sensory neurons [39], rod outer segments [16], mast cells [42] and Swiss 3T3 fibroblasts [14,43].

Relationship between secretion and phospholipase A_2 activation

We have reported that apart from ATP, only UTP, ITP, ATP[S] and App[NH]p could stimulate secretion [2]. We have extended those studies to include other nucleotides, and by monitoring other stimulated events such as inositol phosphate production, a rise in cytosolic Ca^{2+} and phospholipase A_2 activation, a very interesting correlation emerges.

In HL60 cells, of the ten nucleotides that are active in stimulating phospholipase C, only five stimulate secretion. This implies that activation of an additional signalling system must also occur for secretion to take place. Phospholipase A_2 could be the other signalling system since it shows the right characteristics for a transmembrane signalling system, i.e. an enzyme activity coupled to the receptor via a G-protein as discussed above, thus providing the amplification that a signalling system needs. Figs. 3(b) and 3(c) illustrate the relationship between secretion and phospholipase A_2 activation, and it is clear that only those nucleotides that elicit secretion stimulate phospholipase A_2 activation. The rank order (at 100 μM) for stimulation of secretion is $UTP > ATP > ITP > ATP[S] > App[NH]p$, but for stimulation of phospholipase A_2 it is $UTP > ATP = ITP = App[NH]p > ATP[S]$. This anomaly may be due to other intracellular events that are necessary for secretion that are regulated by the receptor (e.g. phospholipase D activation). We have noted that the ability of ATP, UTP, ITP and ATP[S] to stimulate secretion appears to correlate with their ability to stimulate inositol phosphate production at a concentration of 1 μM (see Fig. 3b). This suggests that only those agonists that have a high affinity for the receptor are able to stimulate phospholipase A_2 . However, App[NH]p does not follow this pattern.

In human neutrophils, a similar relationship between secretion (from either specific or azurophilic granules) and phospholipase A_2 is evident. The relative ability of the agonist for stimulation of secretion and phospho-

lipase A_2 activity shows a good correspondence, but this is not the case for the increase in cytosolic Ca^{2+} . We have previously shown that ATP[S] is only slightly less potent than fMetLeuPhe for secretion from azurophilic granules (β -glucuronidase as enzyme marker) (see also Fig. 4c), but it is better than fMetLeuPhe in stimulating secretion from specific granules (Fig. 4d). However, this is not too surprising since secretion from these two granule types do show differential requirements for exocytosis [26]. Nonetheless, ATP[S] is distinctly worse than ATP, ITP or UTP in raising cytosolic Ca^{2+} , and yet it is far more potent in stimulating secretion compared with the other nucleotides.

Thus it is clear that there is a strong relationship between secretion and phospholipase A_2 activation, but not phospholipase C activation. However, secretion is always accompanied by phospholipase C activation. There is one exception and that is in the presence of PMA. Although PMA does inhibit phospholipase C (without affecting phospholipase A_2), secretion is potentiated. Since PMA can substitute for one of the products of phospholipase C, this exception does not invalidate the statement that phospholipase C activation is necessary (but not sufficient) for secretion. The ability of PMA to potentiate secretion may reflect its ability to enhance the affinity for Ca^{2+} and guanine nucleotides for G_e [4–6].

Although the relationship between secretion and phospholipase A_2 when a receptor-directed agonist is used is strong, it should be pointed out that although A23187 is a good activator of phospholipase A_2 (Fig. 2) and phospholipase C [18], it is nonetheless a poor activator of secretion. Clearly this would argue that activation of the secretory mechanism may involve further activation sequences that are not switched on by A23187, or that the uncontrolled rise in cytosolic Ca^{2+} is somehow inhibitory. The inability of A23187 to stimulate secretion also indicates that phospholipase A_2 activation is not a consequence of secretion.

From studies in which the requirements for secretion have been studied in permeabilized cells, we have identified a requirement for a putative G-protein, termed G_e [3–5]. Whether G_e and G_a represent the same entity cannot be ruled out, and this question can only be addressed by experiments that can distinguish between the requirements for phospholipase A_2 activation and secretion by guanine nucleotides.

In conclusion, we have demonstrated that HL60 cells and neutrophils possess a receptor for ATP that is coupled via a pertussis toxin-sensitive G-protein(s) to phospholipases A_2 (this paper) and C [2]. The observed pharmacological specificity is different from any ATP receptor so far characterized and cannot be simply classified into any of the purinergic receptor categories that have been proposed [44,45]. Indeed, the observation that UTP (a pyrimidine) is more potent than ATP (a purine) in HL60 cells (see Fig. 3a) does not permit the categorization of this receptor as a purinergic receptor at all. It is clear that activation of the inositol signalling pathway is not a sufficient mechanism to control secretion and the activation of the phospholipase A_2 pathway appears to be an additional component essential for secretion. This model of activation where a receptor activates two phospholipases is unlikely to be confined to secretory systems only but is likely to apply to other cellular responses such as proliferation or phototransduction.

This work was financed by grants from the Medical Research Council and the Lister Institute. S.C. is a Lister Institute Fellow.

REFERENCES

- Dubyak, G. R., Cowen, D. S. & Mueller, L. M. (1988) *J. Biol. Chem.* **263**, 18108–18117
- Cockcroft, S. & Stutchfield, J. (1989) *FEBS Lett.* **245**, 25–29
- Barrowman, M. M., Cockcroft, S. & Gomperts, B. D. (1986) *Nature (London)* **319**, 504–507
- Cockcroft, S., Howell, T. W. & Gomperts, B. D. (1987) *J. Cell Biol.* **105**, 2745–2750
- Stutchfield, J. & Cockcroft, S. (1988) *Biochem. J.* **250**, 375–382
- Howell, T. W., Cockcroft, S. & Gomperts, B. D. (1987) *J. Cell Biol.* **105**, 191–197
- Hirata, F., Corcoran, B. A., Venkatasubramanian, K., Schiffmann, E. & Axelrod, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 2640–2643
- Okajima, F. & Ui, M. (1984) *J. Biol. Chem.* **259**, 13863–13871
- Bokoch, G. M. & Gilman, A. G. (1984) *Cell* **39**, 301–308
- Cockcroft, S. (1984) *Biochim. Biophys. Acta* **795**, 37–46
- Cockcroft, S. (1986) *Recept. Biochem. Methodol.* **7**, 287–310
- Pai, J., Siegel, M. I., Egan, R. W. & Billah, M. M. (1988) *J. Biol. Chem.* **263**, 12472–12477
- Burch, R. M., Luini, A. & Axelrod, J. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 7201–7205
- Burch, R. M. & Axelrod, J. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6374–6378
- Nakashima, S., Nagata, K.-I., Ueda, K. & Nozawa, Y. (1988) *Arch. Biochem. Biophys.* **261**, 375–383
- Jelsema, C. L. (1987) *J. Biol. Chem.* **262**, 163–168
- Okano, Y., Yamada, K., Yano, K. & Nozawa, Y. (1987) *Biochem. Biophys. Res. Commun.* **145**, 1267–1275
- Meade, C. J., Turner, G. A. & Bateman, P. E. (1986) *Biochem. J.* **238**, 425–436
- Volpi, M., Molski, T. F. P., Naccache, P. H., Feinstein, M. B. & Sha'afi, R. I. (1985) *Biochem. Biophys. Res. Commun.* **128**, 594–600
- Parker, J., Daniel, L. W. & Waite, M. (1987) *J. Biol. Chem.* **262**, 5385–5393
- Ho, A. K. & Klein, D. C. (1987) *J. Biol. Chem.* **262**, 11764–11770
- Touqui, L., Rothut, B., Shaw, A. M., Fradin, A., Vargaftig, B. & Russo-Marie, F. (1986) *Nature (London)* **321**, 177–180
- Pollock, W. K., Rink, T. J. & Irvine, R. F. (1986) *Biochem. J.* **235**, 869–877
- Cockcroft, S. & Gomperts, B. D. (1985) *Nature (London)* **314**, 534–536
- Cockcroft, S. & Stutchfield, J. (1988) *Biochem. J.* **256**, 343–350
- Barrowman, M. M., Cockcroft, S. & Gomperts, B. D. (1987) *J. Physiol. (London)* **383**, 115–124
- Allan, D. & Cockcroft, S. (1982) *J. Lipid Res.* **23**, 1373–1374
- MacIntyre, E., Tatham, P., Abdul-Gaffar, R. & Linch, D. (1988) *Immunology* **64**, 427–432
- Geny, B., Stutchfield, J. & Cockcroft, S. (1989) *Cell. Signalling* **1**, 165–172
- Bell, R. L., Kennerly, D. A., Stanford, N. & Majerus, P. W. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3238–3241
- Mahadevappa, V. G. & Holub, B. J. (1986) *Biochem. Biophys. Res. Commun.* **134**, 1327–1333
- Victor, M., Weiss, J., Klempner, M. S. & Elsbach, P. (1981) *FEBS Lett.* **136**, 298–300
- Matsumoto, T., Tao, W. & Sha'afi, R. I. (1988) *Biochem. J.* **250**, 343–348
- Balsinde, J., Diez, E., Schuller, A. & Mollinedo, F. (1988) *J. Biol. Chem.* **263**, 1929–1936
- Slivka, S. R. & Insel, P. A. (1988) *J. Biol. Chem.* **263**, 14640–14647
- Slivka, S. R. & Insel, P. A. (1987) *J. Biol. Chem.* **262**, 4200–4207
- Jelsema, C. L. & Axelrod, J. (1987) *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) *Nature (London)* **337**, 557–560
- Volterra, A. & Siegelbaum, S. A. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7810–7814
- Cockcroft, S. (1987) *Trends Biochem. Sci.* **12**, 75–78
- Cockcroft, S. & Stutchfield, J. (1988) *Philos. Trans. R. Soc. London B* **320**, 247–265
- Nakamura, T. & Ui, M. (1985) *J. Biol. Chem.* **260**, 3584–3593
- Murayama, T. & Ui, M. (1985) *J. Biol. Chem.* **260**, 7226–7233
- Burnstock, G. & Brown, G. M. (1981) in *Purinergic Receptors* (Burnstock, G., ed.), vol. B12, pp. 1–45, Chapman and Hall, London
- Satchell, D. (1984) *Trends Pharmacol. Sci.* **5**, 340–343

Received 28 March 1989/7 June 1989; accepted 14 June 1989