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The dependence on Ca^{2+} of phosphatidylinositol breakdown and enzyme secretion in rabbit neutrophils stimulated by formylmethionyl-leucylphenylalanine or ionomycin

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1. We have measured the breakdown of [^{3}H]phosphatidylinositol in rabbit neutrophils prelabelled with [^{3}H]glycerol by a pulse–chase procedure. With a view to defining a possible causal relationship between phosphatidylinositol breakdown and enzyme secretion in these cells, we have compared the characteristics of both these processes induced by either the receptor-directed agonist formylmethionyl-leucylphenylalanine (fMet-Leu-Phe) or the Ca^{2+}-ionophore ionomycin. 2. The dependence on fMet-Leu-Phe concentration of phosphatidylinositol breakdown and secretion is identical (half-maximal at 0.3nM). This is 30-fold less than that required for half-maximal occupation of receptors. 3. Both secretion and breakdown of phosphatidylinositol due to fMet-Leu-Phe are modulated by extracellular Ca^{2+}. The sensitivity to Ca^{2+} of both processes is enhanced by pretreatment to deplete cell Ca^{2+}. The concentration of Ca^{2+} required to cause half-maximal effects of both processes in Ca^{2+}-depleted cells on stimulation with 1nM-fMet-Leu-Phe is 100μM. Ionomycin-stimulated secretion and breakdown of phosphatidylinositol are completely dependent on extracellular Ca^{2+} over similar concentration ranges. 4. Both secretion and phosphatidylinositol breakdown due to fMet-Leu-Phe approach completion by 10s. With ionomycin these processes are slower, terminating by 2min. 5. In the presence of [^{32}P]P_i, labelling of [^{32}P]phosphatidic acid reaches a maximum 15min after stimulation with either fMet-Leu-Phe or ionomycin. This precedes the labelling of [^{32}P]phosphatidylinositol and shows the expected precursor–product relationship. 6. We conclude from these results that in rabbit neutrophils a rise in cytosol [Ca^{2+}] is both sufficient and necessary to cause secretion and phosphatidylinositol breakdown. In cells depleted of Ca^{2+}, the occupation of receptors by fMet-Leu-Phe is without effect on these two processes.

The rate of turnover of phosphatidylinositol increases when cells that have Ca^{2+}-mobilizing receptors on their surfaces are stimulated with appropriate ligands. This is initiated by breakdown of phosphatidylinositol, and its resynthesis occurs in a closed cycle of reactions involving phosphorylation of diacylglycerol to give phosphatidic acid and CDP-diacylglycerol as intermediates. It has been proposed that the breakdown of phosphatidylinositol may play a universal role in the mobilization of Ca^{2+} in the cytosol. This hypothesis was based largely on evidence that phosphatidylinositol turnover, though triggered by receptors that control Ca^{2+}, is not itself a consequence of Ca^{2+} translocation (Michell, 1975; Michell *et al.*, 1977).

Abbreviation used: fMet, formylmethionyl.

We have now re-examined this idea by using the secretory response of rabbit peritoneal neutrophils as a well defined example of a tissue activity that is initiated by entry of Ca^{2+} into the cytosol. As an agonist for neutrophil activation we have used the synthetic tripeptide fMet-Leu-Phe (Showell *et al.*, 1976), which has been shown to mimic the soluble bacterial factors that are normal chemotactic agents for these cells (Bennett *et al.*, 1980*a*). In the presence of cytochalasin B, motile functions are suppressed and the response of the cells to stimulation can be measured as the secretion of lysosomal enzymes (Goldstein *et al.*, 1973; Becker & Showell, 1974; Bennett *et al.*, 1980*b*).

Stimulation of neutrophils with fMet-Leu-Phe is accompanied by increases in Ca^{2+} fluxes across the cell membranes (Naccache *et al.*, 1977; Bennett

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et al., 1980*b*; Cockcroft *et al.*, 1980*a*), and the introduction of Ca^{2+} into the cytosol by use of the ionophores A23187 and ionomycin also causes them to secrete (Smith & Ignarro, 1975; Cockcroft *et al.*, 1980*b*). This is the evidence for thinking that interaction of fMet-Leu-Phe with its receptors leads to an increase in intracellular Ca^{2+}, which then provides the signal for secretion to occur.

In the present work we have examined the dependence on Ca^{2+} of fMet-Leu-Phe- and ionomycin-stimulated secretion and phosphatidylinositol breakdown. We have also determined the temporal relationship between secretion and phosphatidylinositol turnover. We have found that the dependence on the concentration of external Ca^{2+} is similar for secretion and phosphatidylinositol breakdown, and we conclude that in these cells phosphatidylinositol breakdown is a consequence of Ca^{2+} entry into the cytosol and is not triggered directly by receptor occupation.

Materials and methods

Neutrophils were obtained from rabbit peritoneal exudates 4h after infusion of 250ml of 0.1% glycogen in 0.15M-NaCl. To prepare these for the measurement of phosphatidylinositol breakdown, they were incubated at 37°C together with [1(3)-^{3}H]glycerol (5–10μCi/ml; 10^{8} cells/ml) for 90min in a buffered salt solution. This comprised 137mM-NaCl, 2.7mM-KCl, 1mM-$MgCl_2$, 20mM-4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid, 5.6mM-glucose and 1mg of bovine serum albumin/ml. Three conditions in the preparation of labelled cells were used. (1) $CaCl_2$ (1.8mM) was present in the experiments illustrated in Figs. 1, 2 and 6, and in expts. A and C of Fig. 4 and Table 1. (2) Ca^{2+} was omitted from the buffer in expt. B of Fig. 4 and Table 1. (3) Ca^{2+} was omitted and replaced by 10μM-EGTA for the experiments of Figs. 5 and 8, and expt. D of Fig. 4 and Table 1. These conditions were then maintained throughout the ensuing preparation of the cells, except in expt. C of Fig. 4 and Table 1, in which the cells were transferred into Ca^{2+}-free medium containing 10μM-EGTA for the chase incubation (see below). Measurements with a Ca^{2+}-specific electrode showed that the concentration of Ca^{2+} in 'Ca^{2+}-free' buffer was about 13μM (mainly contamination by the bovine serum albumin) and this was decreased to 3μM by addition of 10μM-EGTA.

To chase unreacted [^{3}H]glycerol from the cells, they were diluted with an equal volume of buffer containing 10mM-glycerol and sedimented by centrifugation at 400**g** for 5min at room temperature. They were then incubated in 40ml of buffer containing glycerol and incubated at 37°C for 20min. After centrifugation the cells were resuspended at 4×10^{7}/ml and incubated for 20min. Experiments were initiated by the addition of cytochalasin B (5μg/ml final concentration), and samples (1ml) were then transferred to tubes containing equal volumes of solutions containing fMet-Leu-Phe or ionomycin and Ca^{2+} as defined. After 3min, or at other times indicated, the cells were quenched with 5ml of ice-cold 0.15M-NaCl buffered at pH7 with 10mM-sodium phosphate and sedimented by centrifugation at 4°C.

To measure the incorporation of [^{32}P]P_i into phosphatidylinositol and its precursor phosphatidic acid, cells (2×10^{7}/ml) were incubated in the buffered salt solution containing 1.8mM-$CaCl_2$ and [^{32}P]P_i (10–100μCi/ml) for 1h. At this time the radioactivity of the nucleotide pool had reached a plateau level. After addition of cytochalasin B (5μg/ml) the cells were divided into two equal parts, one of which acted as a control. To the other was added either 10nM-fMet-Leu-Phe or 5μM-ionomycin. Samples (2ml) were withdrawn at the times indicated, and quenched and sedimented by centrifugation as described above.

For the measurement of secretion, samples (50μl) of supernatant were removed for the determination of β-glucuronidase as previously described (Bennett *et al.*, 1980*b*). The cell lipids were extracted as previously described (Cockcroft & Gomperts, 1979). ^{32}P-labelled phospholipids were separated by t.l.c. by the procedure of Skipski *et al.* (1964). Lipids were detected by autoradiography and staining with I_2 vapour, and the radioactive spots were scraped into mini-scintillation vials. The lipids were eluted by addition of 0.25ml of methanol followed by 4ml of scintillation fluid and the radioactivity determined by liquid-scintillation counting. ^{3}H-labelled phosphatidylinositol was separated by descending paper chromatography on formaldehyde-treated paper as previously described (Cockcroft & Gomperts, 1979).

The values shown are taken from individual experiments that were carried out on at least three occasions. Data points in the Figures and Table 1 are expressed as means ± S.E.M. of triplicate determinations. Results expressed as a concentration for half-maximal response are means ± S.D. of three independent experiments.

[^{3}H]Glycerol was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). All other materials were obtained from the sources previously specified (Bennett *et al.*, 1979, 1980*b*).

Results

fMet-Leu-Phe-induced breakdown and resynthesis of phosphatidylinositol

Figs. 1(*a*) and 1(*b*) illustrates the dependence on fMet-Leu-Phe concentration of the breakdown of

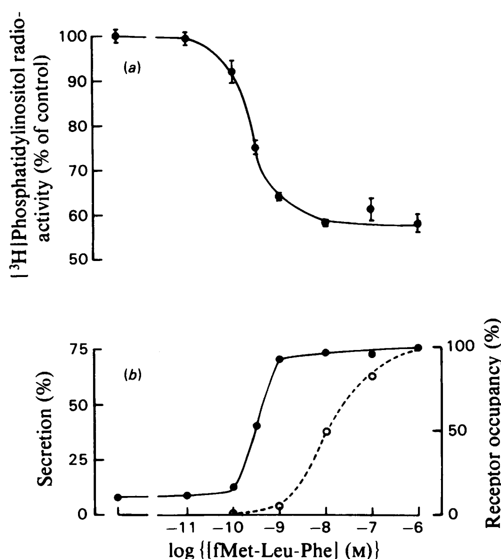

Fig. 1. *Concentration dependence on fMet-Leu-Phe of (a) phosphatidylinositol breakdown and (b) enzyme secretion and receptor occupancy*

Neutrophils were pulse–chased with [^{3}H]glycerol and then stimulated with fMet-Leu-Phe for 3 min. (*a*) The control value (100%) was 6876 ± 260 d.p.m. in phosphatidylinositol (mean ± S.E.M.). (*b*) ●, Enzyme secretion; ○, receptor occupancy. Receptor occupancy data are from Bennett *et al.* (1980*b*).

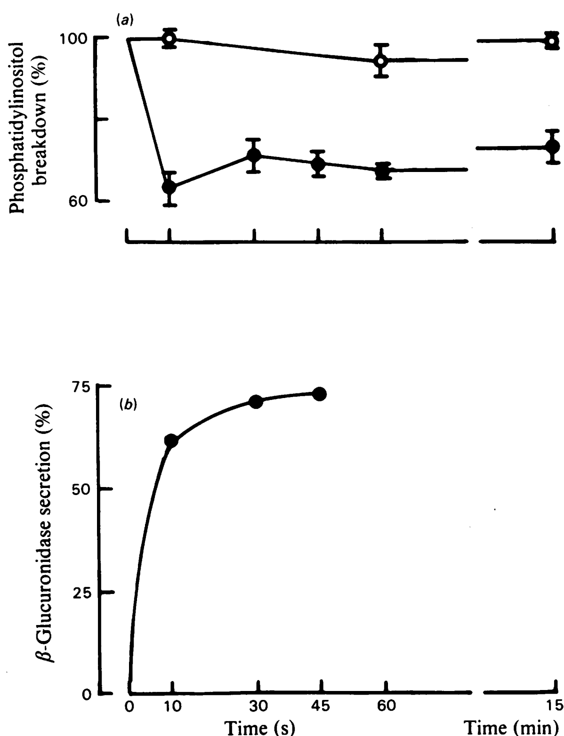

Fig. 2. *Time course of fMet-Leu-Phe (10 nM)-stimulated phosphatidylinositol breakdown (a) and enzyme secretion (b)*

(*a*) ○, Control incubations without fMet-Leu-Phe [control value at 10 s (100%) was 8513 ± 102 d.p.m. in phosphatidylinositol (mean ± S.E.M.)]. ●, Indicates incubations with fMet-Leu-Phe in both (*a*) and (*b*).

[^{3}H]phosphatidylinositol and of secretion of β-glucuronidase in the presence of 1.8 mM-Ca^{2+}. Also shown in this Figure is the extent of receptor occupancy as a function of fMet-Leu-Phe concentration. We find that the concentrations of fMet-Leu-Phe required to induce half-maximal secretion (0.27 ± 0.08 nM, $n = 3$) and phosphatidylinositol breakdown (0.29 ± 0.15 nM, $n = 3$) coincide and are approx. 30-fold less than the concentration required for half-maximal binding of the ligand to its receptor (10 nM).

Figs. 2(*a*) and 2(*b*) show the time course of β-glucuronidase secretion and breakdown of [^{3}H]-phosphatidylinositol after stimulation with fMet-Leu-Phe (10 nM). Both processes are close to completion by 10 s. These data agree closely with our previous results using [^{32}P]P_i to label the phospholipids (Cockcroft *et al.*, 1980*b*), though there is one difference. With ^{32}P-labelled cells the initial decrease in radioactivity is rapidly masked by incorporation of ^{32}P from ATP in the course of phosphatidylinositol resynthesis. In the present experiment using [^{3}H]glycerol to label phospholipids by the pulse–chase procedure, the low level of radioactivity is maintained for a period of at least 15 min.

Fig. 3 illustrates the time course of labelling of phosphatidic acid and phosphatidylinositol with [^{32}P]P_i. Labelling of phosphatidic acid becomes maximal at 15 min and after this there is a slow return to the basal value. Incorporation of [^{32}P]P_i into phosphatidylinositol maximizes at a plateau level after about 30 min. This kinetic pattern is consistent with the idea that phosphatidic acid is the metabolic precursor for the resynthesis of phosphatidylinositol. There was no increase in the incorporation of ^{32}P in phosphatidylcholine (results not shown).

Dependence on Ca^{2+} of fMet-Leu-Phe-induced enzyme secretion and phosphatidylinositol breakdown

Figs. 4(*a*) and 4(*b*) and Table 1 show the results of separate experiments (A, B, C and D) in which we tested the effect of various degrees of Ca^{2+}-deprivation on the dependence of enzyme secretion and

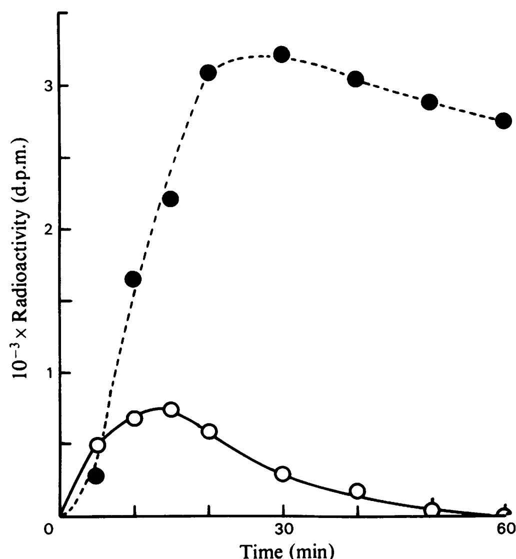

Fig. 3. *Time course of incorporation of ^{32}P in phosphatidic acid and phosphatidylinositol in fMet-Leu-Phe (10 nM)-stimulated neutrophils*

○, Phosphatidic acid; ●, phosphatidylinositol. The radioactivity in the control incubations increased linearly with time (phosphatidic acid, 500 to 1100 d.p.m. in 60 min; phosphatidylinositol, 500 to 2900 d.p.m. in 60 min) and the relevant corrections for this have been made for each point. Enzyme secretion was 75%.

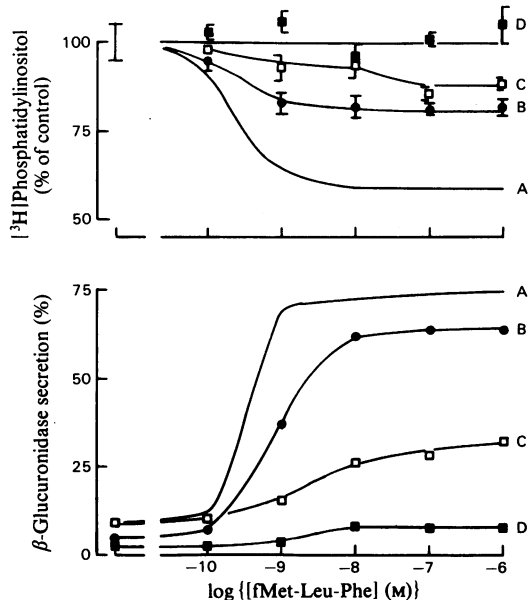

Fig. 4. *Effect of Ca^{2+} deprivation on the dependence of (a) breakdown of phosphatidylinositol and (b) enzyme secretion on fMet-Leu-Phe concentration*

For full experimental details see the text. Expt. A was performed in the presence of 1.8 mM-Ca^{2+} throughout (redrawn from Fig. 1). Expt. B was performed in the absence of added Ca^{2+} or EGTA, throughout. Expt. C was performed by incubating the cells initially in 1.8 mM-Ca^{2+} and finally transferring the cells into a Ca^{2+}-free buffer containing 10 μM-EGTA. Expt. D was performed in the presence of 10 μM-EGTA throughout (no added Ca^{2+}). The control (100%) radioactivity in phosphatidylinositol was: B, 10891 ± 435 d.p.m.; C, 6589 ± 330 d.p.m.; D, 10436 ± 470 d.p.m. (means ± S.E.M.).

phosphatidylinositol breakdown on fMet-Leu-Phe concentration. In agreement with previous results (Cockcroft *et al.*, 1980*a*) we find that omission of Ca^{2+} from the incubation medium both decreases the extent of secretion and also shifts the concentration requirements for the agonist to higher levels (compare expts. A and B). To suppress secretion more extensively we also tested the effect of transferring the cells into Ca^{2+}-free EGTA (10 μM)-containing buffer from 1.8 mM-Ca^{2+} for the chase incubation and all ensuing operations (expt. C), and we also tested the effect of incubating the cells in the presence of 10 μM-EGTA throughout the entire period of labelling and all ensuing operations (expt. D). In this way we modulated the extent of fMet-Leu-Phe-induced secretion over a wide range (Fig. 4*b*) and we also demonstrated that re-introduction of 1.8 mM-Ca^{2+} restored the secretion due to 10 nM-fMet-Leu-Phe to levels within the normal range (Table 1).

It is clear from the experiments of Fig. 4 that Ca^{2+}-deprivation suppresses fMet-Leu-Phe-induced phosphatidylinositol breakdown and secretion to a similar extent. Re-introduction of 1.8 mM-Ca^{2+} also restores the breakdown of phosphatidylinositol (Table 1).

The experiment illustrated in Figs. 5(*a*) and 5(*b*) shows that the dependence on extracellular Ca^{2+} of both secretion and breakdown of [^{3}H]phosphatidylinositol due to fMet-Leu-Phe (1.0 nM) is similar. Half-maximal responses occurred at Ca^{2+} concentrations of 107 ± 3 μM ($n = 3$) for secretion and 99 ± 4 μM ($n = 3$) for phosphatidylinositol breakdown. Maximal effects for both responses were observed with 180 μM-Ca^{2+}.

Ionomycin-induced breakdown and resynthesis of phosphatidylinositol

Figs. 6(*a*) and 6(*b*) show the time course of ionomycin (5 μM)-induced breakdown of [^{3}H]phosphatidylinositol and enzyme secretion. The ionophore-induced processes are slower than those

Table 1. *Effect on secretion and phosphatidylinositol breakdown of restoring 1.8mM-Ca^{2+} to neutrophils previously deprived of Ca^{2+}*

After depriving neutrophils of Ca^{2+} by different procedures B, C and D (for details, see the Materials and methods section and description in the text of Fig. 4), the cells were stimulated with fMet-Leu-Phe (10nM) and Ca^{2+} (1.8mM) as indicated. Data for phosphatidylinositol are calculated as mean percentage breakdown ± the combined S.E.M. of controls and stimulated cells. Significance of differences from controls: *, $P < 0.05$; **, $P < 0.005$; †, not significant.

Conditions of cell preparation	Conditions during stimulation by fMet-Leu-Phe	Phosphatidylinositol breakdown (%)	β-Glucuronidase secretion (%)
(A) 1.8mM-Ca^{2+}	1.8mM-Ca^{2+}	41 ± 3**	73
(B) No added Ca^{2+}	No added Ca^{2+}	18 ± 5*	62
	1.8mM-Ca^{2+}	31 ± 4**	85
(C) 10μM-EGTA during chase period	No added Ca^{2+}	6 ± 6†	17
	1.8mM-Ca^{2+}	31 ± 6**	60
(D) 10μM-EGTA during pulse and chase	No added Ca^{2+}	4 ± 7†	6
	1.8mM-Ca^{2+}	30 ± 5**	40

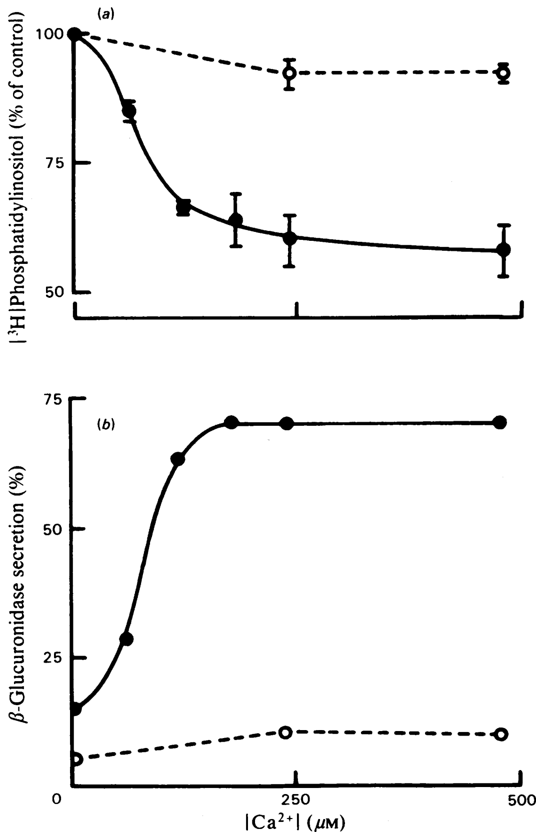

Fig. 5. *Dependence on Ca^{2+} concentration of fMet-Leu-Phe (1nM)-stimulated phosphatidylinositol breakdown (a) and enzyme secretion (b)*

Zero-Ca^{2+} control (100%) was 5154 ± 65 d.p.m. in phosphatidylinositol (mean ± S.E.M.). ○, Control incubations (no fMet-Leu-Phe); ●, incubations with fMet-Leu-Phe.

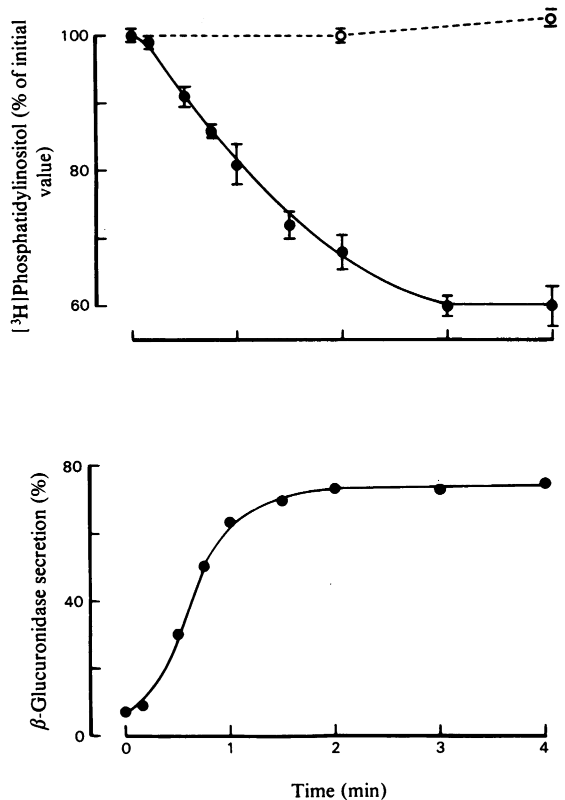

Fig. 6. *Time course of ionomycin (5μM)-stimulated phosphatidylinositol breakdown (a) and enzyme secretion (b)*

(a) ○, Indicates control incubations without ionomycin [control at zero time (100%) was 4635 ± 77 d.p.m. in phosphatidylinositol (mean ± S.E.M.)]. ●, Indicates incubations with ionomycin in both (a) and (b).

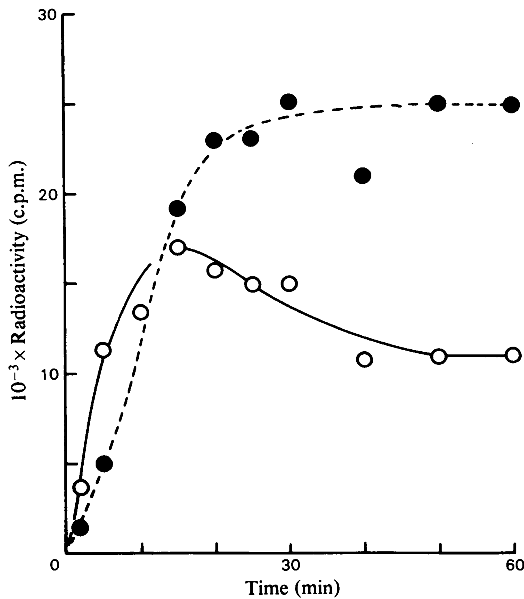

Fig. 7. *Time course of incorporation of ^{32}P in phosphatidic acid and phosphatidylinositol in ionomycin (5 μM) -stimulated neutrophils*

○, Phosphatidic acid; ●, phosphatidylinositol. The radioactivity in the control incubations increased linearly with time (phosphatidic acid, 2500 to 6000 d.p.m. in 60 min; phosphatidylinositol, 2500 to 12500 d.p.m. in 60 min) and the relevant corrections have been made for each point. Enzyme secretion was 79%.

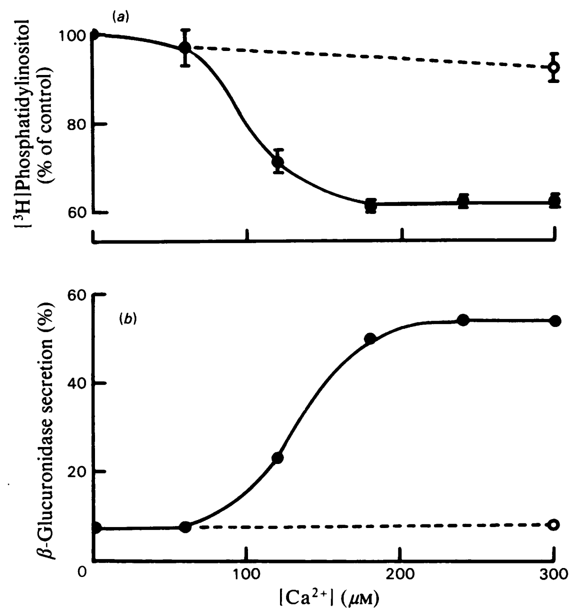

Fig. 8. *Dependence on Ca^{2+} concentration of ionomycin (5 μM)-stimulated phosphatidylinositol breakdown (a) and enzyme secretion (b)*

Zero-Ca^{2+} control (100%) was 3488 ± 28 d.p.m. in phosphatidylinositol. ○, Represents control incubations (no ionomycin) in (*a*) and (*b*); ●, represents incubations with ionomycin in both (*a*) and (*b*).

induced by fMet-Leu-Phe so that it is possible to resolve some kinetic detail. In this and other comparable experiments we have observed that the secretory process terminates before the breakdown of phosphatidylinositol is complete, though the time of onset of both processes appears to be about the same.

The time course of resynthesis of phosphatidylinositol measured in ^{32}P-labelled cells stimulated by ionomycin is shown in Fig. 7. Labelling of phosphatidylinositol is preceded by labelling of phosphatidic acid in a manner similar to that observed with fMet-Leu-Phe (Fig. 3), but with ionomycin we find that the level of phosphatidic acid labelling does not return to control levels by 60 min. In an experiment in which the two stimuli were compared directly, the increase in phosphatidic acid labelling at 15 min was 5-fold for an optimal concentration of fMet-Leu-Phe (10 nM) and 13-fold for an optimal concentration of ionomycin (5 μM), whereas the increase in [^{32}P]phosphatidylinositol was 4-fold for either stimulus.

Fig. 8 shows the dependence on extracellular Ca^{2+} of ionomycin (5 μM)-induced phosphatidylinositol breakdown and β-glucuronidase secretion. These two processes show a similar dependence on Ca^{2+} concentration.

Discussion

The use of [^{3}H]glycerol as a label for phospholipids in the study of phosphatidylinositol metabolism has certain advantages over [^{32}P]P_i. In particular, due to the considerable permeability of the cell membrane to glycerol, the unreacted radioactive glycerol can be easily washed out in a chase procedure.

Our data for rabbit neutrophils are consistent with the idea of a metabolic cycle for phosphatidylinositol breakdown and resynthesis (Michell, 1975). For both fMet-Leu-Phe and ionomycin applied at optimal concentrations, the initial event is the breakdown of 30–40% of the prelabelled phosphatidylinositol. The increase in ^{32}P-labelling of phosphatidylinositol is due to turnover of the phosphorylinositol head-group and not to synthesis *de novo* of the lipid. As evidence for this, we found that in experiments in which the [^{3}H]glycerol was not removed from the cells by the chase procedure,

there was no stimulated incorporation of the label into phosphatidylinositol (results not shown).

The extent of breakdown and labelling of phosphatidylinositol due to fMet-Leu-Phe and ionomycin are similar, but there are two differences in the labelling response of phosphatidic acid. First, the greater extent of labelling due to the ionophore is indicative of more phosphatidic acid formation. Secondly, whereas the labelling of phosphatidic acid after stimulation with fMet-Leu-Phe returns to the control value by 60min, this does not occur with the ionophore, reflecting a partial block in the subsequent metabolism of phosphatidic acid. Although both fMet-Leu-Phe and the ionophore are understood to act by raising the concentration of cytosol Ca^{2+}, long-term receptor-directed processes such as desensitization (O'Flaherty *et al.*, 1978) and endocytosis (Niedel *et al.*, 1979) would terminate the fMet-Leu-Phe-induced signals. In contrast, the ionophore would continue to equilibrate cytosol Ca^{2+} with the extracellular medium, thus causing more prolonged and extensive responses.

One factor that could contribute to the greater magnitude of phosphatidic acid synthesis with the ionophore concerns the activation of triacylglycerol lipase. In neutrophils we have found that loss of label from [^{3}H]triacylglycerol due to ionomycin (50% at 15min) is about twice that due to fMet-Leu-Phe (results not shown). Diacylglycerol from this source could be a substrate for phosphatidic acid formation. A Ca^{2+}-sensitive triacylglycerol lipase has previously been demonstrated in human neutrophils and lymphocytes (Allan & Michell, 1977).

Blockade in the conversion of phosphatidic acid into phosphatidylinositol could be explained in part by the observation that, whereas the total radioactivity in the nucleotide pool (carbon-adsorbed activity of the acid-soluble fraction) is maintained after treatment of neutrophils with fMet-Leu-Phe, it declined by about 50% 15min after treatment with ionomycin (results not shown). Such a loss of nucleotides might limit the availability of CTP required in the conversion of phosphatidic acid into phosphatidylinositol.

On kinetic grounds (see Figs. 3 and 6) the only reaction in the cycle of phosphatidylinositol metabolism that could have any relevance to the control of Ca^{2+} and the secretory process is the breakdown of phosphatidylinositol (and therefore the formation of diacylglycerol and inositol phosphates, which are its immediate breakdown products). It appears from our results (Figs. 4 and 5 and Table 1) that a limited amount of secretion can occur in the absence of added Ca^{2+}, and under these conditions breakdown of phosphatidylinositol probably does not occur (our detection limit for this reaction is 7%). In these circumstances, phosphatidylinositol labelling, although indicative of secondary reactions in the cycle, has the advantage of greater sensitivity. Taken together, the present results, and the data on phosphatidylinositol labelling described previously (Cockcroft *et al.*, 1980*a*), suggest that a limited degree of fMet-Leu-Phe-induced secretion can occur in the absence of any breakdown of phosphatidylinositol.

The results of Fig. 4 strongly support the idea that both secretion and phosphatidylinositol breakdown are modulated by Ca^{2+}. Compared with other systems (for review, see Rubin, 1974) we note that the external requirement for Ca^{2+} in the stimulation by fMet-Leu-Phe of rabbit neutrophils is low, being half-maximal at about 100μM. This is in agreement with the results of others concerning leucocidin-stimulated enzyme secretion from rabbit neutrophils (Woodin & Wieneke, 1963). That Ca^{2+} is actually the trigger for both phosphatidylinositol breakdown and secretion is confirmed by our experiments with ionomycin (Fig. 8) in which neither process occurred in the absence of extracellular Ca^{2+}.

One piece of evidence that has been cited in support of the idea that phosphatidylinositol responses might be mediated directly by activated receptors, and not by Ca^{2+}, is that for the muscarinic cholinergic receptor in some tissues, the extent of phosphatidylinositol breakdown and labelling is regulated by the degree of receptor occupancy. Later events controlled by these receptors maximize with less than 5% of the receptors occupied (Michell *et al.*, 1976). The fMet-Leu-Phe system of rabbit neutrophils is clearly different. Our present experiments show that both phosphatidylinositol breakdown and secretion maximize with less than 10% of receptors occupied, indicating that there is a considerable receptor reserve not coupled to changes in either process.

It has been suggested that 'a central role for phosphatidylinositol breakdown could be envisaged in receptors...probably in the mechanism by which Ca^{2+} channels are opened' (Michell, 1975). In a later development of this idea, it has been suggested that it is phosphatidic acid, an intermediate in the phosphatidylinositol cycle, that is of importance in the regulation of Ca^{2+} movements (Salmon & Honeyman, 1980; Putney *et al.*, 1980). Our results with neutrophils are incompatible with these ideas. In neutrophils, phosphatidylinositol breakdown occurs as a consequence of the elevation of intracellular [Ca^{2+}]; it is not coupled to the occupancy of surface receptors. The generation of phosphatidic acid is slow compared with the rate of secretion and can better be described as a late event. In our view, the breakdown of phosphatidylinositol remains a process in search of a function.

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