Breakdown and synthesis of polyphosphoinositides in fMetLeuPhe-stimulated neutrophils

S. Cockcroft, M.M. Barrowman and B.D. Gomperts

Department of Experimental Pathology, University College London, University Street, London WC1E 6JJ, England

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The interconversions of the inositol-containing lipids (PI, PI-P and PI-P_2) and their products (DG, inositol phosphates and PA) in human and rabbit neutrophils stimulated with fMetLeuPhe and PMA have been examined. PMA causes only the phosphorylation of PI to PI-P whereas fMetLeuPhe causes phosphorylation of both PI and PI-P yielding PI-P_2 and the hydrolysis of all three lipids. While the predominant reaction is breakdown of PI to PA catalysed by phospholipase D, approx. 2% of PI is converted to polyphosphoinositides and then broken down by the phospholipase C route yielding inositol phosphates and DG. The latter reaction occurs without detectable lag and is a function of receptor occupancy. The amount of inositol trisphosphate thus formed would be sufficient to release Ca^{2+} from intracellular stores.

Neutrophil *Receptor* *Polyphosphoinositide* *Inositol phosphate* *Diacylglycerol* *Phospholipase C* *Phospholipase D*

1. INTRODUCTION

Activation of neutrophils with fMetLeuPhe causes aggregation, chemotaxis, exocytosis of the contents of specific and azurophilic granules and a burst of respiratory activity [1,2]. A rise in cytosolic Ca^{2+} has been thought to mediate some of these functional responses [3]. The phorbol ester PMA is also a potent stimulus for the secretion of the specific granules [4,5] and the respiratory burst [6] and this suggests that activation of protein kinase C may mediate these responses [7]. While the rise in cytosol Ca^{2+} is thought to be mediated by IP_3 [8] the activation of protein kinase C is mediated by DG [7]. Both of these are products of phospholipase C action on PI-P_2 consequential on receptor activation [9].

We have characterised in neutrophils a plasma membrane-bound phospholipase C (polyphosphoinositide phosphodiesterase (PPI pde)) which acts on endogenous substrates and we have also demonstrated that the DG thus generated can be phosphorylated to form PA [10]. Although the presence of the PPI pde is clear, we have failed to detect any agonist-induced loss of PPI [10–13]. However, others have reported that this does occur [14–16]. There are extensive changes in PI and PA on activation of neutrophils with fMetLeuPhe but these appear to be a result of phospholipase D action [11].

We now demonstrate that a small fraction of the PI is converted to PPI and hydrolysed by phospholipase C to yield DG and inositol phosphates. It is also shown that the generation of the inositol phosphates is a function of receptor occupancy and this supports the idea that activation of PPI pde is coupled to cell surface activation [9,17].

Abbreviations: PI, phosphatidylinositol; PPI, polyphosphoinositide (includes PI-P, phosphatidylinositol 4-phosphate and PI-P_2, phosphatidylinositol 4,5-bisphosphate); DG, diacylglycerol; PA, phosphatidate; PMA, phorbol myristate acetate; fMet, formylmethionyl; pde, phosphodiesterase; IPs, inositol phosphates (includes IP_2, inositol bisphosphate and IP_3, inositol trisphosphate)

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2. MATERIALS AND METHODS

Human and rabbit neutrophils were obtained as in [2,11] and finally suspended at 10^8 cells/ml in the presence of [^{3}H]inositol (50–100 μCi/ml) for 90 min (rabbit cells) or 150 min (human cells) at 37°C. At the end of this incubation the cells were washed twice in buffer supplemented with 1 mM inositol and 10 mM LiCl and further incubated in this medium for 20 min. LiCl was added to prevent the conversion of inositol phosphates to inositol [18]. A sample (1 ml) of cells was added into tubes containing an equal volume of buffer or agonist as indicated in the individual experiments and the reaction terminated at the indicated times by the addition of acidified chloroform/methanol. Extraction and analysis of lipids and the water soluble components were as described in [19].

For the determination of PPI synthesis, ^{32}P was used to label the rabbit cells for 1 h [11]. The cells were added to tubes containing an equal volume of buffer or fMetLeuPhe (final concentration 10^{-8} M) and stimulated for 1 min. The samples were quenched as described above.

3. RESULTS

The distribution of [^{3}H]inositol activity among the inositol lipids in resting rabbit and human neutrophils is shown in table 1. It is clear that the bulk of the radioactivity is in PI. On stimulation with fMetLeuPhe it was noted that despite the formation of inositol phosphates (table 3), the radioactivity of PPI actually increased (not shown) suggesting that PI was being simultaneously phosphorylated to replenish the PPI pool. Because of the short period used for labelling the neutrophils with [^{3}H]inositol it is likely that the [^{3}H]inositol distribution does not reflect isotopic equilibrium of all the pools of inositol lipid. To circumvent this ambiguity, we used ^{32}P to label the cells.

^{32}P is incorporated into ATP and because the monoester phosphates of the PPI are in equilibrium with ATP it is possible to label the PPI to isotopic equilibrium. The labelling of ATP reaches a plateau by 60 min [11]. Using such labelled neutrophils it was found that the levels of the PPI increased within 1 min of fMetLeuPhe addition (table 2). In contrast to fMetLeuPhe, PMA increased only the levels of PI-P, and this by only a modest amount.

Table 3 illustrates the generation of inositol phosphates in both human and rabbit neutrophils at two different times. It should be stressed that the concentrations of fMetLeuPhe used to stimulate the cells are higher than those required to induce maximal secretion. Earlier efforts to detect inositol phosphate generation at concentrations of fMetLeuPhe optimal for secretion were negative [13] because the dose-response curve is shifted to higher concentrations of the agonist (fig.1). This is

Table 1

Distribution of [^{3}H]inositol activity in the inositol lipids of human and rabbit neutrophils

	Human	Rabbit
PI	94.7 ± 0.8 (4)	88.7 ± 2.2 (5)
PI-P	3.6 ± 1.0 (11)	9.2 ± 1.6 (5)
PI-P_2	1.6 ± 0.5 (4)	2.0 ± 0.6 (5)

Human and rabbit neutrophils (10^8 cells/ml) were incubated with [^{3}H]inositol for 90 or 150 min, respectively. After washing the cells and further incubation for 20 min the distribution of the label in the [^{3}H]inositol lipids was determined. Results are averaged from separate experiments all performed in triplicate (± SE). The number in brackets denotes the number of experiments

Table 2

Increase in the level of polyphosphoinositides on stimulation with fMetLeuPhe or PMA

	PI-P	PI-P_2
Control	(100)	(100)
fMetLeuPhe (10 nM)	131 ± 6 (n = 6)	143 ± 7 (n = 5)
PMA (100 nM)	122 ± 7 (n = 5)	91 ± 6 (n = 5)

Rabbit neutrophils (10^8 cells/ml) were preincubated with ^{32}P for 1 h. Aliquots of cells (50 μl) were incubated with equal volumes of agonist (or medium) for 1 min. The reaction was terminated and the level of PPI determined as described in section 2. Results are pooled from individual experiments all performed in triplicate (± SE). The number in brackets denotes the number of experiments

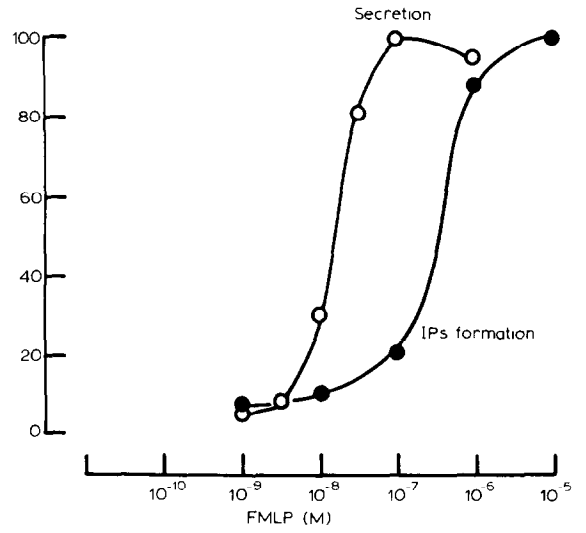

Fig.1. Dose-response relationship between secretion and inositol phosphate generation in human neutrophils. Human neutrophils (10^8 cells/ml prelabelled with [^{3}H]inositol) were stimulated with different concentrations of fMetLeuPhe for 5 min and the production of the total inositol phosphates measured (●—●). Results shown are from a single experiment performed in triplicate and expressed as a percentage of the maximal response. For comparison the dose-response curve for secretion of β-glucuronidase is also shown (○—○) [2].

Table 3

Formation of inositol phosphates in fMetLeuPhe-activated neutrophils

	dpm	
	Human	Rabbit
Control, 30 s	1080 ± 140	1149 ± 176
Control, 5 min	1171 ± 81	1138 ± 28
fMetLeuPhe, 30 s	1476 ± 157	2059 ± 276
fMetLeuPhe, 5 min	1995 ± 85	4693 ± 289

Prelabelled neutrophils were stimulated in the presence or absence of fMetLeuPhe (1 μM for human cells and 0.1 μM for rabbit cells) for either 30 s or 5 min. The generation of total inositol phosphates was measured. Results are shown from a single experiment performed in triplicate (± SD). The dpm in the total inositol lipids in this experiment was 50000. Because of variation between experiments in the number of dpm incorporated in the total inositol lipids, the ratio of stimulated increase in inositol phosphates to the total dpm in inositol lipids was calculated from several experiments. This gives an indication of the extent of inositol lipid hydrolysis. The pooled data of 3 separate experiments show that at 30 s and 5 min the increase in the inositol phosphates could be accounted by hydrolysis of 0.9 (± 0.3)% and 2.4 (± 0.6)% of the inositol lipids, respectively, for human cells. For rabbit cells, data from 4 independent experiments show that at 30 s and 5 min the inositol lipid hydrolysed was 2 (± 0.5)% and 4.7 (± 1.2)%, respectively

precisely what one would expect if inositol phosphate production was coupled to receptor occupancy [9,17]. Fig.2 illustrates the time course of inositol phosphate production: these increase at a constant rate for 3 min after which there is a plateau.

4. DISCUSSION

Our results clearly demonstrate that there is both breakdown and synthesis of PPI on fMetLeuPhe stimulation. Whilst fMetLeuPhe increases the level of PI-P and PI-P_2, PMA appears only to increase the phosphorylation of PI to PI-P. The ability of PMA to increase the levels of the PPI has been reported for platelets [20] and lymphocytes [21]

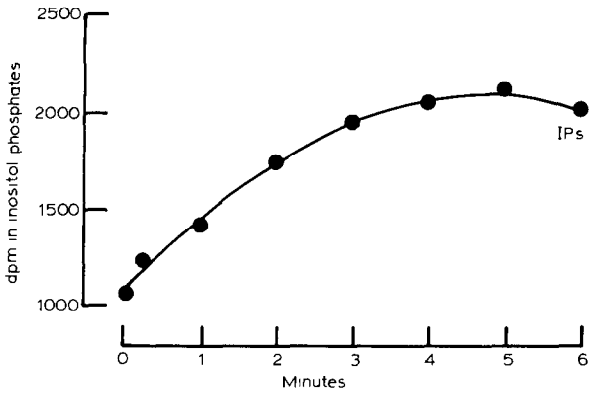

Fig.2. Time course of inositol phosphate generation in human neutrophils stimulated with fMetLeuPhe. [^{3}H]Inositol-labelled human neutrophils were stimulated with 1 μM fMetLeuPhe for the indicated times and the radioactivity in the inositol phosphates determined. A single experiment (one of three) is shown.

and may be indicative of protein kinase C involvement in the activation of inositol lipid kinases.

Inositol phosphate formation in peripheral human cells was always less than the response seen with glycogen-elicited rabbit peritoneal cells. The rabbit neutrophils thus obtained were in a partially activated state (i.e., they had already responded to a chemotactic stimulus). Partial stimulation allows a larger pool of PPI to be labelled (table 1) and would thus explain the larger responses seen in rabbit cells (table 3).

From the magnitude of the changes seen with fMetLeuPhe on human neutrophils at 30 s it can be calculated that about 1% of the total inositol lipid pool is involved in the generation of the two second messengers, DG and IP_3 (see legend to table 3). We have also calculated the amount of PA generated by the phosphorylation of DG from ATP. This calculation (based on experiments previously published [11]) indicates that about 1.5% of the inositol lipid is hydrolysed via the phospholipase C route over a period of 30 s. The amount of DG that would result from this is 0.5 nmol/10^8 cells, most of which would then be rapidly phosphorylated to PA. Thus chemical detection of the increased level of DG generated by the phospholipase C route would be difficult to detect over the background of 2 nmol/10^8 cells present in unstimulated cells [12]. For rabbit neutrophils a similar calculation can be performed and the data again show that about 2% of inositol lipid is hydrolysed by phospholipase C.

Although only a small fraction of the total inositol lipid pool (1–2%) breaks down by the phospholipase C route, we calculate (from the percentage of the inositol lipid hydrolysed (2%), knowledge of the amount of total inositol lipid present in these cells [11], and assuming that most of lipid hydrolysed is PI-P_2) that the concentration of IP_3 generated in the cytosol would be about 20 μM at 30 s; a concentration more than sufficient to release Ca^{2+} from the non-mitochondrial stores of neutrophils [8].

In neutrophils there are two separate routes for the generation of DG. An early phase of DG production derives from PPI hydrolysis by phospholipase C and a second late phase derives from PA which itself is generated by phospholipase D action on PI. Of the ~14% of the total PI lost [11], ~12% is hydrolysed by phospholipase D, whereas the phospholipase C route accounts for only ~2%. DG generation by the phospholipase C route occurs without any detectable lag and is a function of receptor occupancy, whereas DG generation by the phospholipase D route has a 20 s lag and is then sustained for at least 2 min; it is not a function of receptor occupancy.

The calculations are based on maximum responses even though these occur at different concentrations of fMetLeuPhe for the two pathways. This should not distort the results to any considerable extent but the figures thus calculated should therefore only be regarded as an estimate of the relative pathways in operation in the neutrophil. The biphasic nature of DG production may provide a clue as to how the neutrophil can activate its various functional responses independently.

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