Plasma-membrane location of phosphatidylinositol hydrolysis in rabbit neutrophils stimulated with formylmethionyl-leucylphenylalanine

Jonathan P. BENNETT, Shamshad COCKCROFT, Anthony H. CASWELL* and Bastien D. GOMPERTS

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

(Received 16 July 1982/Accepted 12 August 1982)

Rabbit peritoneal neutrophils, disrupted by sonication, were separated into three subcellular fractions by sucrose-step-gradient centrifugation and these were analysed with respect to biochemical markers. They comprised a high-speed supernatant containing the cytosol, a light particulate fraction enriched in Golgi and plasma membranes and a heavy particulate fraction enriched in granules and nuclei. The light particulate fraction was further separated into its components, which were identified as Golgi membranes (galactosyltransferase activity) and plasma membranes {radioactivity derived from labelling intact cells with [125 I]di-iodosulphanilic acid diazonium salt and [3 H]formylmethionyl-leucylphenylalanine ([3 H]fMet-Leu-Phe) binding}. In cells prelabeled with [3 H]glycerol, the hydrolysis of phosphatidylinositol due to cell stimulation with fMet-Leu-Phe (10 nM) was shown to occur in the light particulate fraction. The [32 P]P_i-labelling of phosphatidate, which is an early consequence of phosphatidylinositol hydrolysis, also occurred in this fraction. Analytical sucrose-gradient centrifugation of the light particulate fraction showed that the stimulated increment in [32 P]phosphatidate (and thus by implication the initial phosphatidylinositol breakdown) was localized in the plasma membrane.

A common characteristic of cell-surface stimuli that exert their physiological effects through changes in cytosol [Ca^{2+}] is their ability to increase the metabolic turnover of phosphatidylinositol (Michell, 1975). The initial event is the hydrolysis of phosphatidylinositol to form diacylglycerol, and this is followed by its regeneration via phosphatidate (Cockcroft *et al.*, 1980, 1981). It has been proposed that phosphatidylinositol hydrolysis is intimately associated with the gating of Ca^{2+} (Michell, 1975; Michell *et al.*, 1977, 1981) and a possible mechanism for this has been suggested by indications that phosphatidate might act as a Ca^{2+} -carrying ionophore (Tyson *et al.*, 1976; Putney *et al.*, 1980; Serhan *et al.*, 1981). Our results with rabbit neutrophils have not supported this proposal. In these cells hydrolysis of phosphatidylinositol and the generation of phosphatidate are consequences, not causes, of Ca^{2+} translocation (Cockcroft *et al.*, 1980, 1981). Despite the interest in these processes, the subcellular localization of phosphatidylinositol

hydrolysis and phosphatidate formation remains obscure.

We have developed a method for subcellular fractionation of rabbit neutrophils with the aim of localizing both phosphatidylinositol hydrolysis and phosphatidate formation after stimulation with the chemotactic peptide, fMet-Leu-Phe. This is a synthetic analogue for peptides released by infective bacteria during tissue inflammation (Bennett *et al.*, 1980a). We find that both phosphatidylinositol hydrolysis and phosphatidate formation are localized at the plasma membrane in fMet-Leu-Phe-stimulated neutrophils.

Materials and methods

Preparation and treatment of neutrophils

Rabbit peritoneal exudate neutrophils (>95% pure) were obtained 4 h after intraperitoneal infusion of 0.1% glycogen in 150 mM-NaCl as described previously (Bennett *et al.*, 1980b). On occasions when there was slight visible contamination with erythrocytes these were selectively lysed by using 150 mM-NH₄Cl. The cells were washed twice

Abbreviation used: fMet, formylmethionyl.

* Permanent address: Department of Pharmacology, University of Miami, Miami, FL 33101, U.S.A.

in a buffer (pH 7.3) consisting of 137 mM-NaCl, 2.7 mM-KCl, 1 mM-MgCl₂, 1.8 mM-CaCl₂, 20 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], 5.6 mM-glucose and 1 mg of bovine serum albumin/ml.

For the experiment described in Table 2 cells were pulse-labelled with [1(3)-³H]glycerol and chased with unlabelled glycerol as described previously (Cockcroft *et al.*, 1981). Neutrophils were suspended in the above buffer at 8×10^7 cells/ml and incubated with [³H]glycerol at 20 μ Ci/ml for 60 min at 37°C. The cells were then washed into buffer containing unlabelled 10 mM-glycerol and incubated at 37°C for two periods of 20 min with a wash in between. For the experiments described in Table 3 and Fig. 2 the cells were incubated with [³²P]P_i at 100 μ Ci/ml for 60 min at 37°C before stimulation.

For cell stimulation, cells were added to an equal volume of buffer containing fMet-Leu-Phe (final concentration 10 nM). In control samples no fMet-Leu-Phe was present. After 1 min at 37°C the incubation was terminated by the addition of an equal volume of ice-cold 150 mM-NaCl and centrifugation at 4°C.

Fractionation procedure

After centrifugation the cells were resuspended in 15 ml of ice-cold 150 mM-NaCl, and all subsequent manipulations were carried out in the cold. The cells were treated with 2 mM-di-isopropyl fluorophosphate for 5 min to inactivate endogenous proteinases (Amrein & Stossel, 1980). The cells were centrifuged and resuspended at $(0.5-1.5) \times 10^8$ cells/ml in an ice-cold buffer (pH 7.2) consisting of 250 mM-sucrose, 0.1 mM-EDTA and 20 mM-Hepes. Disruption was accomplished by sonication for 40 s with an MSE probe sonicator with a 4 mm diameter probe set to give an amplitude of 18 μ m. Unbroken cells were removed by centrifugation (600 g for 5 min).

Of the resulting homogenate, 10 ml was layered over a discontinuous sucrose gradient (made up in 0.1 mM-EDTA/20 mM-Hepes, pH 7.2) consisting of 12 ml of 2.2 M-sucrose, 8 ml of 1.6 M-sucrose and 4 ml of 0.6 M-sucrose. This was centrifuged at 4°C for 45 min at 50 000 rev./min (200 000 $g_{av.}$) on a vertical rotor (Sorvall TV 850). The high-speed supernatant fraction was pipetted from the top of the tube. The light particulate fraction was removed from the 0.6 M/1.6 M-sucrose interface by using a hypodermic syringe with a 19G needle bent at a right angle. The heavy particulate fraction (1.6 M/2.2 M-sucrose interface) was removed by using a Pasteur pipette after aspiration of the overlying sucrose.

To locate lipid changes in the light fraction with greater precision, this was subjected to analytical sucrose-density-gradient centrifugation. The light membrane fraction obtained as above was centri-

fuged into a 2.2 M-sucrose cushion, and the material was resuspended in 2.2 M-sucrose and layered under a 0.6–1.6 M-sucrose linear gradient. After centrifugation for 15 h at 200 000 $g_{av.}$, the gradients were fractionated and analysed.

Biochemical assays

Protein concentration was measured by using a modified Lowry procedure (Bennett, 1982) with bovine serum albumin as standard. Lactate dehydrogenase activity was measured by monitoring spectrofluorimetrically the disappearance of NADH (0.1 mM) in the presence of 5 mM-pyruvate, 0.1% Triton X-100, 20 mM-Hepes, pH 7.2, at 25°C. Galactosyltransferase activity was measured at 37°C by the transfer of radioactivity from UDP-[³H]galactose to ovalbumin (Hino *et al.*, 1978). Lysozyme activity was measured at 25°C by monitoring the decrease in A_{450} due to clarification of a suspension of *Micrococcus lysodeikticus* (0.16 mg/ml) in 0.1 M-phosphate buffer, pH 6.5, containing 0.1% Triton X-100 (Becker, 1973). β -Glucuronidase activity was measured spectrofluorimetrically by the hydrolysis of 4-methylumbelliferyl β -D-glucuronide (2.5 mM) at 37°C (Bennett *et al.*, 1980b).

fMet-Leu-Phe binding was measured by a filtration assay (based on that of Sha'afi *et al.*, 1978). Samples were incubated with [³H]fMet-Leu-Phe (sp. radioactivity 50–60 Ci/mmol) at a final concentration of approx. 0.7 μ Ci/ml at 25°C in a buffer (pH 7.2) consisting of 150 mM-NaCl, 1 mg of bovine albumin/ml, 0.1 mM-1-chloro-4-phenyl-3-L-toluene-p-sulphonamidobutan-2-one and 20 mM-Hepes. After 5 $\frac{1}{2}$ min the samples were filtered on pre-wetted Whatman GF/C paper and washed with 5 ml of 150 mM-NaCl, 1 mg of bovine albumin/ml and 20 mM-Hepes, pH 7.2. Non-specific binding was determined in the presence of 1 μ M-fMet-Leu-Phe. The filters were clarified in scintillation fluid and counted for radioactivity.

DNA was measured by a method based on that of Karsten & Wollenberger (1972). Samples (20 μ l) were incubated for 30 min at 37°C with 0.1 ml of a solution containing ribonuclease (20 μ g/ml), proteinase K (80 mg/ml), sodium dodecyl sulphate (10 mg/ml) and 50 mM-Hepes, pH 7.2. After addition of 1 ml of ethidium bromide (10 μ g/ml) in 50 mM-Hepes, pH 7.2, DNA was determined from fluorescence (excitation 365 nm, emission 580 nm) with calf-thymus DNA as standard.

Plasma-membrane identification

Although we routinely identified plasma-membrane enrichment in density-gradient fractions by measuring fMet-Leu-Phe binding to its receptor, we also confirmed this identification by fractionating cells that had previously been labelled with [¹²⁵I]-

di-iodosulphanilic acid diazonium salt, in the cold. This reagent was prepared as described by Edwards *et al.* (1979) and the neutrophils were treated by the methods previously employed for platelets (George *et al.*, 1976). Thus the radioactively labelled diazonium salt, precipitated by centrifugation from water, was shaken in ice-cold 150 mM-NaCl (buffered at pH 7 with 10 mM-potassium phosphate) and the remaining solid material removed by centrifugation. Of the yellow supernatant 10 ml was added to 10^9 neutrophils contained in a minimum volume of 150 mM-NaCl; these had previously been washed five times in cold 150 mM-NaCl (pH 7) to remove all traces of non-cell protein. During the reaction, 50 μ l was removed for measurement of radioactivity to calculate the concentration of the diazonium salt by reference to the specific radioactivity, previously determined from measurements of radioactivity and the absorbance of the α -naphthol adduct. The reaction with the neutrophils was terminated after 20 min with 1.5 equivalents of α -naphthol; the cells were then washed twice in 150 mM-NaCl containing α -naphthol and once again in its absence. After suspension in ice-cold 150 mM-NaCl the cells were treated as described above for fractionation.

Extraction of lipids

Lipids were extracted from the various samples by addition of 3.75 vol. of chloroform/methanol (1:2, v/v) and the resulting single-phase system was partitioned by addition of 1.25 vol. each of chloroform and 2 M-KCl. The chloroform phase from each sample was evaporated to dryness under reduced pressure and the lipids were dissolved in 50 μ l of chloroform for phospholipid analysis.

Chromatography of lipids

[32 P]Phosphatide was separated by t.l.c. by the procedure of Skipski *et al.* (1964) (without addition of bicarbonate to the silica-gel slurry) and was located by autoradiography. Phosphatidylinositol was separated on formaldehyde-impregnated papers as previously described (Cockcroft & Gomperts, 1979; Cockcroft *et al.*, 1981). Total lipid phosphorus was measured as previously described (Allan *et al.*, 1980).

Results and discussion

Characterization of subcellular fractions

A subcellular fractionation scheme was devised to separate the different membrane components of the neutrophils from the cytosol. Table 1 shows the distribution of a number of biochemical markers between three fractions obtained from sonicated neutrophils. The criterion by which we selected sonication was disruption of cells (monitored by release of the cytosol marker enzyme, lactate dehydrogenase) with minimum release of lysosomal hydrolases from within the granules. We chose this

criterion because we wanted to minimize the possibility of degradation of cellular constituents by lysosomal enzymes during fractionation or subsequent storage. For the same reason we treated the intact cells with the serine proteinase inhibitor di-isopropyl fluorophosphate before sonication, as described by Amrein & Stossel (1980). Other devices for cell disruption that we tested were a hand-held glass Dounce homogenizer, a motorized Potter homogenizer and a constant-pressure cell-shearing device (Crompton & Snary, 1974).

The sonicated cells were centrifuged at 2000 rev./min for 5 min. The main effect of this step is to sediment unbroken cells (typically 5–15%) as indicated by a similar yield of protein, lactate dehydrogenase and [3 H]fMet-Leu-Phe-binding in the pellet; however, the pellet was observed to be slightly enriched in granule marker enzymes and in DNA. The supernatant from this centrifugation, designated 'homogenate' in Table 1, was applied to the step sucrose gradient.

The concentrations of sucrose used for the step gradient were selected to provide a fraction that was enriched in the plasma-membrane markers ([3 H]-fMet-Leu-Phe binding activity and labelling with [125 I]di-iodosulphanilic acid diazonium salt), with a low content of granule enzymes. The material that remained in the sample buffer above 0.6 M-sucrose is designated 'high-speed supernatant'; that which formed a band at the 0.6 M/1.6 M-sucrose interface is designated the 'light particulate fraction' and that which sedimented through 1.6 M-sucrose to rest on 2.2 M-sucrose is the 'heavy particulate fraction'.

Certain of the biochemical markers used in Table 1 are conventional, i.e. the use of lactate dehydrogenase (cytosol), galactosyltransferase (Golgi-derived membranes) and DNA (nucleus). Neutrophil granules fall into two main categories (Bainton *et al.*, 1976) and we have measured lysozyme, which is found in both specific and azurophil granules, and β -glucuronidase, which occurs only in azurophil granules. As a plasma-membrane marker we have used the receptor for fMet-Leu-Phe, which is present on the cell surface (Bennett *et al.*, 1980b). We found this preferable to other possible native plasma-membrane markers, such as 5'-nucleotidase and ouabain-inhibitable ($\text{Na}^+ + \text{K}^+$)-dependent ATPase, which are present only at low activity in neutrophils (Shirley *et al.*, 1976; Sha'afi *et al.*, 1976). In separate experiments (results not shown) we found that the distribution of the binding activity for fMet-Leu-Phe is the same as for the β -adrenergic antagonist 3-[125 I]iodohydroxybenzylpindolol, thus lending weight to the conclusion of a plasma-membrane location of this receptor. Further confirmation for this conclusion comes from the finding that the impermeant covalent label, [125 I]di-iodosulphanilic acid diazonium salt distributes among the fractions

Table 1. *Distribution of biochemical markers between subcellular fractions obtained by step-gradient centrifugation*
The results are taken from two similar experiments. In the step-gradient fractionation of [125 I]di-iodosulphanilic acid diazonium-labelled cells, the distribution of protein and of β -glucuronidase was also determined, and closely resembled the presented distribution obtained for the unlabelled cells.

		Homogenate	High-speed supernatant	Light particulate fraction	Heavy particulate fraction
Protein	(mg recovered)	66.0 $\pm$ 1.4	29.6 $\pm$ 0.5	4.4 $\pm$ 0.1	27.0 $\pm$ 0.6
	Yield (%)	(100)	45 $\pm$ 1	7 $\pm$ 1	41 $\pm$ 1
Lipid phosphorus	(nmol/mg of protein)	130 $\pm$ 3	9 $\pm$ 2	739 $\pm$ 17	133 $\pm$ 8
	Yield (%)	(100)	3 $\pm$ 1	38 $\pm$ 1	42 $\pm$ 2
Lactate dehydrogenase (cytosol)	(nmol/min per mg of protein)	394 $\pm$ 16	777 $\pm$ 24	173 $\pm$ 5	59 $\pm$ 4
	Yield (%)	(100)	88 $\pm$ 4	3 $\pm$ 1	6 $\pm$ 1
Galactosyltransferase (Golgi membrane)	(pmol/min per mg of protein)	71.8 $\pm$ 2.9	10.1 $\pm$ 1.7	447.3 $\pm$ 10.8	47.4 $\pm$ 0.1
	Yield (%)	(100)	6 $\pm$ 1	42 $\pm$ 2	27 $\pm$ 1
[3 H]fMet-Leu-Phe binding (plasma membrane)	(pmol/mg of protein)	1.53 $\pm$ 0.15	0.00 $\pm$ 0.07	10.95 $\pm$ 0.14	1.33 $\pm$ 0.11
	Yield (%)	(100)	0 $\pm$ 2	48 $\pm$ 5	36 $\pm$ 5
Lysozyme (specific and azurophilic granules)	(A_{450} /min per mg of protein)	0.142 $\pm$ 0.003	0.007 $\pm$ 0.004	0.245 $\pm$ 0.067	0.304 $\pm$ 0.010
	Yield (%)	(100)	2 $\pm$ 1	12 $\pm$ 3	87 $\pm$ 2
β -Glucuronidase (azurophilic granules)	(nmol/min per mg of protein)	40.9 $\pm$ 1.6	3.7 $\pm$ 0.3	17.7 $\pm$ 0.9	80.0 $\pm$ 2.1
	Yield (%)	(100)	4 $\pm$ 1	3 $\pm$ 1	80 $\pm$ 4
DNA (nuclei)	(μ g/mg of protein)	126 $\pm$ 3	16 $\pm$ 2	162 $\pm$ 4	264 $\pm$ 8
	Yield (%)	(100)	6 $\pm$ 1	9 $\pm$ 1	85 $\pm$ 2
[125 I]Di-iodosulphanilic acid diazonium salt (plasma membrane)	(pmol/mg of protein)	52 $\pm$ 2.4	14 $\pm$ 0.2	146 $\pm$ 0.2	33 $\pm$ 0.4
	Yield (%)	(100)	10	50	18.7

Table 2. *Distribution of biochemical markers between subcellular fractions in cells stimulated with fMet-Leu-Phe*
The results are taken from two similar experiments. In the step-gradient fractionation of [125 I]di-iodosulphanilic acid diazonium-labelled cells, the distribution of protein and β -glucuronidase was also determined, and closely resembled the presented distribution obtained for the unlabelled cells.

		High-speed supernatant	Light particulate fraction	Heavy particulate fraction
Protein	Control cells	43 $\pm$ 1	8 $\pm$ 1	37 $\pm$ 1
	Stimulated cells	43 $\pm$ 1	6 $\pm$ 2	38 $\pm$ 1
Lipid phosphorus	Control cells	1 $\pm$ 2	34 $\pm$ 2	46 $\pm$ 3
	Stimulated cells	0 $\pm$ 3	39 $\pm$ 1	47 $\pm$ 1
fMet-Leu-Phe binding	Control cells	1 $\pm$ 1	48 $\pm$ 3	31 $\pm$ 5
	Stimulated cells	2 $\pm$ 3	47 $\pm$ 6	32 $\pm$ 6
β -Glucuronidase	Control cells	3 $\pm$ 1	6 $\pm$ 1	76 $\pm$ 4
	Stimulated cells	3 $\pm$ 1	8 $\pm$ 1	77 $\pm$ 5
[125 I]Di-iodosulphanilic acid diazonium salt	Control cells	10 $\pm$ 1	50 $\pm$ 2	19 $\pm$ 2
	Stimulated cells	7 $\pm$ 2	58 $\pm$ 2	22 $\pm$ 2

of the step gradient in a manner very similar to the native plasma-membrane markers that we have used (obviously the 125 I-labelled membranes could not be used in experiments concerned with lipid metabolism, so binding measurements with radioactive ligands were routinely used in this work).

We have not examined the distribution of biochemical markers for endoplasmic reticulum or mitochondria, both of which are very sparse in

rabbit neutrophils. The neutrophil is a fully differentiated cell with little synthetic activity (we have attempted to measure protein synthesis in our cells and found less than 0.8 pmol of [3 H]lysine incorporated/ 10^6 cells per h at 37°C). In this light it is not surprising that endoplasmic reticulum is present in small amounts, whether detected by electron microscopy (Bainton *et al.*, 1971) or by enzyme markers [we measure 0.68 $\pm$ 0.13 μ mol/min per mg of pro-

tein for NADPH-cytochrome *c* reductase in the homogenate, as assayed by the method of Sottocasa *et al.* (1967)]; nevertheless Golgi cisternae are readily observed by electron microscopy. We have found the activity of the mitochondrial marker enzyme succinate dehydrogenase (Earl & Korner, 1965) to be $0.96 (\pm 0.13)$ nmol/min per mg of protein in the homogenate, and few mitochondria are seen in thin sections (Bainton *et al.*, 1971).

The data of Table 1 indicate that the high-speed supernatant contains largely cytosol material. The light particulate fraction contains most of the Golgi material and the plasma membrane, and the heavy particulate fraction comprises largely the granules (both classes) and nuclei, though with appreciable contamination by plasma membrane and Golgi. The distribution of biochemical markers on the step gradient is not significantly affected by stimulation of the cells with fMet-Leu-Phe (10 nM) (Table 2).

Light membranes derived from cells that had been labelled with [125 I]di-iodosulphanilic acid diazonium salt were further fractionated by analytical sucrose-density-gradient centrifugation. Fig. 1 shows the results of such a fractionation in which the data are in the form of frequency-density histograms (Beaufay *et al.*, 1964). Plasma membranes (containing 125 I-labelled trichloroacetic acid-precipitable radioactivity) form a discrete peak centred at around 1.135, and in general account for the majority of the protein. The contaminating β -glucuronidase present in the light membrane fraction derived from the step gradient is clearly separated. A similar fractionation of light membranes derived from 32 P-labelled cells is illustrated in Fig. 2. The distribution of the fractions having binding activity for fMet-Leu-Phe closely resembles that for the plasma-membrane marker, illustrated in Fig. 1. On stimulation the density of the fractions binding fMet-Leu-Phe increased somewhat (Fig. 2b): a parallel increase in the density of fractions labelled with [125 I]di-iodosulphanilic acid diazonium salt after treatment of labelled cells with fMet-Leu-Phe also occurred (results not shown). This may reflect a small amount of fusion of granule membranes with plasma membrane [even in the absence of cytochalasin B, fMet-Leu-Phe causes a small degree of exocytotic secretion (Bennett *et al.*, 1980b)], or possibly attachment of clathrin to the cytoplasmic surface of the membrane before internalization of the fMet-Leu-Phe receptor, which occurs 2–5 min after stimulation (Niedel *et al.*, 1979). The marker for Golgi membranes (galactosyltransferase) is found as a fairly broad peak in the gradient, possibly representing the somewhat heterogeneous nature of this organelle.

Distribution of phosphatidylinositol hydrolysis and phosphatidate formation in subcellular fractions

The above fractionation procedure was used to

Fig. 1. Frequency-density histograms of some membrane markers from rabbit neutrophil light particulate fraction analysed by linear-sucrose-density-gradient centrifugation

examine the subcellular location of the changes in lipid metabolism after stimulation of rabbit neutrophils with fMet-Leu-Phe (10 nM).

The results of Table 3 indicate that the extent of fMet-Leu-Phe-induced phosphatidylinositol hydrolysis observed for intact cells is also observed in their homogenate. The small increase in specific radioactivity in phosphatidylinositol (relative to total lipid phosphorus) might be because the nuclear and granule material that sediments along with unbroken cells is relatively depleted in labelled phosphatidylinositol.

Phosphatidylinositol hydrolysis is apparent only in the light and not in the heavy particulate fraction. In principle, one would expect to see an enrichment of breakdown in the light fraction compensating for the decreased breakdown elsewhere. However, we find there is a greater proportion of phosphatidylinositol in the light compared with the heavy fraction, the percentages of the total phospholipid being 4.5 ± 0.3 ($n = 5$) and 2.9 ± 0.4 ($n = 5$) respectively [this also explains why the specific radioactivity of light-fraction phosphatidylinositol (relative to total lipid phosphorus) for control samples is higher than that in the heavy fraction]. This means that the expected degree of enrichment of hydrolysis is not great, and probably is too small for detection.

Fig. 2. Frequency-density histograms of some membrane markers and labelling of phosphatidate from resting and stimulated neutrophils
(a) Control cells; (b) cells stimulated with fMet-Leu-Phe (10 nM).

Table 3. Subcellular distribution of [^3H]glycerol-labelled phosphatidylinositol in control and stimulated cells

	Radioactivity in phosphatidylinositol (d.p.m./nmol of total lipid phosphorus)		Ratio of stimulated/ control $\times 100$ (%)
	Control cells	Stimulated cells	
Cells	26.8 ± 0.9	21.8 ± 2.4	81 ± 9
Homogenate	34.6 ± 0.6	28.2 ± 1.0	82 ± 3
Light particulate fraction	31.9 ± 1.5	25.3 ± 2.3	79 ± 8
Heavy particulate fraction	20.0 ± 0.7	20.5 ± 0.8	103 ± 5

We do not show data for the high-speed supernatant where the low amounts of lipid phosphorus present (see Table 1) (which is presumably due to contamination during reorientation of the sucrose gradient on the vertical rotor) reduced the accuracy of the specific radioactivity to the point where our data were uninterpretable.

In these experiments the extent of phosphatidylinositol hydrolysis measured in intact cells was less than previously reported (Cockcroft *et al.*, 1981). This is because cytochalasin B was omitted from the incubations in the present experiments [phosphatidylinositol hydrolysis due to fMet-Leu-Phe (10 nM) is $12.5 \pm 1.5\%$ ($n = 12$) in the absence and $34 \pm 1.3\%$ ($n = 12$) in the presence of cytochalasin B ($5 \mu\text{g/ml}$)]. We omitted the cytochalasin B so as to prevent the fusion of the plasma membrane

with that of the granules which occurs during exocytosis and which leads to the release of 20% of the total cell protein into the surrounding medium. Under circumstances in which exocytotic secretion is permitted we find that the majority of the plasma-membrane marker (fMet-Leu-Phe binding) occurs in the heavy particulate fraction so that the comparison of lipid changes in the different fractions that occur on stimulation cannot be meaningfully interpreted. In the absence of cytochalasin B we find that the stimulation of neutrophils with fMet-Leu-Phe (10 nM) does not change the distribution of the marker enzymes on the step gradient (Table 2).

An alternative method of detecting phosphatidylinositol hydrolysis is to study the appearance of the intermediates of phosphatidylinositol resyn-

Table 4. Subcellular distribution of [^{32}P]phosphatidate in control and stimulated cells

	Radioactivity in phosphatidate (d.p.m./nmol of total lipid phosphorus)		Ratio of stimulated/ control $\times 100$ (%)
	Control cells	Stimulated cells	
Cells	6.9 ± 0.5	13.8 ± 0.3	199 ± 15
Homogenate	6.5 ± 0.3	12.7 ± 0.6	195 ± 14
Light particulate fraction	7.4 ± 0.2	16.6 ± 0.9	225 ± 7
Heavy particulate fraction	3.8 ± 0.3	7.5 ± 0.5	197 ± 20

thesis. The immediate product of phosphatidylinositol hydrolysis, diacylglycerol, is rapidly phosphorylated to phosphatidate. In cells that have been pre-incubated with [^{32}P]P_i, the phosphatidate thus formed is labelled to high specific radioactivity. Table 4 shows the distribution of [^{32}P]phosphatidate after stimulation of neutrophils with 10 nM fMet-Leu-Phe for 1 min. The light fraction contains the most metabolically active pool of phosphatidate in both control and stimulated cells.

Localization of phosphatidate formation

To localize these lipid changes more precisely, we subjected the light particulate fraction from ^{32}P -labelled cells to analytical sucrose-density-gradient centrifugation. The results are shown in Fig. 2, together with data, described above, defining Golgi and plasma membranes.

^{32}P -Labelling of phosphatidate in unstimulated cells is broadly spread out in the gradient. It appears not to correlate with plasma membrane (fMet-Leu-Phe binding), but approximates to the shape and position of Golgi membranes (galactosyltransferase). Presumably ^{32}P incorporation into phosphatidate in resting cells occurs mainly as an intermediate in lipid synthesis, and in rabbit neutrophils (where there is little endoplasmic reticulum) the lipid synthetic machinery is probably principally localized in the Golgi.

In stimulated cells the profile of [^{32}P]phosphatidate is considerably altered. It now reaches a peak at the same sucrose density as fMet-Leu-Phe binding, although with a detectable shoulder at the position of galactosyltransferase, presumably ascribable to the labelling activity observed in control cells (which would account for only about one-third of the total radioactivity in this experiment). Thus the increment of increased [^{32}P]phosphatidate labelling in fMet-Leu-Phe-stimulated cells appears to be localized at the plasma membrane.

Conclusion

If one makes the assumption that phosphatidylinositol hydrolysis in stimulated neutrophils occurs in the same subcellular site as the conse-

quent phosphatidate labelling, then that site is the plasma membrane. We cannot, of course, exclude the possibility that a small proportion of the phosphatidate labelling could occur as a consequence of polyphosphoinositide breakdown (Michell *et al.*, 1981). The assumption is probably justified, since there is no evidence that cells possess a mechanism for rapid transport of diacylglycerol (the immediate product of phosphatidylinositol hydrolysis) from one membrane to another; furthermore the enzyme diacylglycerol kinase, which is responsible for phosphatidate synthesis from diacylglycerol, is thought to be present at the plasma membrane (Michell, 1975).

Studies of the intracellular site of phosphatidylinositol hydrolysis and/or phosphatidate synthesis in other tissues have led to various conclusions. These events have been thought to occur at the secretory vesicles in islets of Langerhans (Clements *et al.*, 1977) and synaptosomes (Pickard & Hawthorne, 1978), at the rough endoplasmic reticulum in exocrine pancreas (Hokin-Neaverson, 1977) and to be distributed throughout the cellular membranes in hepatocytes (Kirk *et al.*, 1981) and in adrenal medulla (Azila & Hawthorne, 1982). In lymphocytes (Fisher & Mueller, 1971) and avian salt gland (Hokin-Neaverson, 1977) the site of the lipid changes were found to be the plasma membrane, in agreement with the present study.

In the case of islets of Langerhans and synaptosomes, cell stimulation will have been accompanied by exocytotic secretion involving extensive fusion between secretory vesicle membranes and plasma membranes. In neither study do the authors demonstrate that their cell fractionation procedure (in each case defined for unstimulated cells) remains valid after secretion has occurred. Our own data on neutrophils show that after secretion has occurred [i.e. in the presence of cytochalasin B (Bennett *et al.*, 1980b)] most of the plasma membrane marker (fMet-Leu-Phe binding) is found in the heavy particulate fraction, which in unstimulated cells is the fraction containing the secretory granules. Thus the finding of phosphatidylinositol breakdown in a secretory vesicle fraction (Clements *et al.*, 1977; Pickard & Hawthorne, 1978) may not necessarily

preclude the possibility that it occurs at the plasma membrane.

In the study on hepatocytes (Kirk *et al.*, 1981) it is suggested that the lack of apparent localization of phosphatidylinositol hydrolysis may be due to rapid redistribution of phosphatidylinositol throughout the cell by lipid exchange proteins. A similar explanation could account for the localization at the endoplasmic reticulum of phosphatidylinositol breakdown in pancreas (Hokin-Neaverson, 1977), since a majority of the total phosphatidylinositol was found in the rough endoplasmic reticulum in those cells.

The possibility that hydrolysis of phosphatidylinositol occurs as an intermediate reaction, after stimulation of cell-surface receptors, in the mobilization of cytosol Ca^{2+} has been widely discussed (Michell, 1975; Michell *et al.*, 1977, 1981). In some tissues [though the neutrophils do not fall into this category (Cockcroft *et al.*, 1981)] it has been found that the extent of phosphatidylinositol hydrolysis is tightly coupled to the degree of receptor occupancy, leading to the proposal that phosphatidylinositol hydrolysis probably has a plasma-membrane location. Although our results are in agreement with this proposition, we should stress that in neutrophils both phosphatidylinositol hydrolysis and phosphatidate formation are consequences of the rise in cytosol $[\text{Ca}^{2+}]$ that occurs after stimulation (Cockcroft *et al.*, 1980, 1981). There is therefore no reason *a priori* for expecting these reactions to occur at the plasma membrane, nor is it likely that either of them are relevant to the mechanism of Ca^{2+} mobilization.

References

- Allan, D., Thomas, P. & Limbrick, A. R. (1980) *Biochem. J.* **188**, 881–887
- Amrein, P. C. & Stossel, T. P. (1980) *Blood* **56**, 442–447
- Azila, N. & Hawthorne, J. N. (1982) *Biochem. J.* **204**, 291–299
- Bainton, D. F., Ullyot, J. O. & Farquhar, M. G. (1971) *J. Exp. Med.* **134**, 907–934
- Bainton, D. F., Nichols, B. A. & Farquhar, M. G. (1976) in *Lysosomes in Biology and Pathology* (Dingle, J. T. & Dean, R. T., eds.), vol. 5, Chapter 1, pp. 3–32, Elsevier, Amsterdam
- Beaufay, H., Jaques, P., Baudhuin, P., Selinger, O. Z., Berthet, J. & de Duve, C. (1964) *Biochem. J.* **92**, 184–205
- Becker, E. L. (1973) in *Mechanisms in Allergy* (Goodfriend, L., Schon, A. H. & Orange, R. P., eds.), pp. 339–356, Decker, New York
- Bennett, J. P. (1982) in *Techniques in Lipid and Membrane Biochemistry* (Hesketh, T. R. & Metcalfe, J. C., eds.), vol. 84/1, pp. 1–22, Elsevier, Amsterdam
- Bennett, J. P., Hirth, K. P., Fuchs, E., Sarvas, M. & Warren, G. B. (1980a) *FEBS Lett.* **116**, 57–61
- Bennett, J. P., Cockcroft, S. & Gomperts, B. D. (1980b) *Biochim. Biophys. Acta* **601**, 584–591
- Clements, R. S., Rhoten, W. B. & Starnes, W. R. (1977) *Diabetes* **26**, 1109–1116
- Cockcroft, S. & Gomperts, B. D. (1979) *Biochem. J.* **178**, 681–687
- Cockcroft, S., Bennett, J. P. & Gomperts, B. D. (1980) *Nature (London)* **288**, 275–277
- Cockcroft, S., Bennett, J. P. & Gomperts, B. D. (1981) *Biochem. J.* **200**, 501–508
- Crompton, M. J. & Snary, D. (1974) *Contemp. Top. Mol. Immunol.* **3**, 27–56
- Earl, D. C. N. & Korner, A. (1965) *Biochem. J.* **94**, 721–734
- Edwards, R. M., Kempson, S. A., Carlson, G. L. & Dousa, T. P. (1979) *Biochim. Biophys. Acta* **553**, 54–65
- Fisher, D. B. & Mueller, G. C. (1971) *Biochim. Biophys. Acta* **248**, 434–448
- George, J. N., Potterf, R. D., Lewis, P. C. & Sears, D. A. (1976) *J. Lab. Clin. Med.* **88**, 232–246
- Hino, Y., Asano, A., Sato, R. & Shimizu, S. (1978) *J. Biochem. (Tokyo)* **83**, 909–923
- Hokin-Neaverson, M. (1977) *Adv. Exp. Med. Biol.* **83**, 429–446
- Karsten, U. & Wollenberger, A. (1972) *Anal. Biochem.* **46**, 135–148
- Kirk, C. J., Michell, R. H. & Hems, D. A. (1981) *Biochem. J.* **194**, 155–165
- Michell, R. H. (1975) *Biochim. Biophys. Acta* **415**, 81–148
- Michell, R. H., Jafferji, S. & Jones, L. M. (1977) *Adv. Exp. Med. Biol.* **83**, 447–465
- Michell, R. H., Kirk, C. J., Jones, L. M., Downes, C. P. & Creba, J. A. (1981) *Philos. Trans. R. Soc. London Ser. B* **296**, 123–137
- Niedel, J. E., Kahane, I. & Cuatrecasas, P. (1979) *Science* **205**, 1412–1414
- Pickard, M. R. & Hawthorne, J. N. (1978) *J. Neurochem.* **30**, 145–155
- Putney, J. W., Jr., Weiss, S. J., van de Walle, C. M. & Haddas, R. A. (1980) *Nature (London)* **284**, 345–347
- Serhan, C., Anderson, P., Goodman, E., Dunham, P. & Weissmann, G. (1981) *J. Biol. Chem.* **256**, 2736–2741
- Sha'afi, R. I., Naccache, P., Raible, D., Krepcio, A., Showell, H. J. & Becker, E. L. (1976) *Biochim. Biophys. Acta* **448**, 638–641
- Sha'afi, R. I., Williams, K., Wacholtz, M. C. & Becker, E. L. (1978) *FEBS Lett.* **91**, 305–309
- Shirley, P. S., Wang, P., DeChatelet, L. R. & Waite, M. (1976) *Biochem. Med.* **15**, 289–295
- Skipiski, V. P., Peterson, R. F. & Barclay, M. (1964) *Biochem. J.* **90**, 374–378
- Sottocasa, G. L., Kuylenstierna, B., Ernster, L. & Bergstrand, A. (1967) *J. Cell Biol.* **32**, 415–428
- Tyson, C. A., van Zande, H. & Green, D. E. (1976) *J. Biol. Chem.* **251**, 1326–1332