Correlation between secretion and phospholipase D activation in differentiated HL60 cells

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Receptor-directed agonists including *N*-formylmethionyl-leucyl-phenylalanine (fMetLeuPhe), C5a, ATP and UTP all activate phospholipase D (PLD), which is accompanied by secretion in differentiated HL60 cells. Interference in the production of phosphatidase (PA) by the PLD pathway by diverting it towards the production of phosphatidylethanol (PEt) in the presence of ethanol leads to near-total inhibition of the secretion evoked by ATP and UTP and a partial inhibition of that evoked by fMetLeuPhe and C5a. In streptolysin-O-permeabilized cells, fMetLeuPhe is able to activate PLD, and this is dependent on the presence of a low concentration of guanosine 5'-[γ -thio]-triphosphate (GTP[S]). Ca^{2+} (10 μM) and GTP[S] individually or in combination are also able to activate PLD and secretion. The

stimulation of secretion in permeabilized cells stimulated by Ca^{2+} alone or fMetLeuPhe or GTP[S] is also abrogated when the production of PA is diverted to PEt by the presence of ethanol. Activation of PLD by GTP[S] or fMetLeuPhe is decreased if the cells are permeabilized first and GTP[S] or fMetLeuPhe is added subsequently. This corresponds well with the loss of the secretory response. We conclude that the ability of GTP[S] or fMetLeuPhe to stimulate secretion from permeabilized cells is dependent on a prior activation of the PLD signalling pathway. PA, generated as a consequence of PLD activation, acts as second messenger that can provide an initiating signal for secretion and is not required for exocytosis itself.

INTRODUCTION

Occupation of receptors on neutrophils and HL60 cells leads to the generation of multiple intracellular messenger molecules, e.g. Ca^{2+} , diacylglycerol (DG), phosphatidic acid (PA) and arachidonic acid, which control the functional response of the cell. Many of these intracellular messengers are formed predominantly from the action of three phospholipases, A_2 , C and D, acting on membrane phospholipids (for review, see [1]). In neutrophils and differentiated HL60 cells, there are several receptors that belong to the seven transmembrane-spanning segment family that couple to heterotrimeric G-proteins. These include receptors for *N*-formylmethionyl-leucylphenylalanine (fMetLeuPhe), ATP, platelet-activating factor (PAF), leukotriene B_4 , C5a and interleukin-8. It is widely accepted that phospholipase C (PLC) is linked to some receptors via G-proteins [2,3]. More recently, mechanisms for similar G-protein-linked regulation of phospholipase A_2 and phospholipase D (PLD) have also been proposed (for review, see [1]). The main evidence indicating that PLD regulation in neutrophils and HL60 cells may be via a G-protein rests on the observation that guanosine 5'-[γ -thio]-triphosphate (GTP[S]) can stimulate PLD activity in permeabilized cells as well as in cell-free systems [4–8].

One of the functional responses of the neutrophil and the HL60 cell is secretion; this has been examined in great detail in permeabilized cell preparations [9–15]. Secretion from neutrophils and HL60 cells has been demonstrated to involve at least two G-proteins [9,14]. The first G-protein implicated is the one which regulates PLC, leading to the generation of the second messengers inositol trisphosphate (involved in Ca^{2+} mobilization) and DG (activator of protein kinase C). Support for the existence of a second G-protein regulating secretion has come from many other cell types, including eosinophils [16], mast cells [17] and

platelets [18] (see [19] for review). Evidence for the involvement of a second G-protein in HL60 cells and neutrophils includes the following observations [9,14,15]: (1) secretion can be stimulated by the non-hydrolysable analogue, GTP[S], under conditions where PLC is only marginally activated; (2) Ca^{2+} and protein kinase C activation (by phorbol esters) do not mimic the extent of secretion induced by Ca^{2+} and GTP[S]; (3) exposure to a low concentration of phorbol ester inhibits PLC, but enhances secretion stimulated by GTP[S], in particular the Ca^{2+} -independent component [14,20].

The identity of the second G-protein and the catalytic activity that it may regulate have not yet been defined. So far it has been possible to eliminate phospholipase A_2 as the catalytic activity regulated by this G-protein [15,21,22]. The aim of this study was to investigate the relationship between secretion and PLD activation in intact and streptolysin-O-permeabilized HL60 cells differentiated with dibutyryl cyclic AMP. We demonstrate that PLD activation is part of the transmembrane signalling process coupled to the fMetLeuPhe receptor via a G-protein. In addition, PLD activation contributes to the activation of the secretory process in conjunction with other second messengers.

MATERIALS AND METHODS

Materials

Culture media and supplements were purchased from Flow Laboratories, guanine nucleotides and BSA were from Boehringer Mannheim, and streptolysin O was from Wellcome Diagnostics, [^3H]Alkyl-lyso-PC (92 Ci/mmol) was purchased from Amersham. All other reagents, including cytochalasin B, fMetLeuPhe, C5a, PA, peanut PLD and phosphatidylcholine (PC), were purchased from Sigma. Phosphatidylethanol (PEt) was obtained from Avanti.

Abbreviations used: PLD, phospholipase D; PLC, phospholipase C; DG, diacylglycerol; PEt, phosphatidylethanol; PA, phosphatidic acid; PC, phosphatidylcholine; fMetLeuPhe, *N*-formylmethionyl-leucylphenylalanine; GTP[S], guanosine 5'-[γ -thio]triphosphate.

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Preparation of PA and PEt

PC was incubated with peanut PLD in the presence of ethanol (2%) to make a mixture of PA and PEt. PC (4 mg) was incubated with 1000 units of PLD in 1 ml of acetate buffer (0.2 M, pH 5.6) containing Ca^{2+} (10 mM) in the presence of ether (400 μl) overnight at room temperature. At the end of the incubation, the ether was evaporated and the lipids were extracted with 3.75 ml of chloroform/methanol (1:2, v/v), followed by addition of 1.25 ml of chloroform and 1.25 ml of water. After phase separation, the chloroform phase was recovered. The sample was run on t.l.c. and two spots corresponding to PA and PEt were observed. Authentic PA and PEt were run in parallel for confirmation.

Culture and labelling of HL60 cells

Culturing and dibutyl-AMP-induced differentiation of HL60 cells were carried out as previously described [14,23]. Before labelling with [^3H]alkyl-lyso-PC, HL60 cells were washed and resuspended at 5×10^7 cells/ml in Buffer A, comprising 20 mM Hepes, 137 mM NaCl, 2.7 mM KCl, 1 mM CaCl_2 , 1 mM MgCl_2 , 5.6 mM glucose and 1 mg/ml fatty-acid-free BSA (pH 7.2). Cells were labelled with 5 $\mu\text{Ci}/\text{ml}$ [^3H]alkyl-lyso-PC for 30 min at 37 °C as described previously [24].

Determination of PLD activation in intact HL60 cells

The labelled cells were washed twice and resuspended in Buffer A and equilibrated for 30 min at 37 °C. Cytochalasin B was added to the cells (5 μM final concn.) and 100 μl samples were transferred to tubes containing the agonist with or without ethanol (2% final concn.) as indicated. The cells were incubated for 10 min at 37 °C, transferred to ice, and centrifuged at 4 °C for 5 min at 600 *g*. The supernatant was assayed for enzyme release (50 μl) and the remainder of the incubation was quenched with chloroform/methanol (1:2, v/v) followed by vortex-mixing. A mixture of unlabelled PA and PEt was added at this stage for localization of the reaction products by t.l.c. Phase separation was achieved by sequential addition of 2 M KCl and chloroform.

The chloroform phase was collected, dried under vacuum and then redissolved in 50 μl of chloroform. Samples were spotted on Whatman LK6TLC silica plates previously impregnated with 1% potassium oxalate solution, dried at room temperature overnight and activated for 1 h at 110 °C. The plates were developed in chloroform/methanol/acetic acid/water (75:45:3:1, by vol.), dried at room temperature, and the lipid spots were revealed with iodine vapour. R_f values for the lipids were: sphingomyelin, 0.08; PC, 0.135; PA, 0.445; phosphatidylethanolamine, 0.49; PEt, 0.67. The spots corresponding to PA and PEt standards were excised after iodine sublimation at room temperature. The silica was put into scintillation vials, and the lipids were extracted with 250 μl of methanol and counted for radioactivity after addition of scintillation liquid.

Experiments with permeabilized HL60 cells

For experiments where the cells were to be permeabilized, the labelled cells were washed and resuspended in the permeabilization buffer (20 mM Pipes, 137 mM NaCl, 2.7 mM KCl, 5.6 mM glucose and 1 mg/ml fatty-acid-free BSA; pH 6.8) and equilibrated for 30 min. Portions (100 μl) of cells were then transferred to tubes containing 100 μl of reaction mixture containing streptolysin O (final concn. 0.4 i.u./ml), a range of Ca^{2+} concentrations buffered with 3 mM EGTA, ethanol (2%),

MgATP (1 mM), GTP[S] and fMetLeuPhe as indicated. After the incubation period of 10 min (unless stated otherwise), the tubes were centrifuged at 800 *g* for 5 min at 4 °C. A 50 μl portion of the supernatant was removed for measurement of secretion, and the remainder of the cells were processed as described for the intact cells.

In experiments where the effect of MgATP or fMetLeuPhe was monitored, glucose was omitted from the permeabilization buffer, and after the equilibration period the cells were metabolically inhibited with 5.6 mM 2-deoxyglucose and 5 μM antimycin A for a further 5 min before being added to the tubes containing the reaction mixture. Under these conditions, the metabolically inhibited cells are unresponsive to external stimulation [15].

Measurement of secretion

Secretion of β -glucuronidase was measured as previously described [14]. In some experiments, secretion of hexosaminidase was measured by incubating at 37 °C a 50 μl portion of the incubation supernatant with an equal volume of 1 M 4-methylumbelliferyl *N*-acetyl- β -D-glucosaminide in 0.2 M citrate (pH 4.5), a fluorescent substrate for hexosaminidase, in a black multi-welled dish for 1 h. The reaction was then quenched with 1 M Tris and the resulting fluorescence measured with a Titertek Fluoroskan II fluorescence plate-reader. The secretion of β -glucuronidase and hexosaminidase, both from the same granules in the HL60 cells, followed an identical pattern.

Presentation of results

Assays were done either in duplicate or triplicate. Each set of experiments were performed at least twice under identical conditions, and most observations were verified on 3–4 occasions with slight modifications in the protocol. The data shown are from representative experiments.

RESULTS

Receptor-mediated stimulation of secretion and PLD activation in HL60 cells

Incubation of intact differentiated HL60 cells with fMetLeuPhe shows concentration-dependent stimulation of secretion (Figure 1a) and PLD activation (Figure 1b). In order to measure PLD activation, the cells were prelabelled with [^3H]alkyl-lyso-PC, which is converted into PC by the cell. Over 90% of the lipid-soluble radioactivity was associated with PC (results not shown). Activation of PLD generates PA (results not shown), but, when cells are stimulated in the presence of 2% ethanol, PEt is formed at the expense of PA. The amount of labelled PA formed (in the absence of ethanol) is less than PEt in the presence of ethanol, due to the labile nature of PA. PA is converted into DG in a time-dependent manner by the enzyme PA phosphohydrolase. PEt, on the other hand, is a more stable product. In the presence of ethanol, secretion stimulated by fMetLeuPhe is inhibited, but by only 50% (Figure 1a). The time course of secretion and PEt formation was also monitored; both responses reached a peak maximally at approx. 30 s and remained at that level for 10 min (results not shown).

Stimulation of secretion by activation of the C5a or purinergic receptors is also accompanied by activation of PLD (Table 1). To investigate the relationship between secretion and PA production, ethanol was used to divert the PA to PEt. In the presence of ethanol, secretion was inhibited nearly completely when ATP, UTP or adenosine 5'-[γ -thio]triphosphate (ATP[S]) was used as agonist, and only partially when fMetLeuPhe or C5a was used

Figure 1 Effect of ethanol on secretion and PEt formation in fMetLeuPhe-stimulated HL60 cells

Intact HL60 cells were treated with cytochalasin B and then stimulated with increasing concentration of fMetLeuPhe in the absence or presence of 2% ethanol; $\circ$, control cells; $\bullet$, 2% ethanol. (a) Secretion and (b) PEt were measured in the same cell preparation. Values are averages of two determinations which were within 5% of the mean.

Table 1 Relationship between PLD activation and secretion by different agonists

Intact differentiated HL60 cells were stimulated with the agonist in the absence (—) or presence (+) of 2% ethanol (EtOH) and secretion of β -glucuronidase was measured. PEt formation was also assessed in those cells that were treated with ethanol. Cells were treated with cytochalasin B (5 $\mu\text{g/ml}$ final concn.) before activation with an agonist. Radioactivity in PEt and secretion were measured in the same cell preparation. Abbreviation: ATP[S], adenosine 5'-[γ -thio]-triphosphate.

Agonist	PEt (d.p.m.)	Secretion (%)	
		— EtOH	+ EtOH
Control	346	2	2
fMetLeuPhe (1 μM)	2453	45	24
C5a (10 nM)	1850	32	19
UTP (100 μM)	834	22	4
ATP (100 μM)	944	8	2
ATP[S] (100 μM)	760	4	2

Table 2 PLD activation stimulated by GTP[S] at two concentrations of Ca^{2+} in the absence or presence of MgATP

HL60 cells were metabolically inhibited and then permeabilized with streptolysin O in the absence or presence of MgATP (1 mM) and GTP[S] (50 μM) as indicated. Ca^{2+} was present at either pCa 7 or pCa 5 as indicated. Ethanol (2%) was present in all the incubations. The formation of PEt and PA was monitored as indicators of PLD activation.

	PEt (d.p.m.)		PA (d.p.m.)	
	Control	+ GTP[S]	Control	+ GTP[S]
pCa 7	131	1089	372	581
pCa 5	1099	6793	548	1499
pCa 7 + MgATP	106	2747	291	1460
pCa 5 + MgATP	2947	15785	2104	7571

Table 3 Effect of cytochalasin B and dGDP on GTP[S] stimulated PLD and secretion from permeabilized HL60 cells

Differentiated HL60 cells were permeabilized with streptolysin O in the presence of pCa 7 or pCa 5, MgATP (1 mM) and 2% ethanol in the presence or absence of dGDP (1 mM) or cytochalasin B (cyto B; 5 μM) for 1 min. GTP[S] (40 μM) was then added and the cells were incubated for 10 min. Radioactivity in PEt and secretion were measured in the same cell preparation.

	PEt (d.p.m.)	Secretion (%)
pCa 7	420	4
pCa 5	1722	6
pCa 5 + GTP[S] (40 μM)	15455	45
pCa 5 + dGDP (1 mM)	946	8
pCa 5 + GTP[S] (40 μM) + dGDP (1 mM)	1743	14
pCa 5 + cyto B	1694	6
pCa 5 + GTP[S] (40 μM) + cyto B	15505	44

as agonist (Table 1). The extent of PLD activation varies with the agonist. (The maximal concentration of agonist was used for this comparison.) The degree of secretion does not correspond to the extent of PEt formation by the different agonists. For example, the degree of secretion elicited by UTP is half that with fMetLeuPhe, and yet the amount of PEt formed is only one-third of the response elicited by fMetLeuPhe. In addition, although the extent of PLD activation by the various purinergic-receptor-directed agonists is similar, the degree of secretion with UTP is more extensive.

To investigate the mechanism of activation of PLD, HL60 cells were permeabilized with streptolysin O to clamp Ca^{2+} levels at known values and to control phosphorylation by manipulating MgATP levels. Here we show that the activation of PLD in differentiated cells can be stimulated by GTP[S], and the effect is enhanced by MgATP (Table 2). Table 2 illustrates the production of PEt and PA, determined in the presence of 2% ethanol. Stimulation with GTP[S] can be observed at pCa 7 and is further enhanced at pCa 5 alone, the extent of PLD activation is similar to that stimulated by GTP[S] at pCa 7. The extent of PLD activation stimulated by GTP[S] at pCa 7 is similar in magnitude to the response observed in intact cells stimulated with fMetLeuPhe. Since GTP[S] is unable to stimulate PLC or phospholipase A_2 in the absence of MgATP, this excludes any indirect effects of these two phospholipases in PLD activation. It also excludes protein phosphorylation as essential for regulating PLD activity. Since MgATP enhances the responses to GTP[S]

Figure 2 fMetLeuPhe enhances the rate of GTP[S]-stimulated PLD activation

Intact HL60 cells were metabolically inhibited before transfer to tubes containing streptolysin O, ethanol (2%) and Ca^{2+} buffered at pCa 7. The concentration of GTP[S] used was 1 μM and that of fMetLeuPhe (FMLP) was 1 μM .

and to Ca^{2+} present alone or in combination, this may reflect the involvement of phosphorylation. GTP[S] is able to stimulate PLC (and hence protein kinase C activation) as well as tyrosine phosphorylation in the presence of MgATP.

GTP[S]-mediated activation of PLD can be inhibited by dGDP (Table 3). In addition, it was also observed that dGDP inhibited PLD activation by Ca^{2+} alone.

The mechanism of fMetLeuPhe-stimulated PLD activation was also studied in permeabilized cells. Figure 2 shows that fMetLeuPhe increases the rate of PLD activation stimulated by GTP[S]. In permeabilized cells, the effect of fMetLeuPhe is only observed in the presence of GTP[S]. The extent of PLD activation by fMetLeuPhe in permeabilized cells is similar in magnitude to the response observed in intact cells (compare data in Table 1 and Figure 3). The experiment presented in Table 3 was carried out at pCa 7, the resting level of Ca^{2+} present in most cells, and in the absence of MgATP. The observation that fMetLeuPhe can activate PLD only in the presence of GTP[S] supports the notion that PLD is directly coupled to the fMetLeuPhe receptor via a G-protein.

Cytochalasin B treatment is not required for secretion or PLD activation in permeabilized cells

PLD activation and secretion are greatly potentiated by the presence of cytochalasin B in intact cells [25–27]. In contrast with intact cells, cytochalasin B is not required for secretion or PLD activation in permeabilized cells (Table 3).

Effects of ethanol on exocytosis from permeabilized cells

When Ca^{2+} , buffered between pCa 8 and pCa 5, is introduced into permeabilized cells, some stimulation of secretion occurs at pCa 5. This Ca^{2+} -dependent secretion is enhanced in the presence of fMetLeuPhe (Figure 3). Ethanol was found to inhibit both the secretion induced by Ca^{2+} alone or that induced by fMetLeuPhe. In contrast with intact cells, ethanol inhibits fMetLeuPhe-induced secretion from permeabilized cells completely (compare Figure 1a with Figure 3).

Secretion can also be elicited from permeabilized HL60 cells by GTP[S]. Three separate components of secretion can be distinguished depending on the absence or presence of MgATP (Figures 4a and 4b):

Figure 3 Ethanol inhibits fMetLeuPhe-stimulated secretion in permeabilized HL60 cells

Intact HL60 cells were metabolically inhibited before transfer to tubes containing streptolysin O, Ca^{2+} buffered at the indicated concentrations with 3 mM EGTA, and 1 mM MgATP. Ethanol (2%) was present where indicated. The concentration of fMetLeuPhe (FMLP) was 1 μM .

Figure 4 Ethanol inhibits Ca^{2+} -dependent secretion, GTP[S]-dependent secretion (in the absence of MgATP) and Ca^{2+} -independent secretion stimulated by GTP[S]

Intact HL60 cells were metabolically inhibited. Secretion was stimulated in the absence or presence of GTP[S] and MgATP as a function of Ca^{2+} in (a) the absence or (b) the presence of ethanol.

- (1) Ca^{2+} -dependent secretion, which requires MgATP and Ca^{2+} at pCa 5;
- (2) GTP[S]-dependent secretion (at pCa 8, referred to as Ca^{2+} -independent secretion);

Figure 5 Secretion from permeabilized cells is dependent on the concentration of GTP[S] with or without MgATP: effect of ethanol

Permeabilized cells were stimulated in the presence of pCa 5, with or without MgATP (1 mM), with or without ethanol (2%) and indicated concentrations of GTP[S].

Table 4 Comparison of GTP[S], XTP- and ITP-mediated stimulation of PLD and secretion from permeabilized HL60 cells

Differentiated HL60 cells were permeabilized with streptolysin O in the presence of 10 μ M Ca^{2+} , MgATP (1 mM) and 2% ethanol in the presence of the indicated concentrations of GTP[S], XTP or ITP, and the cells were incubated for 10 min. Radioactivity in PEt and secretion were measured in the same cell preparation.

		PEt (d.p.m.)	Secretion (%)
pCa 5		2059	5.5
GTP[S]	1 μ M	7549	25
	10 μ M	15434	43
	100 μ M	24457	47
XTP	0.1 mM	3253	6
	1 mM	4195	17.5
	10 mM	5967	48.5
ITP	0.1 mM	4166	8
	1 mM	4635	18.5
	10 mM	5184	45

(3) secretion evoked by GTP[S] plus Ca^{2+} (pCa 5), which does not require MgATP.

In all these cases, the secretion induced is sub-maximal. The secretion elicited at pCa 5 averaged around 15–20%. Ca^{2+} -independent secretion showed the greatest variability and was about 5–20%. GTP[S]-dependent secretion was approx. 20% and showed the least variability. We have examined the effect of ethanol on all these different components of secretion (Figure 4b). All three components of secretion observed in permeabilized HL60 cells are inhibited nearly completely by the presence of ethanol.

Maximal secretion occurs when Ca^{2+} , GTP[S] and MgATP are present simultaneously (Figure 4a). Maximal secretion ranged from 40 to 60%. With this combination of effectors, the action of ethanol is minimal (Figure 4b). In Figure 5, we examined the effect of ethanol on secretion elicited by different concentrations

Figure 6 Loss of PLD activation as the time between permeabilization and addition of stimulus is increased

HL60 cells were permeabilized for different lengths of time (designated as the pre-permeabilization interval) at pCa 7 in the absence of MgATP or ethanol. The pre-permeabilized cells were stimulated with pCa 7 or pCa 5 alone, pCa 5 + 30 μ M GTP[S] or pCa 5 + 1 μ M fMetLeuPhe (FMLP) for 10 min in the presence of 2% ethanol.

of GTP[S] at pCa 5 in the absence or presence of MgATP. In the absence of MgATP, ethanol inhibits secretion due to pCa 5 alone and that due to GTP[S]. However, when the three effectors of secretion are present at optimal concentration, ethanol has no effect on secretion.

In permeabilized cells, XTP and ITP can stimulate secretion to a similar extent to GTP[S]. In order to understand further the relationship between secretion and PLD activation, we have compared the effects of ITP and XTP with GTP[S] on both parameters. Both ITP and XTP were found to stimulate PEt formation, but to a much smaller extent than that observed with GTP[S] (Table 4). Despite the presence of 2% ethanol, XTP and ITP were still able to stimulate secretion (Table 4). In the presence of 2% ethanol, the amount of PA formed by XTP or ITP was not significant (results not shown).

Role of cytosolic components for PLD activation in permeabilized cells

Recent studies have indicated that activation of PLD by GTP[S] requires the presence of both cytosolic and membrane components [5,6,8]. We have examined the requirement for cytosolic components for Ca^{2+} -, GTP[S]- and fMetLeuPhe-dependent activation of PLD. We exploited the observation that streptolysin O allows the efflux of cytosolic proteins such as lactate dehydrogenase from these cells within 5 min [14]. HL60 cells were pre-permeabilized for different lengths of time with streptolysin O in the presence of pCa 7 and no MgATP. Under these conditions, PLD activation does not occur. The pre-permeabilized cells were then stimulated with pCa 5, pCa 5 plus fMetLeuPhe, or pCa 5 plus GTP[S] for 10 min to assess PLD activation. Figure 6 shows a decline in PLD activation as the pre-permeabilization interval is increased. A parallel decline in secretion is also seen (results not shown). Not only does the response to GTP[S] decline, but so does the response to fMetLeuPhe. These results clearly show that both GTP[S] and fMetLeuPhe are dependent on the presence of cytosolic components for PLD activation and for secretion.

DISCUSSION

Evidence that PLD is regulated by a G-protein

First we review the evidence that supports the view that PLD activation is stimulated via a G-protein. When intact HL60 cells are stimulated by a receptor-directed agonist, the activation of three phospholipases (C, D and A_2) can be demonstrated which cannot be resolved on a temporal basis. Studies in permeabilized cells allow control of the intracellular environment, in particular, concentration of Ca^{2+} and the availability of MgATP (required for phosphorylation reactions).

The most unambiguous observation pointing to G-protein control of PLD activation comes from experiments in streptolysin-O-permeabilized cells, which demonstrate that fMetLeuPhe can only trigger PLD activation provided that a low concentration of GTP[S] is also present. In this experiment, the Ca^{2+} was clamped at pCa 7 and MgATP was excluded. This result indicates that phosphorylation events may not be essential for the fMetLeuPhe receptor to couple to PLD. Other studies have suggested that tyrosine phosphorylation may be important for regulating PLD activation. This was demonstrated with the use of pharmacological inhibitors; it was shown that fMetLeuPhe-stimulated PLD activation was inhibited by inhibitors of tyrosine phosphorylation in intact human neutrophils [28]. Since many 'specific' inhibitors have other cellular effects, these data are open to interpretation.

In the presence of MgATP, GTP[S]-stimulated and pCa 5-stimulated PLD activation is greatly enhanced. Since MgATP supports phosphorylation events, it would imply that phosphorylation can have a profound effect on the activation of the PLD pathway. We can therefore speculate that there are multiple PLD enzymes, and/or that the state of phosphorylation greatly affects the activity of the PLD enzyme.

Relationship between PLD activation and secretion

In studies in cells of haematopoietic origins (e.g. neutrophils and HL60 cells, mast cells, eosinophils), it has been clearly shown that at least two G-proteins participate in the regulation of secretion. One of the G-proteins is identified as the G-protein that regulates the activation of phosphoinositide-specific PLC. This results in an increase in cytosol Ca^{2+} and protein kinase C activation. The second G-protein has been defined as G_{β} , and is considered to operate downstream of Ca^{2+} [9,29]. Since PLD can be regulated by a G-protein and is considerably influenced by Ca^{2+} , we have explored the possibility that the target for G_{β} may be PLD.

Activation of the PLD pathway will generate choline and PA. Of the two products, PA is a possible candidate as a second messenger. The involvement of PA in secretion was analysed by diverting the production of PA to PET by addition of ethanol. Secretion is compromised in both intact and permeabilized cells when stimulated by the receptor-directed agonist. In intact cells, stimulation by fMetLeuPhe or C5a is only partially inhibited by ethanol, indicating that PA production is not an absolute requirement for secretion to take place. Similar results have been found with fMetLeuPhe-, PAF- and LTB_4 -stimulated secretion in rabbit neutrophils [27]. Activation of secretion via purinergic agonists such as UTP and ATP is much more sensitive to ethanol, suggesting that the PLD pathway plays a more prominent role in supporting secretion via the ATP receptor.

fMetLeuPhe is also able to stimulate secretion from permeabilized cells, but, in comparison with intact cells, secretion is more sensitive to the presence of ethanol (compare Figure 1a

with Figure 3). We would propose that in the intact cell fMetLeuPhe triggers the transient production of Ca^{2+} , arachidonate, DG and PA, and these four messengers then control the functional events, i.e. secretion and NADPH oxidase. If PA production is interrupted by the presence of ethanol, arachidonate, Ca^{2+} and DG are still able to allow some downstream events to proceed, implying some redundancy at the level of second messengers. In permeabilized cells, there is a greater level of dependency on PA, since secretion is inhibited to a greater extent.

In permeabilized cells, GTP[S] can by-pass the receptors and directly trigger secretion. In this case several independent components for triggering secretion can be discerned, and all appear to be associated with PLD activation to differing extents. These components will be discussed individually.

Ca^{2+} -dependent secretion

Ca^{2+} alone in the presence of MgATP can stimulate secretion and activate PLD. Although Ca^{2+} -dependent secretion is sensitive to the presence of ethanol, it is not the only determining factor. We have previously shown that undifferentiated HL60 cells do not secrete in response to Ca^{2+} alone [14], and yet Ca^{2+} at pCa 5 will stimulate the PLD pathway [8]. Clearly, the expression of Ca^{2+} -dependent secretion is dependent on additional factors that are induced when the cells are differentiated. It should be stressed that differentiated HL60 cells are more similar to human neutrophils than are the undifferentiated cells. Both human neutrophils and differentiated HL60 cells secrete in the presence of micromolar levels of Ca^{2+} [15].

Ca^{2+} -independent secretion stimulated with GTP[S]

This component of secretion is small and does show greater experimental variation. This component of secretion is accompanied by PLD activation and is inhibited by ethanol.

Ca^{2+} + GTP[S]-dependent secretion in the absence of MgATP

Under these conditions, PLD activation appears to be essential for secretion to occur, since secretion is very sensitive to the presence of ethanol.

These three components of secretion are each clearly sensitive to the presence of ethanol. However, secretion is barely affected when it is stimulated by Ca^{2+} + GTP[S] in the presence of MgATP (Figures 4 and 6). This would imply that PA is not essential for secretion when all three effectors are present at maximal levels. However, under maximal stimulation, a substantial amount of PA is formed, despite the presence of ethanol (see Table 2), which may be sufficient to support secretion. Therefore, despite the lack of effect of ethanol on secretion when triggered by Ca^{2+} + GTP[S] in the presence of MgATP, this does not disprove the hypothesis that PLD activation is required for secretion.

The conditional inhibition of secretion by ethanol argues strongly for a role for PA as one of the second messengers, rather than an intrinsic requirement for PA in the secretory process itself. This conclusion is strengthened by two other observations: (1) ethanol inhibits receptor-stimulated NADPH oxidase in intact cells, and it has been suggested that PA functions as a second messenger for the activation of the NADPH oxidase in neutrophils [30–33]; (2) XTP and ITP are good activators of secretion, but are poor activators of PLD activation.

What is the second-messenger role of PA? In principle, since PA can be converted into DG, it is conceivable that the main role of the PLD pathway is provision of DG via PA phosphohydrolase

for stimulation of protein kinase C. There are several reasons why it could be argued that it is PA rather than DG which is important. First, there is a good temporal relationship between the increase in PA and secretion [34–36]. The rise in PA and secretion reach a peak at 20–30 s, whereas the increase in DG occurs over a period of 2–5 min [35,37]. Secondly, secretion can be stimulated in permeabilized cells by GTP[S] in the absence of MgATP, which is accompanied by PLD activation. Ethanol inhibits secretion under these conditions very effectively. This indicates that the role of PA is not related to protein phosphorylation, either directly [38] or via protein kinase C. In the absence of MgATP, the number of biochemical events that can occur is limited, and one possible candidate is that PA may promote dephosphorylation of a regulatory protein by activating a protein phosphatase.

In conclusion, PA should be considered as one of many second messengers formed during receptor-mediated activation of neutrophils. Its production is also regulated by a G-protein, similarly to the production of inositol trisphosphate and DG. These second messengers may participate in further recruiting G-proteins downstream to stimulate secretion or NADPH oxidase. In the case of the NADPH oxidase, it is clear that the G-proteins recruited downstream are *rac1* and *rac2* [39,40]. By analogy with the oxidase system, small GTP-binding proteins may also be involved in secretion. A considerable body of evidence has accumulated indicating that small GTP-binding proteins of the *rab* family participate in vesicle fusion [41–44]. More specifically, exocytotic fusion is activated by Rab3a peptides in mast cells [45], pancreatic acinar cells [46] and adrenal chromaffin cells [47].

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REFERENCES

- Cockcroft, S. (1992) *Biochim. Biophys. Acta* **1113**, 135–160
- Cockcroft, S. and Thomas, G. M. H. (1992) *Biochem. J.* **288**, 1–14
- Rhee, S. G. and Choi, K. D. (1992) *J. Biol. Chem.* **267**, 12393–12396
- Tettenborn, C. S. and Mueller, G. C. (1988) *Biochem. Biophys. Res. Commun.* **155**, 249–255
- Anthes, J. C., Wang, P., Siegel, M. I., Egan, R. W. and Billah, M. M. (1991) *Biochem. Biophys. Res. Commun.* **175**, 236–243
- Olson, S. C., Bowman, E. P. and Lambeth, J. D. (1991) *J. Biol. Chem.* **266**, 17236–17242
- Xie, M. and Dubyak, G. R. (1991) *Biochem. J.* **278**, 81–89
- Geny, B. and Cockcroft, S. (1992) *Biochem. J.* **284**, 531–538
- Barrowman, M. M., Cockcroft, S. and Gomperts, B. D. (1986) *Nature (London)* **319**, 504–507
- Barrowman, M. M., Cockcroft, S. and Gomperts, B. D. (1987) *J. Physiol. (London)* **383**, 115–124
- Smolen, J. E. and Stoehr, S. J. (1986) *Biochim. Biophys. Acta* **889**, 171–178
- Smolen, J. E., Stoehr, S. J. and Boxer, L. A. (1986) *Biochim. Biophys. Acta* **886**, 1–17
- Smolen, J. E., Stoehr, S. J., Traynor, A. E. and Sklar, L. A. (1987) *J. Leukocyte Biol.* **41**, 8–13
- Stutchfield, J. and Cockcroft, S. (1988) *Biochem. J.* **250**, 375–382
- Cockcroft, S. (1991) *Biochem. J.* **275**, 127–131
- Cromwell, O., Bennett, J. P., Kay, A. B., Hide, I. and Gomperts, B. D. (1991) *J. Immunol.* **147**, 1905–1911
- Cockcroft, S., Howell, T. W. and Gomperts, B. D. (1987) *J. Cell Biol.* **105**, 2745–2750
- Wheeler-Jones, C. D. P., Saermark, T., Kakkar, V. V. and Authi, K. S. (1992) *Biochem. J.* **281**, 465–472
- Gomperts, B. D. (1990) *Annu. Rev. Physiol.* **52**, 591–606
- Geny, B., Stutchfield, J. and Cockcroft, S. (1988) *Cell. Signalling* **1**, 165–172
- Morgan, A. and Burgoyne, R. D. (1990) *Biochem. J.* **269**, 521–526
- Churcher, Y., Allan, D. and Gomperts, B. D. (1990) *Biochem. J.* **266**, 157–163
- Stutchfield, J. and Cockcroft, S. (1991) *Eur. J. Biochem.* **197**, 119–125
- Pai, J.-K., Siegel, M. I., Egan, R. W. and Billah, M. M. (1988) *Biochem. Biophys. Res. Commun.* **150**, 355–364
- Mullman, T. J., Siegel, M. I., Egan, R. W. and Billah, M. M. (1990) *J. Immunol.* **144**, 1901–1908
- Xie, M., Jacobs, L. S. and Dubyak, G. R. (1991) *J. Clin. Invest.* **88**, 45–54
- Kanaho, Y., Kanoh, H., Saitoh, K. and Nozawa, Y. (1991) *J. Immunol.* **146**, 3536–3541
- Uings, I. J., Thompson, N. T., Randall, R. W., Spacey, G. D., Bonser, R. W., Hudson, A. T. and Garland, L. G. (1992) *Biochem. J.* **281**, 597–600
- Lillie, T. H. W. and Gomperts, B. D. (1992) *Philos. Trans. R. Soc. London B* **336**, 25–34
- Yuli, I., Tomonaga, A. and Snyderman, R. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 5906–5910
- Bonser, R. W., Thompson, N. T., Randall, R. W. and Garland, L. G. (1989) *Biochem. J.* **264**, 617–620
- Rossi, F., Grzeskowiak, M., Della Bianca, V., Calzetti, F. and Gandini, G. (1990) *Biochem. Biophys. Res. Commun.* **168**, 320–327
- Agwu, D. E., McPhail, L. C., Sozzani, S., Bass, D. A. and McCall, C. E. (1991) *J. Clin. Invest.* **88**, 531–539
- Cockcroft, S. (1984) *Biochim. Biophys. Acta* **795**, 37–46
- Cockcroft, S. and Allan, D. (1984) *Biochem. J.* **222**, 557–559
- Billah, M. M., Eckel, S., Mullman, T. J., Egan, R. W. and Siegel, M. I. (1989) *J. Biol. Chem.* **264**, 17069–17077
- Preiss, J. E., Bell, R. M. and Neidel, J. E. (1987) *J. Immunol.* **138**, 1542–1545
- Bocckino, S. B., Wilson, P. B. and Exton, J. H. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 6210–6213
- Abo, A., Pick, E., Hall, A., Totty, N., Teahan, C. G. and Segal, A. W. (1991) *Nature (London)* **353**, 668–670
- Knaus, U. G., Heyworth, P. G., Evans, T., Curnutte, J. T. and Bokoch, G. M. (1991) *Science* **254**, 1512–1515
- Salminen, A. and Novick, P. J. (1987) *Cell* **49**, 527–538
- Segev, N., Mulholland, J. and Botstein, D. (1988) *Cell* **52**, 915–924
- Goud, B., Salminen, A., Walworth, N. C. and Novick, P. J. (1988) *Cell* **53**, 753–768
- Zahraoui, A., Touchot, N., Chardin, P. and Tavitan, A. (1989) *J. Biol. Chem.* **264**, 12394–12401
- Oberhauser, A. F., Monck, J. R., Balch, W. E. and Fernandez, J. M. (1992) *Nature (London)* **360**, 270–273
- Padfield, P. J., Balch, W. E. and Jamieson, J. D. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 1656–1660
- Senyshyn, J., Balch, W. E. and Holz, R. W. (1992) *FEBS Lett.* **309**, 41–46