Phospholipid metabolism in human neutrophils activated by *N*-formyl-methionyl-leucyl-phenylalanine

Degranulation is not required for release of arachidonic acid: studies with neutrophils and neutrophil-derived cytoplasts

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Neutrophils respond to chemoattractants by aggregating, degranulating, remodelling of phospholipids and releasing arachidonic acid. To determine whether ligand-induced remodelling of phospholipids depends on redistribution of intracellular organelles (degranulation), we compared phospholipid remodelling of human neutrophils with that of neutrophil-derived cytoplasts. Cytoplasts, organelle-depleted vesicles of cytosol surrounded by plasmalemma, cannot degranulate. Without a stimulus, [³H]arachidonate was incorporated preferentially into phosphatidylinositol (PI) and phosphatidylcholine (PC). Exposure of cytoplasts and neutrophils prelabelled with [³H]arachidonate or [¹⁴C]glycerol to fMet-Leu-Phe (10⁻⁷ M) induced rapid changes in distribution of label and mass of individual phospholipids: [³H]arachidonate in phosphatidic acid (PA) increased 500% (120 s), [¹⁴C]glycerol incorporation and mass of PA approached 200% of unstimulated values, and [³H]arachidonate in PI decreased continuously; these data are compatible with activity of a PI/PA cycle. However, the mass of PI in both preparations and [¹⁴C]glycerol label in intact neutrophils increased initially (5 s), suggesting net synthesis and mobilization of more than one pool of PI. Heterogeneity of PC pools was also observed: [³H]arachidonate was lost from PC immediately upon addition of stimulus, whereas mass and [¹⁴C]glycerol values increased. Thus, net phospholipid synthesis, redistribution of arachidonate and activation of the PI/PA cycle are immediate responses of the neutrophil to receptor occupancy by chemoattractants. Furthermore, the similarity in response to fMet-Leu-Phe of neutrophils and granule-free cytoplasts indicates that these processes are independent of degranulation.

INTRODUCTION

Neutrophils respond to a variety of stimuli with extensive remodelling of membrane phospholipids [1,2,32] accompanied by release of arachidonic acid [10]. Arachidonate is oxygenated by lipoxygenases to active products which influence neutrophil function, e.g. chemotaxis [3], degranulation [4] and ion movements [5]. Thus, arachidonic acid metabolites not only mediate inflammatory responses but also enhance stimulus-response coupling of the human neutrophil. Two pathways have been proposed for mobilization of arachidonate from membrane phospholipids: either directly via phospholipase A₂, to yield a lysophospholipid and arachidonic acid, or indirectly via phospholipase C followed by lipase action on the resulting diacylglycerol [6]. Alternatively, diacylglycerol is phosphorylated by a kinase to generate PA as part of the PI/PA cycle [7].

When human neutrophils are stimulated with opsonized zymosan or the calcium ionophore A23187, arachidonic acid is selectively released from PI and PC [10]. Neither lysoPI nor lysoPC were detected in such experiments, indicating rapid reacylation of the lyso-compounds by fatty acids via the 'Lands' pathway [13]. In platelets,

arachidonate may be released from diacylglycerol by diacylglycerol lipase [6] following degradation of PI by PI-specific phospholipase C [30].

In the human neutrophil, rapid and selective changes in endogenous phospholipids are evoked by a variety of stimuli. In response to ligands such as fMet-Leu-Phe, the calcium ionophore A23187, and phorbol myristate acetate, changes have been found in phospholipid mass, [³H]arachidonic acid and ³²P [2,32,34]. However, these studies have not rigorously documented the changes in the specific activity of individual phospholipids early in the course of receptor occupancy, nor, indeed, whether these changes are dependent on the primary functional response of the neutrophil: degranulation. In human neutrophils, a phospholipase A₂ is associated with plasmalemma [9] as well as the membranes of the granules [8] and in rabbit neutrophils, phospholipase A₂ is secreted from the granules in response to fMet-Leu-Phe [33].

To eliminate the confusion between degranulation and ligand-induced lipid remodelling *per se*, we prepared organelle-depleted cytoplasts from human neutrophils. Cytoplasts are granule-free vesicles of cytoplasm surrounded by an intact plasma membrane, which can phagocytose, generate superoxide anion and H₂O₂ and

Abbreviations used: fMet-Leu-Phe, *N*-formyl-methionyl-leucyl-phenylalanine; PA, phosphatidic acid; PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; BSA, bovine serum albumin.

consume oxygen on stimulation [14,15]. Thus, stimulus-response coupling mechanisms remain intact for each of these functions.

In the studies reported below, we examined the extent to which neutrophil granule phospholipases and the primary functional response of the cell (degranulation) are involved in phospholipid remodelling. Consequently, we compared incorporation and redistribution of [^3H]arachidonic acid as well as changes in the [^{14}C]glycerol content and mass of individual phospholipids of stimulated human neutrophils with similar ligand-induced changes in cytoplasts.

MATERIALS AND METHODS

Preparation of neutrophil suspensions

Cell suspensions were prepared by means of Hypaque/Ficoll gradients [16] followed by dextran sedimentation and hypo-osmotic lysis of erythrocytes. Cells were suspended in pH 7.45 Hepes buffer (150 mM- Na^+ , 5 mM- K^+ , 1.3 mM- Ca^{2+} , 1.2 mM- Mg^{2+} , 155 mM- Cl^- , 10 mM-Hepes and 5.5 mM-glucose).

Preparation of cytoplasts

Cytoplasts were prepared from human neutrophils suspended in 12.5% (w/v) Ficoll 70 containing cytochalasin B (5 $\mu\text{g}/\text{ml}$) [14]. The cells were layered on a discontinuous Ficoll gradient and centrifuged for 30 min at 35 °C. The band of cytoplasts obtained was harvested and washed three times to remove cytochalasin B.

Labelling of neutrophils and cytoplasts

[^3H]Arachidonic acid in 0.1% ethanol was diluted in Hepes buffer containing 0.01% BSA, and added at a concentration of $(3-5) \times 10^5$ c.p.m./ 30×10^6 cells or equal number of cytoplasts. The same quantity of unlabelled arachidonic acid was added to the cells and cytoplasts used for studies in which only phospholipid mass was determined. After 30 min of incubation at 37 °C, cells were washed twice in Hepes/BSA at 4 °C and resuspended in buffer. Uptake of [^3H]arachidonic acid in neutrophils and cytoplasts was 65% and 62% respectively. [^{14}C]Glycerol in water was diluted in Hepes buffer and added at a concentration of $(3-5) \times 10^5$ c.p.m./ 30×10^6 cells or equal number of cytoplasts, followed by 30 min incubation at 37 °C.

Enzyme assays

Lactate dehydrogenase, a cytoplasmic enzyme, was determined by measuring the supernatant of suspensions lysed with Triton X-100 (0.2%, v/v) [17]. Lactate dehydrogenase activity was measured using Statzyme (Worthington Biochemical Co., Freehold, NJ, U.S.A.). The granule enzymes lysozyme and β -glucuronidase were measured as described previously [18]; β -glucuronidase was determined by incubation with phenolphthalein glucuronidate as substrate, and lysozyme was determined as the rate of lysis of *Micrococcus lysodeikticus*. Activities of rotenone-insensitive NADH-cytochrome *c* reductase (a microsomal marker) and succinate-cytochrome *c* reductase (a mitochondrial marker) were measured by continuous recording of reduction of ferricytochrome *c* (horse heart type III; Sigma Chemical Co.) in the presence of 0.1 mM-NADH or 3 mM-succinate. Assay mixture contained 0.1 mM-cytochrome *c*, 0.3 mM-KCN,

50 mM-phosphate buffer and, when indicated, 1.5 μM -rotenone [19,20]. Aliquots of neutrophils or cytoplasts were suspended in water and cooled on ice for 60 min. Cells or cytoplasts were added to assay mixture and kept at room temperature for 30 min to deplete endogenous substrate. The reaction was started by addition of exogenous substrate.

Degranulation

The extracellular release from cytochalasin B-treated neutrophils and cytoplasts of the azurophil granule marker elastase was monitored continuously by determining the release of *p*-nitrophenol from the substrate *N*-t-butoxycarbonyl-L-alanine *p*-nitrophenyl ester as described in [39]. Neutrophils ($1.25 \times 10^6/\text{ml}$) or an equivalent number of cytoplasts were preincubated at 37 °C for 5 min with 5 μg of cytochalasin B/ml. The reaction was started by the addition of 10^{-7} M-fMet-Leu-Phe to the sample cuvette. *p*-Nitrophenol was hydrolysed from the substrate at 430.1 nmol/min per mg of protein by stimulated neutrophils, versus 17.1 nmol/min per mg of protein by stimulated cytoplasts [15,37].

Neutrophil activation and phospholipid analysis

Aliquots of neutrophils or cytoplasts in 0.9 ml of buffer were incubated at 37 °C for 5 min before addition of 0.1 ml of buffer containing fMet-Leu-Phe (10^{-7} M) or buffer alone (control). All incubations were performed in duplicate or triplicate, precisely timed and stopped by rapid addition of 3.5 ml of chloroform/methanol (2:5, v/v) before lipid extraction [21]. For the ^{14}C -labelled suspensions, the chloroform phase of extracted lipids was backwashed twice with 0.2 vol. of methanol and 0.2 vol. of unlabelled glycerol (1 mM) to remove any [^{14}C]glycerol not incorporated. Two-dimensional t.l.c. was performed with chloroform/methanol/ NH_4OH (65:25:6, by vol.) as solvent system for the first dimension, and, after drying under N_2 , chloroform/acetone/methanol/acetic acid/water (3:4:1:1:0.5, by vol.) in the second dimension. To determine label content, phospholipids were identified, scraped and placed in scintillation vials containing 10 ml of Filtron X (National Diagnostics) and radioactivity was determined in a Beckman model LS 7000 scintillation counter. For analysis of mass, phospholipids were digested with 70% HClO_4 at 180 °C for 30 min [21]. Phospholipid content was measured by phosphorus determination [11,21,27] with a sensitivity of 0.15 ± 0.05 nmol of P.

Protein

Protein was determined by the method of Lowry *et al.* [12] using lysozyme as the standard.

Reagents

Cytochalasin B (Aldrich Chemical Co.) and fMet-Leu-Phe (Vega Biochemicals, Tucson, AZ, U.S.A.) were each prepared as concentrated stock solutions in dimethyl sulphoxide (Sigma) and diluted in Hepes buffer. Standards for lipid analysis and t.l.c. supplies were from Supelco, Bellefonte, PA, U.S.A. T.l.c. solvents were of pesticide grade (Nanograde) purchased from Mallinckrodt, St. Louis, MO, U.S.A. [^3H]Arachidonic acid (83.9 Ci/mmol) was from New England Nuclear, and [^{14}C]glycerol (170 mCi/mmol) was from Amersham International. BSA was from Sigma.

Table 1. [³H]Arachidonic acid labelling and endogenous phospholipid content of resting human neutrophils and neutrophil-derived cytoplasts

For labelling experiments, neutrophils and cytoplasts were prelabelled with 0.5 μ Ci of [³H]arachidonic acid/ 30×10^6 neutrophils or 90×10^6 cytoplasts for 30 min at 37 °C. Distribution of label was determined as described in the Materials and methods section. Results are expressed as percentage of label in each compound relative to total counts recovered ($\pm$ S.E.M., $n = 4$). Total counts recovered include free fatty acids. Phospholipids were quantified as described in the Materials and methods section and expressed as percentage of total phosphorus recovered ($\pm$ S.E.M., $n = 4$). Actual d.p.m. of ³H or nmol of phosphorus/mg of protein are given in the legends to Figs. 1–5.

Phospholipid	[³ H]Arachidonic acid (%)		Lipid phosphorus (%)	
	Neutrophils	Cytoplasts	Neutrophils	Cytoplasts
PA	0.3 (0.1)	0.5 (0.1)	0.5 (0.1)	1.6 (0.2)
PI	66.1 (2.8)	62.1 (2.1)	7.2 (0.3)	7.6 (0.3)
PC	20.1 (1.5)	21.0 (1.4)	39.2 (2.1)	41.5 (1.2)
PE	7.8 (1.1)	7.6 (0.9)	32.1 (0.7)	30.7 (1.0)
PS	2.6 (0.1)	3.1 (0.5)	11.8 (0.4)	10.4 (0.7)

Table 2. Enzyme activity in neutrophils and cytoplasts

Results are of a representative experiment of three or more.

Enzyme	Activity	
	Neutrophils	Cytoplasts
Lactate dehydrogenase*	47.7	25.9
β -Glucuronidase†	69 200	15 380
Lysozyme‡	2490	553
NADH–cytochrome <i>c</i> reductase§	2.42	0.12
Succinate–cytochrome <i>c</i> reductase§	0.19	0.02

* nmol of NAD⁺ reduced/min per 10^6 neutrophils or cytoplasts.

† nmol of phenolphthalein released/ 10^6 neutrophils or cytoplasts

‡ ng of egg white lysozyme/ 10^6 neutrophils or cytoplasts

§ nmol of cytochrome *c* reduced/min per 10^6 neutrophils or cytoplasts

RESULTS

Phospholipid composition and [³H]-arachidonic acid incorporation of resting human neutrophils and cytoplasts

The phospholipid composition of resting cytoplasts was determined and compared with that of intact neutrophils (Table 1). PC and PE accounted for more than 70% of the total phospholipids in resting neutrophils and cytoplasts, whereas lesser amounts of PA, PI and PS were found; these data are in accord with previous studies in human neutrophils [2,22].

Cytoplasts fully retained the capacity to incorporate exogenously added arachidonic acid, demonstrating intact de- and re-acylating systems, as shown by the similarity of distribution of incorporated label between cytoplasts and intact cells (Table 1). [³H]Arachidonic acid was incorporated preferentially in PI and PC in unstimulated neutrophils and cytoplasts. As has been reported for platelets [29,38], [³H]arachidonic acid incorporation into PE was low in both preparations.

Activity of enzymes in neutrophils and cytoplasts

In cytoplasts, the amount of the cytoplasmic marker lactate dehydrogenase was reduced by about one-half from that of intact cells (Table 2), as reported by Roos *et al.* [14]. Although the specific and azurophil granule enzyme lysozyme and the azurophil granule marker

β -glucuronidase were detectable in cytoplasts lysed with 0.2% Triton X-100, release of these enzymes is not elicited by the complete secretagogue fMet-Leu-Phe [15]. Thus, cytoplasts are incapable of degranulation. Ultrastructural studies have shown a small but significant number of mitochondria (524/100) in resting neutrophils and virtually none (6/100) in cytoplasts [15]. In accordance with these studies, activity of the mitochondrial enzyme succinate–cytochrome *c* reductase was very low in intact neutrophils and barely detectable in cytoplasts (Table 2). Activity of the microsomal enzyme rotenone-insensitive NADH–cytochrome *c* reductase was greatly diminished in cytoplasts when compared with neutrophils. The data indicate that cytoplasts are depleted of microsomes and mitochondria.

Activation of prelabelled neutrophils and cytoplasts by fMet-Leu-Phe

Changes in [³H]arachidonic acid and lipid phosphorus.

Prelabelled, washed neutrophils or cytoplasts were exposed to fMet-Leu-Phe (10^{-7} M) for various periods of time during which changes in endogenous phospholipids were also monitored. Labelled PA (Figs. 1a and 1d) increased to nearly 500%, while mass (Figs. 1b and 1e) approached 200% of unstimulated values. Consequently, specific radioactivity (Figs. 1c and 1f), expressed as the

Fig. 1. Phosphatidic acid remodelling in human neutrophils and neutrophil-derived cytoplasts stimulated with 10^{-7} M-fMet-Leu-Phe: time course of changes in mass and $[^3\text{H}]$ arachidonic acid content

Neutrophils and cytoplasts were preincubated (30 min, 37°C) with either $[^3\text{H}]$ arachidonic acid (AA) ($0.5 \mu\text{Ci}/3 \times 10^7$ neutrophils or 9×10^7 cytoplasts) or an equivalent amount of unlabelled arachidonic acid. Cells were washed twice in Hepes/BSA before addition of 10^{-7} M-fMet-Leu-Phe. Ordinates in panels *a, b, d* and *e* are expressed as percentage of d.p.m. of ^3H or nmol of phosphorus/mg of protein ($\pm$ S.E.M.) at zero time [= 100%: (*a*), 233 ± 9 d.p.m., $n = 3$; (*b*), 0.30 ± 0.03 nmol, $n = 5$; (*d*), 326 ± 101 d.p.m., $n = 3$; (*e*), 0.70 ± 0.11 nmol, $n = 4$]. Specific radioactivity (S.A.) (*c* and *f*) represents the ratio of % d.p.m./% nmol of phosphorus. All experiments were performed in duplicate or triplicate. *P* values were determined by one-tailed Student *t*-test compared with control incubations: **P* < 0.05, ***P* < 0.02, ****P* < 0.004.

ratio $[^3\text{H}]$ arachidonic acid/lipid phosphorus, also increased for PA.

Whereas $[^3\text{H}]$ arachidonic acid in PA increased in both preparations, this was not the case for PI. In intact neutrophils (Fig. 2*a*) $[^3\text{H}]$ arachidonic acid was incrementally lost from PI for the entire 2 min. Increase in labelled PA and decrease in labelled PI is consistent with activation of the PI/PA cycle. However, the loss of $[^3\text{H}]$ arachidonate in PI (4000 d.p.m./mg of protein at 120 s) far exceeded the gain in PA (1000 d.p.m./mg of protein at 120 s), indicating that the PI/PA cycle is not a closed cycle. Phospholipase C activity may have been followed by that of diacylglycerol lipase [6] and/or labelled PI was phosphorylated to polyphosphoinositides [26]. After a small, but significant, increase at 5 s, label was lost from cytoplast PI as well (Fig. 2*d*). Loss of $[^3\text{H}]$ arachidonate from PI, however, was not immediately accompanied by loss of mass. After stimulation of intact cells and cytoplasts for 5 and 15 s respectively, endogenous PI (Figs. 2*b* and 2*e*) increased by 5%. These data suggest that more than one pool of PI was mobilized in both neutrophils and cytoplasts. Over the remaining time course (15–120 s), the mass of PI decreased. The specific radioactivity of PI (Figs. 2*c* and 2*f*) returned to baseline after an initial decrease, indicating re-equilibration of labelled and endogenous pools.

Upon stimulation, PC (Figs. 3*a* and 3*d*) showed a loss of approx. 10% of labelled arachidonic acid compared with resting values in both neutrophils and cytoplasts

(0–120 s). In sharp contrast, endogenous PC (Figs. 3*b* and 3*e*) increased rapidly (5% above resting values at 5 s) in both preparations and remained above unstimulated values (8% at 120 s). No lysoPC was found under resting or stimulated conditions, in agreement with previous studies [10], indicating rapid reacylation. However, reacylation of lysoPC alone cannot account for the observed gain in endogenous PC and the data suggest net synthesis. Since $[^3\text{H}]$ arachidonic acid decreased in PC while mass increased, specific radioactivity (Figs. 3*c* and 3*f*) decreased in stimulated neutrophils and cytoplasts.

In PE, $[^3\text{H}]$ arachidonic acid (Fig. 4*a*) increased immediately and greatly (30% of total resting PE at 5 s), in activated neutrophils and remained elevated. Endogenous PE (Fig. 4*b*) increased as well and stayed above unstimulated values, rendering transmethylation [35] unlikely as a pathway for the gain in mass of PC. On addition of fMet-Leu-Phe to cytoplasts, changes in $[^3\text{H}]$ arachidonate as well as mass in PE (Figs. 4*d* and 4*e*) followed the pattern of the intact cell. For both stimulated neutrophils and cytoplasts, the increase in labelled PE was greater than the increase in its mass.

Therefore, specific radioactivity (Figs. 4*c* and 4*f*) increased and remained elevated as well.

Approx. 3% of total recovered $[^3\text{H}]$ arachidonic acid was found in PS in unstimulated neutrophils and cytoplasts, and although label increased at 15 s (Figs. 5*b* and 5*e*), values returned to baseline by 120 s. Endogenous PS (Figs. 5*b* and 5*e*) increased slightly on stimulation and

Fig. 2. Phosphatidylinositol remodelling in human neutrophils and neutrophil-derived cytoplasts stimulated with 10^{-7} M-fMet-Leu-Phe: time course of changes in mass and $[^3\text{H}]$ arachidonic acid content

Experimental conditions are described in Fig. 1. Ordinates in panels *a, b, d* and *e* are expressed as percentage of d.p.m. of ^3H or nmol of phosphorus/mg of protein ($\pm$ s.e.m.) at zero time [= 100%: (*a*), 35000 ± 1060 d.p.m., $n = 4$; (*b*), 3.96 ± 0.15 nmol, $n = 4$; (*d*), 16700 ± 3850 d.p.m., $n = 3$; (*e*), 2.91 ± 0.17 nmol, $n = 3$]. * $P < 0.05$, ** $P < 0.03$, *** $P < 0.006$.

remained elevated, resulting in an overall decrease in specific radioactivity in PS (Figs. 5c and 5f).

Changes in $[^{14}\text{C}]$ glycerol. To examine the extent to which the increase in lipid phosphorus could be attributed to newly synthesized phospholipid, neutrophils and cytoplasts were preincubated with $[^{14}\text{C}]$ glycerol before addition of fMet-Leu-Phe.

Upon stimulation, $[^{14}\text{C}]$ glycerol label in PA nearly doubled from control values in neutrophils (from 650 d.p.m./mg of protein to 1200 d.p.m./mg of protein, 0–120 s) and increased significantly in cytoplasts (Table 3). Specific radioactivity, expressed as the ratio of $[^{14}\text{C}]$ glycerol over lipid phosphorus (Table 4), decreased sharply in the neutrophil during the first 15 s but returned toward baseline by 120 s. Specific radioactivity decreased in PA in the cytoplasm as well, although less extensively than in the intact cell. Decrease in the $[^{14}\text{C}]$ glycerol/lipid phosphorus ratio (0–15 s) points toward increased synthesis of PA via dihydroxyacetone phosphate [24,25] and/or increased recycling of unlabelled diacylglycerol, the breakdown product of phospholipase C. Whereas the pattern of incorporation of labelled glycerol in PA was similar in both preparations, this was not the case in labelled PI (Table 3): 5 s after addition of fMet-Leu-Phe to neutrophils, $[^{14}\text{C}]$ glycerol increased significantly in PI (2500 d.p.m./mg of protein to 2750 d.p.m./mg of

protein, 0–5 s) but decreased rapidly over the remaining time course (2200 d.p.m./mg of protein, 120 s). In the cytoplasm, however, label increased from 1600 d.p.m./mg of protein to 1900 d.p.m./mg of protein (0–120 s), after an initial decrease at 5 s. Thus, opposing specific radioactivity patterns resulted (Table 4): cytoplasmic specific radioactivity decreased at 5 s followed by an increase, whereas in neutrophils an increase at 5 s was followed by a decrease. The rise in lipid phosphorus and $[^{14}\text{C}]$ glycerol content in PI in the neutrophil at 5 s points toward acceleration of synthesis *de novo*. The increase in the specific activity of PA between 15 and 120 s and the reciprocal decrease of the specific activity of PI (15–120 s) are consistent with activity of the PI/PA cycle. Phosphorylation of PI to polyphosphoinositides [26] may also have contributed to loss of $[^{14}\text{C}]$ glycerol label after 15 s of fMet-Leu-Phe stimulation. Specific activity of PI in the cytoplasm rose between 15 and 120 s (Table 4). $[^{14}\text{C}]$ Glycerol-labelled PC and PE increased at 5 s and remained elevated in neutrophils and cytoplasts (Table 3). The ratio of $[^{14}\text{C}]$ glycerol to lipid phosphorus remained relatively unchanged in PC and PE (Table 4) in both preparations. Therefore, the gain of mass in these phospholipid species is most probably due to synthesis *de novo*. ($[^{14}\text{C}]$ Glycerol values for PS were barely above background levels and are therefore omitted from the Table.)

Fig. 3. Phosphatidylcholine remodelling in human neutrophils and neutrophil-derived cytoplasts stimulated with 10^{-7} M-fMet-Leu-Phe: time course of changes in mass and $[^3\text{H}]\text{arachidonic acid content}$

Experimental conditions are described in Fig. 1. Ordinates in panels *a, b, d* and *e* are expressed as percentage of d.p.m. of ^3H or nmol of phosphorus/mg of protein ($\pm$ S.E.M.) at zero time [= 100%]: (*a*), 13160 ± 1990 d.p.m., $n = 4$; (*b*), 20.10 ± 1.97 nmol, $n = 4$; (*d*), 6950 ± 1423 d.p.m., $n = 4$; (*e*), 16.64 ± 0.61 nmol, $n = 4$. * $P < 0.05$, ** $P < 0.03$, *** $P < 0.005$.

DISCUSSION

Similar changes in $[^3\text{H}]\text{arachidonic acid}$, $[^{14}\text{C}]\text{glycerol}$ and lipid phosphorus content were observed both in neutrophils, which are capable of degranulation, and in cytoplasts, which are not. Since the data suggest that phospholipid metabolism of human neutrophils proceeds unimpaired in the absence of granules, it is likely that ligand-induced degranulation, which involves fusion of granule membranes with plasma membrane [36], cannot account for arachidonic acid turnover and phospholipid remodeling in cells exposed to fMet-Leu-Phe.

Studies of arachidonic acid metabolism in platelets indicate two major enzymic mechanisms for release of arachidonate from membrane phospholipids, one mediated by phospholipase A_2 and the other via phospholipase C followed by diacylglycerol lipase [21,29,31,6]. Both mechanisms suggest that there is more than one pool from which arachidonate is derived, and also imply activation of a closed cycle, (i.e. $\text{PI} \rightarrow \text{phosphatidylinositol 4,5-bisphosphate} \rightarrow \text{diacylglycerol} \rightarrow \text{PA} \rightarrow \text{PI}$). Our data are compatible with release of arachidonic acid from the phospholipids of human neutrophils by several pathways. Certainly, these data support the origin of PA from PI: $[^3\text{H}]\text{arachidonate}$ in PA increased rapidly while that in PI decreased. Furthermore, the initial sharp decrease in the ratio of $[^{14}\text{C}]\text{glycerol/lipid phosphorus}$ in PA is compat-

ible with recycling of 1,2-diacylglycerol as a consequence of phospholipase C activity.

Large increases in endogenous PA after fMet-Leu-Phe stimulation of human neutrophils have been reported previously [2,40]. Increase in mass of PI has also been observed in other tissues [24,41,42]. Polyphosphoinositide turnover occurs extremely rapidly in human neutrophils [43,44] and an increase in PI at 5 s may derive, in part, from dephosphorylation of polyphosphoinositides back to PI [45].

The specific radioactivity of PI and PA differed in organelle-depleted cytoplasts from that of neutrophils: it is, therefore, possible that these cell fragments augment synthesis *de novo* to compensate for pools normally available to intact cells.

Nevertheless, our data cannot be entirely explained by invoking phospholipase C activity in the course of PI/PA cycling. For example, loss of $[^3\text{H}]\text{arachidonate}$ from PC without loss of $[^{14}\text{C}]\text{glycerol}$ or mass is compatible with phospholipase A_2 activity and the immediate reacylation by an unlabelled fatty acid of lysoPC. Considering the foregoing, it is likely that ligand-induced changes in phospholipids proceed by a variety of pathways, and that 'closed' systems may reflect the usual oversimplification of an emerging field. This novel suggestion for the neutrophil will require rigorous confirmation with other ligands.

Fig. 4. Phosphatidylethanolamine remodelling in human neutrophils and neutrophil-derived cytoplasts stimulated with 10^{-7} M-fMet-Leu-Phe: time course of changes in mass and $[^3\text{H}]$ arachidonic acid content

Experimental conditions are described in Fig. 1. Ordinates in panels *a, b, d* and *e* are expressed as percentage of d.p.m. of ^3H or nmol of phosphorus/mg of protein ($\pm$ s.e.m.) at zero time [= 100%: (*a*), 5285 ± 628 d.p.m., $n = 3$; (*b*), 15.95 ± 2.81 nmol, $n = 4$; (*d*), 4169 ± 446 d.p.m., $n = 3$; (*e*), 12.33 ± 0.55 nmol, $n = 3$]. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.006$.

Table 3. Phospholipid changes in fMet-Leu-Phe-stimulated neutrophils and cytoplasts labelled with $[^{14}\text{C}]$ glycerol

Neutrophils and cytoplasts were prelabelled with $0.5 \mu\text{Ci}$ of $[^{14}\text{C}]$ glycerol/ 30×10^6 cells or 90×10^6 cytoplasts for 30 min at 37°C . Distribution of label was determined as described in the Materials and methods section. Results are expressed as percentage change in d.p.m. from control incubations (paired samples) $\pm$ s.e.m. ($n = 4$ or more); *** $P < 0.001$, ** $P < 0.02$, * $P < 0.05$. For neutrophils zero time (100%) = 650 d.p.m./mg of protein in PA, 2500 d.p.m./mg of protein in PI, 2000 d.p.m./mg of protein in PC, 1500 d.p.m./mg of protein in PE. For cytoplasts 100% = 360 d.p.m./mg of protein in PA, 1600 d.p.m./mg of protein in PI, 600 d.p.m./mg of protein in PC, and 625 d.p.m./mg of protein in PE.

Time(s)	PA		PI		PC		PE	
	Neutrophils	Cytoplasts	Neutrophils	Cytoplasts	Neutrophils	Cytoplasts	Neutrophils	Cytoplasts
5	108.23** (2.98)	104.86 (3.33)	107.74* (1.53)	91.82 (12.41)	104.96 (3.35)	105.00 (2.72)	103.91 (0.75)	104.23 (14.22)
15	126.25 (8.63)	137.46** (6.69)	97.36 (2.93)	101.05 (11.64)	104.32 (1.71)	104.53 (1.36)	104.31 (2.57)	104.47 (23.67)
120	184.62*** (7.12)	163.23 (11.51)	93.43 (3.19)	111.28 (8.58)	106.05 (0.51)	107.09 (1.36)	103.82 (1.85)	102.19 (18.83)

The net increase in endogenous amounts and $[^{14}\text{C}]$ glycerol labelling of PC suggests that fMet-Leu-Phe induces synthesis of new PC, both in neutrophils and cytoplasts. Transmethylation, by which PE is converted to PC upon cell stimulation [35] is an unlikely explanation for the increase in the mass of PC since neither label nor mass was lost from PE. That PC synthesized *de novo* differs from 'existing' PC in susceptibility to phospholipase A_2 has been shown in platelet preparations in which arachidonate is not incorporated in newly synthesized

phospholipids [23]. Our observation that hydrolysis of $[^3\text{H}]$ arachidonate-labelled PC was not the same as for $[^{14}\text{C}]$ glycerol-labelled PC indicates the presence of heterogeneous pools of PC in neutrophils and their cytoplasts.

$[^3\text{H}]$ Arachidonate of PE increased sharply and immediately in neutrophils and to a lesser extent in cytoplasts. Similar observations have been made in other tissues: in murine bone marrow macrophages, a direct CoA-mediated transfer of arachidonic acid from PC or PI to

Fig. 5. Phosphatidylserine remodelling in human neutrophils and neutrophil-derived cytoplasts stimulated with 10^{-7} M-fMet-Leu-Phe: time course of changes in mass and $[^3\text{H}]$ arachidonic acid content

Experimental conditions are described in Fig. 1. Ordinates in panels *a, b, d* and *e* are expressed as percentage of d.p.m. of ^3H or nmol of phosphorus/mg of protein ($\pm$ S.E.M.) at zero time [= 100%]: (*a*), 1800 ± 280 d.p.m., $n = 4$; (*b*), 6.76 ± 0.28 nmol, $n = 3$; (*d*), 1850 ± 205 d.p.m., $n = 4$; (*e*), 3.79 ± 0.31 nmol, $n = 3$). * $P < 0.05$, ** $P < 0.03$.

Table 4. Specific activity of individual phospholipids in fMet-Leu-Phe-stimulated $[^{14}\text{C}]$ glycerol-labelled neutrophils and cytoplasts

Specific radioactivity represents the ratio of percentage d.p.m. of $[^{14}\text{C}]$ glycerol/percentage nmol of phosphorus. D.p.m./mg of protein for each phospholipid species is given in Table 3; nmol of phosphorus/mg of protein for each phospholipid species is given in Figs. 1–5.

Time(s)	PA		PI		PC		PE	
	Neutrophils	Cytoplasts	Neutrophils	Cytoplasts	Neutrophils	Cytoplasts	Neutrophils	Cytoplasts
5	0.75	0.97	1.04	0.89	0.99	0.98	0.98	1.00
15	0.75	0.99	0.95	0.97	1.02	1.00	0.98	1.02
120	0.97	0.98	0.96	1.19	0.98	0.99	0.99	1.02

exogenously added lysoPE has been reported in which free fatty acid is not an intermediate [28]. An increase in $[^3\text{H}]$ arachidonate in plasmalogen PE, released from PI and PC, has been reported in thrombin-stimulated human platelets [29]. Our data support the presence of similar pathways in neutrophils: acyltransferase activity may account for the immediate and significant increase in labelled PE. In addition, rapid exchange of 2-position fatty acids between PC and PE appears to be induced by fMet-Leu-Phe. PE or lysoPE may therefore act as the phospholipid acceptor of arachidonate that is not used as substrate for lipoxygenases or incorporated in neutral lipids. PS was the least active phospholipid with respect

to $[^3\text{H}]$ arachidonate and $[^{14}\text{C}]$ glycerol incorporation and turnover, but the pattern of PS metabolism in cytoplasts was still comparable with that of neutrophils.

From the rapid changes in specific activity of phospholipids in stimulated neutrophils and cytoplasts, we conclude the following. (1) Ligand-induced degranulation is not required for activation of the enzymes responsible for arachidonic acid turnover (phospholipase C, phospholipase A_2 and the acylating enzymes). This conclusion is based upon the close similarity in patterns of $[^3\text{H}]$ arachidonic acid turnover and mass changes in the cytoplasm, which cannot degranulate, and in the intact cell, which can. (2) On stimulation with fMet-Leu-Phe,

human neutrophils rapidly remodel their membrane phospholipids, a process accompanied by redistribution of labelled arachidonic acid. PI and PC appear to be the major sources of [³H]arachidonate release, while PE appears to be an acceptor. In contrast, an unlabelled pool of fatty acids is preferred for reacylation of lysoPC. (3) Phospholipid synthesis is an immediate response by human neutrophils to stimulation with chemoattractants.

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