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Relationship between arachidonate release and exocytosis in permeabilized human neutrophils stimulated with formylmethionyl-leucyl-phenylalanine (fMetLeuPhe), guanosine 5′-[γ-thio]triphosphate (GTP[S]) and Ca^{2+}

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The role of two G-proteins, G_p and G_e, in the stimulus–secretion pathway has been proposed on the basis of studies where GTP analogues have been introduced into permeabilized cell preparations. In this study, evidence is provided that two G-proteins are also involved when a receptor-directed agonist is used. Intact human neutrophils were made refractory to formylmethionyl-leucyl-phenylalanine (fMetLeuPhe) stimulation by metabolic inhibition and then permeabilized with streptolysin O to compare the intracellular requirements for exocytosis from specific and azurophilic granules and arachidonate release. In the presence of 1 μM-Ca^{2+} and 1 mM-MgATP, fMetLeuPhe or guanosine 5′-[γ-thio]triphosphate (GTP[S]) induce secretion from both granule types as well as arachidonate release. Secretion and arachidonate release owing to fMetLeuPhe can occur in the absence of ATP, conditions under which G-protein-mediated activation of phospholipase C is suppressed. GTP[S]-induced secretion can also occur in the absence of MgATP, but GTP[S]-induced arachidonate release cannot. It is concluded that fMetLeuPhe, like GTP[S], stimulates secretion by interacting with another G-protein-mediated reaction apart from G_p. Evidence is provided that a possible target for the second G-protein-mediated reaction involved in fMetLeuPhe-induced secretion (but not GTP[S]-induced secretion) is phospholipase A_2.

INTRODUCTION

The use of permeabilized cell systems has permitted the identification of intracellular effectors that trigger and modulate the secretory process [1–13]. In particular, the role of Ca^{2+} and guanine nucleotides as effectors, as well as the modulatory role for ATP in sustaining exocytosis, have been identified. From studies with permeabilized neutrophils [8], HL60 cells [12], mast cells [10,11]. RINm5F cells [13] and adrenal medulla [3,14], the involvement of two GTP-binding proteins in the stimulus-secretion pathway has been suggested: G_p, the G-protein that couples to the inositol-lipid-specific phospholipase C is involved in the early transduction of receptor-mediated events, and a second G-protein, G_e, whose target is not known. Where the role of MgATP has been investigated, it has been observed that there is not always an obligatory requirement for ATP in the terminal steps of the secretory pathway. However, ATP does modulate the secretory process, (1) by enhancing the effective affinity for both of the effectors, Ca^{2+} and guanine nucleotides [10–12], and (2) by increasing the absolute amount of secretion [12], probably by means of phosphorylation reactions mediated by protein kinase C [11,15].

Intact neutrophils respond to extracellular ATP and fMetLeuPhe by stimulating secretion from two granule types, specific and azurophilic granules [16,17]. One of the early reactions stimulated by fMetLeuPhe or ATP is the activation of phospholipase C via a pertussis-toxin-sensitive G-protein, G_p. An obligatory reaction that also appears to be required for exocytosis to take place from intact neutrophils by receptor-mediated stimulation is the activation of phospholipase A_2 [17]. Phospholipase A_2 activation has been shown to be regulated by a pertussis-toxin-sensitive G-protein in many cell types, including neutrophils [17–19], FRTL-5 cells [20], Swiss 3T3 fibroblasts [21], rod outer segments [22], mast cells and its related cell line, RBL cells [23–25], and *Aplysia* sensory neurons [26]. The question thus arises whether the activation of phospholipase A_2 is an essential step in the secretory pathway possibly mediated by G_e, or whether it is another receptor-mediated pathway for the generation of either intra- or extra-cellular signals.

Although Ca^{2+}- and guanine nucleotide-induced exocytosis have been studied in neutrophils and HL60 cells [4,7,8,12], no studies have yet investigated whether receptor-mediated exocytosis can be retained in permeabilized neutrophil preparations. In the experiments reported here we have used human neutrophils permeabilized with streptolysin O to investigate the intracellular requirements for secretion and arachidonate release induced by either a receptor-directed agonist, fMetLeuPhe or GTP[S]. It is shown that both secretion and arachidonate release can be stimulated by fMetLeuPhe or GTP[S] in the presence of Ca^{2+} (1 μM). However, secretion induced by GTP[S] can be dissociated from arachidonate release, but secretion stimulated by fMetLeuPhe cannot. It is concluded that activation of phospholipase A_2 is closely associated with fMetLeuPhe-stimulated secretion, but is not the target for G_e.

MATERIALS AND METHODS

Materials

[5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid (201.5 Ci/mmol) was obtained from Amersham International. Pertussis toxin was obtained from Porton Products, CAMR, Porton Down, Salisbury, Wilts., U.K. All the nucleotides were obtained from Boehringer Mannheim Biochemicals, and streptolysin O was obtained from Wellcome Diagnostics U.K.

Methods

Neutrophils were isolated from human blood by dextran precipitation of erythrocytes, centrifugation through Ficoll/

Abbreviations used: G-protein, guanine nucleotide regulatory protein; fMetLeuPhe, formylmethionyl-leucyl-phenylalanine; GTP[S], guanosine 5′-[γ-thio]triphosphate; G_p, the G-protein that couples to phospholipase C; G_e, a putative G-protein that regulates exocytosis.

sodium metrizoate, and haemolytic lysis of residual erythrocytes. The cells were washed twice and finally suspended in buffer A, consisting of 137 mM-NaCl, 2.7 mM-KCl, 20 mM-Hepes, 1 mM-$CaCl_2$, 1 mM-$MgCl_2$, 5.6 mM-glucose and 1 mg of fatty-acid-free BSA/ml (pH 7.2), at 2×10^7 cells/ml and incubated with 1 μCi of [^{3}H]arachidonic acid/ml for 1 h. For pretreatment of neutrophils with pertussis toxin, the cells were incubated with pertussis toxin (500 ng/ml) for 2 h at 37 °C.

For permeabilization studies, the labelled cells were washed three times in permeabilizing buffer (consisting of 137 mM-NaCl, 2.7 mM-KCl, 20 mM-Pipes and 1 mg of fatty-acid-free BSA/ml, pH 6.8), and resuspended finally at 2×10^7 cells/ml. After incubation of the cells for 30 min at 37 °C, the cells were metabolically inhibited by addition of 5 μM-antimycin A and 6 mM-deoxyglucose for 5 min, treated with cytochalasin B (5 μg/ml final concn.), and 100 μl portions were transferred to tubes containing an equal volume of buffer supplemented with the permeabilizing agent streptolysin O (0.4 i.u./ml final concn.), Ca^{2+} (in the range of pCa 8–5 buffered with 3 mM-EGTA) [12], guanine nucleotides, fMetLeuPhe and MgATP as indicated in the individual Figure legends. In all experiments the effect of fMetLeuPhe on intact cells was routinely assessed to confirm that metabolic inhibition was totally effective. After 10 min at 37 °C, the cells were centrifuged for 5 min at 4 °C at 1000 *g*. Portions (50 μl) of the supernatant were harvested for determination of β-glucuronidase and lysozyme as described previously [9,27] and for the release of labelled arachidonate (and its metabolites) [17].

All determinations were carried out in duplicate. Data are expressed from individual experiments conducted on at least three different occasions. Secretion was routinely monitored in the same cell preparation used for the measurement of arachidonate release. Release of arachidonate is expressed as recovered radioactivity (c.p.m.) in the supernatant as a percentage of the total radioactivity incorporated in the cell, determined after lysis with 1% Triton X-100.

RESULTS

When intact neutrophils are metabolically inhibited, they are unresponsive to external stimulation by a receptor-directed agonist such as fMetLeuPhe [17]. This observation was exploited to study the intracellular requirements for both fMetLeuPhe- and GTP[S]-mediated secretion and arachidonate release from streptolysin-O-permeabilized human neutrophils.

Secretion stimulated from permeabilized cells

When metabolically inhibited cells are incubated with Ca^{2+} in the range of pCa 8—5 in the presence of the permeabilizing agent streptolysin O and MgATP, neutrophils secrete β-glucuronidase (a marker of azurophil granules) (Fig. 1*a*) and lysozyme (a marker for specific granules) (Fig. 1*b*). The threshold for Ca^{2+}-dependent secretion is pCa 6, and the level of secretion from either granule type is in the region of 25–35% of the total granule content when Ca^{2+} is increased to pCa 5.

Addition of fMetLeuPhe or GTP[S] to the permeabilized cells shifts the Ca^{2+} requirement for secretion to lower values as well as increasing the extent of secretion (Figs. 1*a* and 1*b*). The maximal extent of secretion of both β-glucuronidase (Fig. 1*a*) and lysozyme (Fig. 1*b*) stimulated with fMetLeuPhe obtained in permeabilized cells is comparable with that observed in intact cells [17]. The concentration-dependence of fMetLeuPhe-induced secretion in permeabilized cells (Fig. 2) is also similar to that observed in intact cells [17]. In contrast with fMetLeuPhe, GTP[S] is a more effective stimulator of secretion (Figs. 1*a* and 1*b*).

Effect of MgATP on secretion

In the absence of MgATP, secretion owing to Ca^{2+} alone is completely inhibited (Fig. 3), whereas fMetLeuPhe-dependent secretion is still apparent (Fig. 2*a*). MgATP-independent secretion stimulated by fMetLeuPhe requires a higher level of Ca^{2+} (pCa 5) (cf. Figs. 2*a* and 2*b*). GTP[S]-dependent secretion is also observed in the absence of MgATP, but the concentration of Ca^{2+} and GTP[S] required is shifted to higher concentrations compared with that required to stimulate the same level of secretion in the presence of MgATP. The maximal secretion observed with GTP[S] plus Ca^{2+} in the absence of MgATP never achieves the same level as that seen in the presence of MgATP (cf. Figs. 4*a* and 4*b*). In contrast with fMetLeuPhe, GTP[S] also stimulates secretion at pCa 8, but this effect of GTP[S] is totally dependent on the presence of MgATP (Fig. 4*b*).

In human neutrophils, ATP can also interact with a cell-surface receptor which is coupled to phosphoinositide-specific phospholipase C via a pertussis-toxin-sensitive G-protein [17,28]. ATP is a very poor agonist for secretion compared with fMetLeuPhe. Maximal secretion of β-glucuronidase observed with ATP is about 10%, compared with 30–40% with fMetLeuPhe. To evaluate the role of ATP in supporting secretion in permeabilized cells, it was necessary to investigate whether the primary effect of ATP observed in permeabilized cells was due to

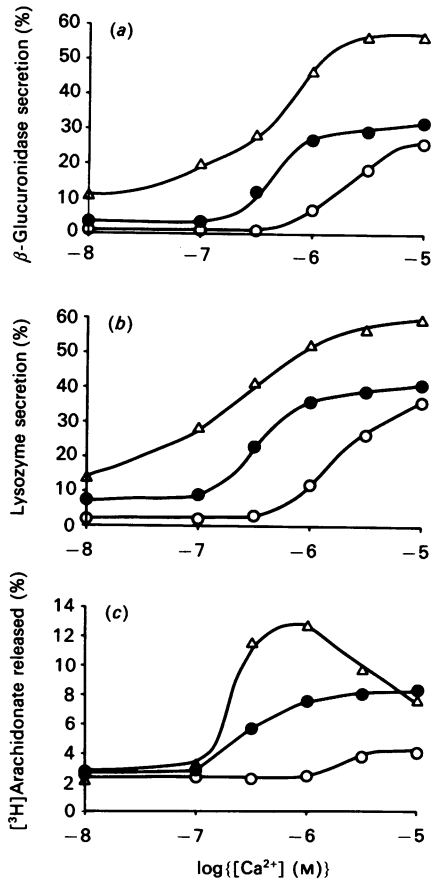

Fig. 1. Effect of fMetLeuPhe and GTP[S] on (*a*) β-glucuronidase secretion, (*b*) lysozyme secretion and (*c*) arachidonate release from streptolysin-O-permeabilized human neutrophils

Neutrophils were metabolically inhibited for 5 min and then transferred to tubes containing the permeabilizing agent streptolysin O (0.4 i.u./ml), MgATP (1 mM) and the indicated concentrations of Ca^{2+} buffered with 3 mM-EGTA. ○, control; ●, fMetLeuPhe (1 μM); △, GTP[S] (60 μM).

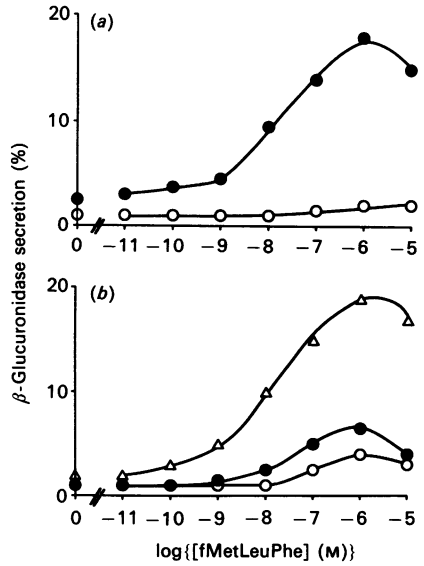

Fig. 2. fMetLeuPhe-stimulated secretion (*a*) at pCa 5 in the absence of MgATP from permeabilized and non-permeabilized neutrophils and (*b*) at pCa 6 in the presence of MgATP from permeabilized neutrophils

Neutrophils were metabolically inhibited for 5 min and then transferred to tubes containing the indicated concentrations of fMetLeuPhe, Ca^{2+} and MgATP. (*a*) ○, no streptolysin O (intact cells); ●, streptolysin O (0.4 i.u./ml) (permeabilized cells). (*b*) ○, no MgATP; ●, 100 μM-MgATP; △, 1000 μM-MgATP. Streptolysin O was present at 0.4 i.u./ml.

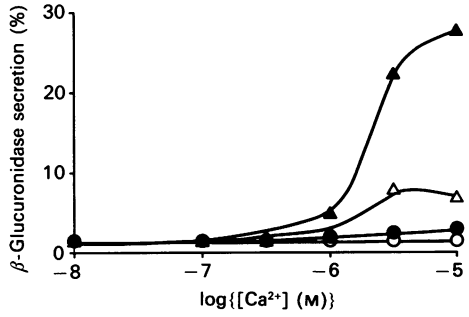

Fig. 3. Effect of MgATP on Ca^{2+}-stimulated secretion from streptolysin-O-permeabilized human neutrophils

Metabolically inhibited neutrophils were transferred to tubes containing streptolysin O (0.4 i.u./ml), and the indicated concentrations of Ca^{2+} and MgATP. ○, no MgATP; ●, MgATP (10 μM); △, MgATP (100 μM); ▲, MgATP (1000 μM).

ATP acting at its receptor, or whether it was exerted intracellularly. Two independent approaches were used to address this question.

The first one was to compare the concentration of MgATP that was required for secretion in intact cells and permeabilized cells. Maximal secretion with external ATP occurs at 100 μM-Ca^{2+} [28]. However, the concentration to support optimal secretion in permeabilized cells is 1 mM (Figs. 2*b* and 3). This was true for secretion stimulated by fMetLeuPhe (at pCa 6) (Fig. 2*b*) or stimulated by Ca^{2+} alone (pCa 5) (Fig. 3).

In the second approach that was used to establish that MgATP was not operating predominantly as a receptor-directed agonist, use was made of the observation that the receptor-mediated stimulation of secretion is susceptible to inhibition by ADP-

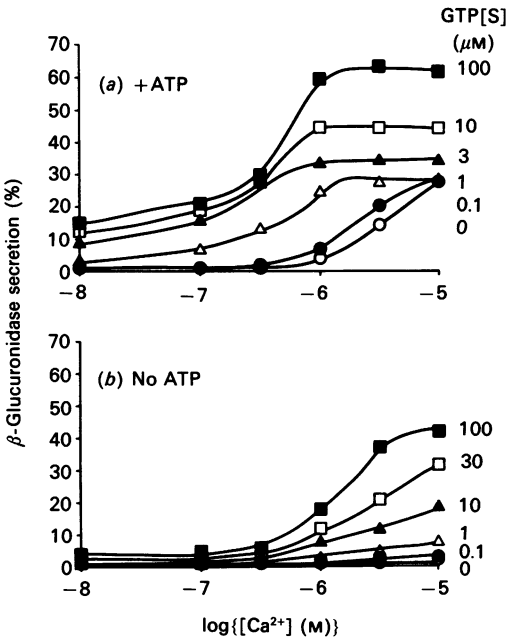

Fig. 4. Effect of MgATP on β-glucuronidase secretion induced by various concentrations of GTP[S]

Metabolically inhibited cells were transferred to tubes containing streptolysin O (0.4 i.u./ml) and the indicated concentrations of Ca^{2+}. (*a*) The GTP[S] concentration was varied from 0 to 100 μM in the presence of MgATP (1 mM): ○, zero GTP[S]; ●, 0.1 μM-GTP[S]; △, 1 μM-GTP[S]; ▲, 3 μM-GTP[S]; □, 10 μM-GTP[S]; ■, 100 μM-GTP[S]. (*b*) The GTP[S] concentration was varied from 0 to 100 μM in the absence of MgATP: ○, zero GTP[S]; ●, 0.1 μM-GTP[S]; △, 1 μM-GTP[S]; ▲, 10 μM-GTP[S]; □, 30 μM-GTP[S]; ■, 100 μM-GTP[S].

Table 1. Effect of pertussis-toxin pretreatment on Ca^{2+}-, fMetLeuPhe- and GTP[S]-induced secretion from permeabilized human neutrophils

Human neutrophils were incubated for 2 h with pertussis toxin (500 ng/ml). The cells were washed, metabolically inhibited and transferred to tubes containing streptolysin O (0.4 i.u./ml), MgATP (1 mM), Ca^{2+} (pCa 6 or pCa 5 as indicated), fMetLeuPhe (1 μM) or GTP[S] (10 μM).

	β-Glucuronidase secretion (%)	
	Control cells	Pertussis-toxin-treated cells
pCa 6	2	1
pCa 5	24	22
pCa 6 plus fMetLeuPhe	25	1
pCa 6 plus GTP[S]	51	38

ribosylation by pertussis toxin [28]. Secretion was triggered in the presence of 1 mM-MgATP by Ca^{2+} only (pCa 5), fMetLeuPhe plus Ca^{2+} (pCa 6) or GTP[S] plus Ca^{2+} (pCa 6) (Table 1). pCa 6 does not stimulate secretion alone, but supports secretion when fMetLeuPhe or GTP[S] is also present. In cells which were pretreated with pertussis toxin, only fMetLeuPhe-dependent secretion was totally inhibited. Both the Ca^{2+}-stimulated secretion and GTP[S]-stimulated secretion were only marginally affected, implying that the primary effect of MgATP was not at the receptor level.

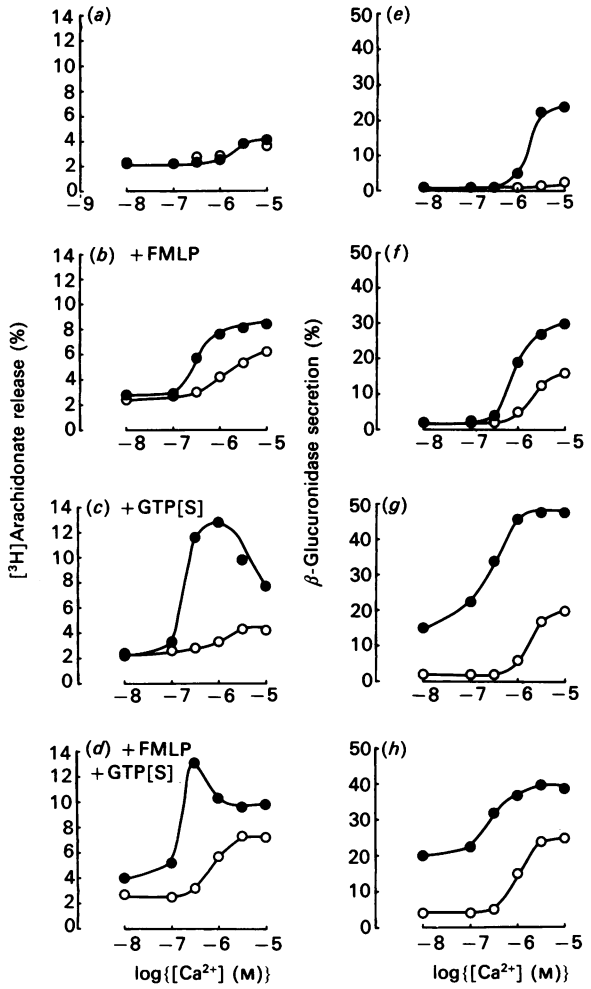

Fig. 5. Effect of MgATP on secretion (*a–d*) and arachidonate release (*e–h*) stimulated by Ca^{2+} alone, fMetLeuPhe, GTP[S] and fMetLeuPhe plus GTP[S]

Metabolically inhibited cells were transferred to tubes containing streptolysin O and Ca^{2+} at the indicated concentrations: (*a*) and (*e*), no additions; (*b*) and (*f*), fMetLeuPhe (FMLP; 1 μM); (*c*) and (*g*), GTP[S] (10 μM); (*d*) and (*h*), fMetLeuPhe (1 μM) plus GTP[S] (10 μM). ○, Incubations in the absence of MgATP; ●, incubations in the presence of 1 mM-MgATP.

Stimulation of arachidonate release

Arachidonate release was used as a measure of phospholipase A_2 activation. Arachidonate release is only marginally stimulated by Ca^{2+} alone in the range of pCa 8–5 (Fig. 1*c*). Optimal activation of arachidonate release occurs at pCa 6 with either fMetLeuPhe or GTP[S]. At pCa 7, the resting level of Ca^{2+} in most cells, neither GTP[S] nor fMetLeuPhe stimulated any release of arachidonate. Arachidonate release with GTP[S] is inhibited when the Ca^{2+} concentration is increased to pCa 5 (Fig. 1*c*). This effect of Ca^{2+} was also apparent with two other GTP analogues, the βγ-imido and the βγ-methylene (results not shown).

To investigate the relationship between secretion and arachidonate release, the effect of MgATP (1 mM) was examined for both processes when stimulated by Ca^{2+} alone, fMetLeuPhe, GTP[S] or fMetLeuPhe plus GTP[S]. (Fig. 5). It is clear that the MgATP-dependence for the two processes is different. Whereas Ca^{2+}-dependent and GTP[S]-dependent secretion can be dissociated from arachidonate release, fMetLeuPhe-dependent secretion and arachidonate release are correlated. fMetLeuPhe can stimulate both secretion and arachidonate release in the absence of MgATP; in its presence both processes are enhanced (Figs. 5*b* and 5*f*). In contrast, arachidonate release stimulated by GTP[S] is dependent on the presence of MgATP, unlike secretion (Figs. 5*c* and 5*g*). The opposite is true for Ca^{2+}-dependent secretion. Thus secretion, but not arachidonate release, is dependent on the presence of MgATP (cf. Figs. 5*a* and 5*e*).

Figs. 5(*d*) and 5(*h*) illustrate the effect of adding fMetLeuPhe plus GTP[S] on arachidonate release and secretion. Synergy between fMetLeuPhe and GTP[S] is apparent both for secretion as well as for arachidonate release in the absence of MgATP. In the presence of MgATP, arachidonate release is now observed at pCa 7. It was always observed that secretion due to GTP[S] alone was always greater at pCa 5 than when fMetLeuPhe plus GTP[S] were added together (cf. Figs. 5*g* and 5*h*), i.e. fMetLeuPhe suppressed GTP[S]-induced secretion.

DISCUSSION

In this paper it is demonstrated that the exocytotic process is still functionally coupled to the fMetLeuPhe receptor in streptolysin-O-permeabilized human neutrophils. Secretion can be initiated by either a single effector or a dual-effector system dependent on the presence or absence of MgATP. Thus a Ca^{2+}-only stimulus (here the amount of Ca^{2+} required is over 1 μM) or a GTP[S]-only stimulus both stimulate secretion. In both cases there is an obligatory requirement for MgATP, and the extent of secretion is sub-maximal. Secretion can also be triggered by a dual effector system, in which case the requirement for MgATP is not obligatory. Thus GTP[S] plus Ca^{2+} or fMetLeuPhe plus Ca^{2+} both provoke sub-optimal secretion, and when MgATP is provided the extent of secretion is increased.

Compared with fMetLeuPhe, GTP[S] stimulates the greatest extent of secretion in either the presence or the absence of MgATP. This suggests that the extent of secretion stimulated by a receptor-directed ligand is restricted by some internal switch-off mechanism which does not operate when GTP[S] is used. This is supported by the observation that fMetLeuPhe inhibits GTP[S]-mediated secretion (cf. Figs. 5*g* and 5*h*). The difference between GTP[S]- and fMetLeuPhe-stimulated secretion could also be due to the different sites of action. Although GTP[S] can directly activate the exocytotic machinery intracellularly, fMetLeuPhe can only exert its effect by activating transmembrane signalling system(s) that would control the production of diffusible and non-diffusible second messengers.

The role of MgATP in supporting secretion in permeabilized neutrophils must be primarily exerted intracellularly rather than extracellularly, despite the presence of a cell-surface receptor for ATP on these cells [17,28]. This was established in two ways. (1) By studying the concentration-dependence for ATP to stimulate secretion in permeabilized cells and intact cells. The optimal concentration required to support secretion from permeabilized neutrophils is 1 mM-MgATP (Figs. 2*b* and 3) compared with 100 μM in intact cells [17,28]. (2) Pertussis-toxin pretreatment inhibits secretion in intact cells when stimulated with ATP [17,28], but in permeabilized cells only secretion stimulated by fMetLeuPhe is inhibited by pertussis-toxin pretreatment, but not when GTP[S] plus Ca^{2+} (pCa 6) or Ca^{2+} (pCa 5) alone is used as a trigger.

MgATP will maintain the polyphosphoinositide levels (and thus make available substrate for phospholipase C) as well as allowing protein phosphorylations to take place. Stimulation of phospholipase C, and hence diacylglycerol formation, would lead to protein kinase C activation. In metabolically inhibited cells the level of polyphosphoinositides falls at the expense of phosphatidylinositol (S. Cockcroft, unpublished work). We have

previously shown that GTP[S]-stimulated InsP_3 formation is inhibited in cells metabolically inhibited for 5 min before permeabilization. Provision of MgATP in the millimolar range (1 mM and above) was required to restore the GTP[S]-stimulated InsP_3 production [12]. The effect of MgATP on exocytosis from permeabilized cells observed here is most likely related to substrate provision for phospholipase C, judging by the level of MgATP required. GTP[S] (in the presence of 1 mM-MgATP) by stimulating phospholipase C would generate diacylglycerol, and hence activation of protein kinase C-mediated phosphorylations, a conclusion also supported by studies on mast cells [11,15]. The lack of absolute requirement for MgATP for GTP[S]- and fMetLeuPhe-mediated secretion would indicate that protein kinase C-mediated phosphorylations play a modulatory rather than an obligatory role. In contrast, Ca^{2+}-dependent secretion was found to be totally dependent on MgATP. This would suggest that stimulation of phospholipase C, and hence activation of protein kinase C, is necessary. Another possibility is that there is a requirement for the presence of the polyphosphoinositides themselves, as suggested recently for adrenal chromaffin cells [29] for Ca^{2+}-dependent secretion (but not obligatory for fMetLeuPhe- or GTP[S]-induced secretion).

Two G-proteins involved in exocytosis

The observation that GTP[S] and fMetLeuPhe can both stimulate secretion under conditions where G_p-mediated phospholipase C activation is curtailed would indicate that a second G-protein is involved in regulating exocytosis. Another catalytic activity that is also regulated by G-proteins is phospholipase A_2, and evidence is provided here that it is also the case in human neutrophils. Thus it is shown that (1) GTP[S] can stimulate arachidonate release (an indicator of phospholipase A_2 activation), and (2) fMetLeuPhe and GTP[S] can synergistically stimulate arachidonate release.

A comparison of the MgATP-dependence for secretion and arachidonate release when GTP[S] is used as a trigger allows the following conclusions to be drawn. The G-protein (G_e) previously suggested to be involved in the exocytotic mechanism cannot be acting via phospholipase A_2 activation, since Ca^{2+}-dependent and GTP[S]-dependent secretion can be dissociated from arachidonate release. Dissociation between GTP[S]-induced secretion and arachidonate release is also apparent in Fig. 1, where secretion increases as a function of Ca^{2+}, whereas arachidonate release appears to be maximally stimulated at pCa 6, followed by inhibition as Ca^{2+} is increased to pCa 5. Such a conclusion was also reached from recent studies on mast cells [24] and adrenal medulla [14]. Thus the target for G_e in exocytosis still remains to be identified. Potential candidates are phospholipase D [30] or a phosphoprotein phosphatase [31].

From the studies with intact neutrophils, there is a strong correlation between the ability of agonists to induce exocytosis and arachidonate release [17]. Such a correlation is also found with fMetLeuPhe-dependent secretion from streptolysin-O-permeabilized neutrophils. Thus phospholipase A_2 activation is possibly involved in the induction of exocytosis stimulated by fMetLeuPhe. That this reaction is under G-protein control is supported by the observation that pertussis toxin inhibits arachidonate release. The identity of the G-protein remains to be elucidated, but is likely to be either G_{i2} or G_{i3}, the two pertussis-toxin substrates found in these cells [32,33]. A role for the involvement of G_e in fMetLeuPhe-stimulated secretion cannot be ruled out by our experiments, however.

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