

Effects of Protein I of *Neisseria gonorrhoeae* on Neutrophil Activation: Generation of Diacylglycerol from Phosphatidylcholine via a Specific Phospholipase C Is Associated with Exocytosis

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Abstract. Upon engagement of chemoattractant receptors, neutrophils generate inositol trisphosphate and diacylglycerol (DG) by means of a phosphatidylinositol-specific phospholipase C (PI-PLC) which is regulated by a GTP-binding protein(s). We have previously reported (Reibman, J., H. M. Korchak, L. B. Vosshall, K. A. Haines, A. M. Rich, and G. Weissmann. 1988. *J. Biol. Chem.* 263:6322-6328) a biphasic rise in DG after exposure of neutrophils to the chemoattractant FMLP: a rapid (≤ 15 s) phase ("triggering") and a slow (≥ 30 s) phase ("activation"). These derive from distinct intracellular lipid pools. To study the source of rapid and slow DG, we have used a unique probe, protein I, a porin that is the major outer membrane protein of *Neisseria gonorrhoeae*. Treatment of neutrophils with protein I inhibits exocytosis and homotypic cell adhesion provoked by FMLP without inhibiting assembly of the NADPH oxidase responsible for O_2^- generation. DG turnover in PMN labeled with [3H]arachidonate and [^{14}C]glycerol was profoundly altered by protein I. Whereas the rapid peak of DG was only modestly diminished (FMLP vs. FMLP plus protein I = DG labeled with [3H]arachidonic acid (3H -a.a.-DG): $142 \pm 14\%$ SEM vs. $125 \pm 22\%$; DG labeled with the glycerol backbone with [^{14}C]glycerol (D- ^{14}C -G): $125 \pm 10\%$ SEM vs. $107 \pm 8.5\%$ SEM), the slow rise in both 3H -a.a.-DG and D- ^{14}C -G was essentially abolished. Moreover, treatment of neutrophils with 4-4'-

diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), which, like protein I, inhibits exocytosis without affecting O_2^- generation also inhibited slow DG. However, protein phosphorylation and dephosphorylation (47_{phox} , 66_{phox}) were unaffected in the absence of slow DG. To determine the source of the slow DG, we have analyzed radiolabeled phospholipid (PL) turnover after FMLP \pm protein I (P.I.). Treatment of PMN with FMLP ($0.1 \mu\text{M}$) resulted in breakdown of phosphatidylcholine (PC), beginning at 30 s, and reaching a nadir at 60 s (3H -PC = $59 \pm 10.2\%$ SEM of resting, ^{14}C -PC = $57 \pm 6.4\%$). Protein I ($0.25 \mu\text{M}$) significantly inhibited PC turnover after FMLP (3H]PC = $95 \pm 5.6\%$ and [^{14}C]PC = $86 \pm 8.4\%$ of resting at 60 s), but failed to alter the metabolism of 3H - or ^{14}C -phosphatidylinositol after FMLP (91 ± 19.6 and $88 \pm 16.5\%$ vs. 92 ± 9.2 and $91 \pm 16\%$ at 60 s). Increases of [3H]- and [^{14}C]phosphatidate (PA) provoked by FMLP were unimpaired in the presence of P.I. (341 ± 99 and $210 \pm 73\%$ vs. 358 ± 42 and $187 \pm 20\%$). We conclude that FMLP triggers generation of distinct second messengers for exocytosis and O_2^- generation. Degranulation is tightly correlated with, and may thus require production of the slow DAG from PC by a PC-specific PLC and whereas generation of O_2^- requires phosphatidic acid which can be formed de novo.

IN response to phlogistic agents such as FMLP, neutrophils aggregate, move toward chemoattractants, generate active oxygen species and release digestive enzymes (exocytosis) (66). These functional responses are preceded by a discrete series of intracellular signals: activation of the GTP-dependent protein (G_{pc}), and stimulation of phosphatidylinositol 4,5 bisphosphate (PIP_2)-specific phospholipase C, resulting in generation of the second messengers inositol

1,4,5 trisphosphate (IP_3) and 1,2-*sn*-diacylglycerol (DG)¹ (45, 61). IP_3 and DG provoke increments in intracellular

1. *Abbreviations used in this paper:* [3H]a.a., [3H]arachidonic acid; [^{14}C]Gly, [^{14}C]glycerol; [3H]a.a.-DG, diacylglycerol labeled with [3H]a.a., D-[^{14}C]G, diacylglycerol labeled in the glycerol backbone with [^{14}C]Gly; H-Z buffer, HEPES-buffered saline with 0.02% wt/vol Zwittergent (Z-3, 14); PA, phosphatidic acid; PC, phosphatidylcholine; PI, phosphatidylinositol; P.I., protein I.

calcium ($[Ca]_i$), and activation of the Ca^{2+} , phospholipid-dependent kinase, protein kinase C (PKC) (7, 8, 39, 43), respectively. Concomitantly, the cells undergo multiple ion movements resulting in changes in intracellular pH and membrane depolarization (18, 32, 54, 67).

Protein I of *Neisseria gonorrhoeae* is the major outer membrane protein of the organism, and a voltage-dependent porin that the bacterium uses to transfer ions and small sugars from the external milieu to its cytosol. In addition, protein I can be transferred from the intact gonococcus into artificial lipid bilayers and human cells (12, 40). When purified protein I is added to a suspension of human neutrophils, it associates with these cells as demonstrated by immunoblotting (30). We have previously demonstrated that treatment of neutrophils with protein I of *N. gonorrhoeae* inhibits FMLP-induced homotypic cell/cell adhesion of neutrophils, addition of membrane to the cell surface, and exocytosis of granule enzymes (30). However, assembly of the multicomponent NADPH oxidase was possible for superoxide anion generation proceeds in an unimpaired fashion as does calcium movements—measured by changes in the fluorescent dye, Fura-2. Protein I did not inhibit exocytosis from neutrophils treated with phorbol myristate acetate, which directly activates PKC rather than acting through a receptor bound to the cell surface. We have continued to use this unique probe to dissect the divergent signaling mechanisms necessary for exocytosis and generation of O_2^- provoked by chemoattractants.

We (50), and others (4, 5, 59) have previously reported that treatment of neutrophils with FMLP generates diacylglycerol from at least two distinct intracellular pools. We have termed these distinct peaks of DG as follows: (a) a “rapid” peak, or that which reaches maximal at ≤ 15 s after receptor ligation and then declines, and (b) a “slow” peak, or that which contributes to rapid DG but continues to rise throughout activation. In this report we demonstrate that protein I inhibits slow DG generation after treatment of cells with FMLP but has no effect on rapid diacylglycerol. DIDS, an anion transport inhibitor that alters neutrophil functions in a manner identical to protein I (24, 33) similarly inhibited DG formation after FMLP. Other investigators, by specifically labeling the pool of 1-*O*-alkyl, 2-acyl-phosphatidylcholine in neutrophils by means of the lyso derivative of EA-PC have demonstrated that slow 1-*O*-alkyl, 2-acylglycerol derives from EA-PC by activity of a phospholipase D. In contrast, our studies have analyzed the pool(s) of phospholipids which have ester-linked fatty acids in both the *sn*-1,2 positions. To determine the source of the slow diacylglycerol, we measured radiolabeled phospholipid turnover provoked by FMLP in the presence and absence of protein I. We have found that protein I inhibited metabolism of phosphatidylcholine without affecting generation of phosphatidic acid or turnover of phosphatidylinositol. Moreover, phosphorylation of 47_{phox} , a component of the NADPH oxidase (44, 64) and of pp66, a late-appearing phosphoprotein which may be 66_{phox} , proceeded normally despite a lack of prolonged elevation of diacylglycerol, the endogenous activator of protein kinase C (39).

These data confirm that rapid and slow diacylglycerol are derived from different phospholipid pools. Furthermore, the data demonstrate that (a) generation of “slow” diacylglycerol, associated closely with exocytosis derives from metabolism of phosphatidylcholine, most likely via activity of a phos-

pholipase C; (b) diacyl-linked phosphatidate produced after activation of neutrophils by FMLP is generated de novo (c) “slow” DG is not required to provoke activation of the C kinase and subsequent phosphorylation of 47_{phox} and 66_{phox} ; and (d) supports the hypothesis published by us (35) and confirmed by others (6, 52) that generation and maintenance of PA rather than generation of slow DG may be an important signal for assembly of the NADPH oxidase.

Materials and Methods

Buffers and Reagents

Hepes buffer, 150 mM Na^+ , 5 mM K^+ , 1.2 mM Mg^{2+} , 1.3 mM Ca^{2+} , 155 mM Cl^- , 10 mM Hepes, pH 7.45; PBS containing 0.01 M PO_4 , pH 7.4, Hepes buffer containing N-tetradecyl-*N,N*-dimethyl ammonium-1-propane-sulfonate (Zwittergent; Z-3, 14, (0.02% wt/vol) (H-Z buffer), cytochalasin B (Aldrich Chemical Co., Milwaukee, WI) FMLP (Vega Biochemicals, Tucson, AZ).

Neutrophil Separation

Polymorphonuclear leukocytes from normal adult volunteers were isolated by centrifugation (200 g, 40 min) over a Ficoll-Hypaque cushion (specific gravity 1.078–1.080). The pellet was removed and subjected to dextran sedimentation according to the method of Boyum (15). Remaining erythrocytes were removed by hypotonic lysis.

Cell Viability

Cell viability was measured as release of the cytosolic enzyme, lactate dehydrogenase according to the method of Wacker (65) using the commercially available assay (228-10; Sigma Chemical Co., St. Louis, MO). Average lactate dehydrogenase release in both treated and untreated cell populations were $\leq 5\%$ for the experiment to be considered valid.

Bacterial Culture and Preparation of Protein I

Gonococcal strain R10 were grown on solid typing medium defined by Swanson (58). Nonpiliated organisms of the transparent colonial phenotype were used. Bacteria were grown in liquid medium (11) and harvested by centrifugation at 17,000 g. Protein purification was performed as reported previously (30). Excess detergent was removed by applying this to a Sephacryl S-200 column equilibrated with H-Z buffer. The solution was rendered sterile by filtering through a 0.22- μ m filter.

Diacylglycerol Determination

Diacylglycerol generation was examined as previously reported (50). Briefly, neutrophils ($75 \times 10^6/ml$) were suspended in Hepes buffer containing fatty acid-free BSA (0.1%), [3H]arachidonate (3 μ Ci/ml) and [^{14}C]glycerol (7 μ Ci/ml) and incubated (30 min, 37°C). Unincorporated label was removed by washing cells twice in Hepes BSA. Neutrophils (17×10^6) were incubated (5 min, 37°C) in the presence of cytochalasin B (5 μ g/ml) and treated with FMLP (0.1 μ M) for various times. The reaction was terminated by the addition of 3.5 ml of chloroform/methanol (2:5, vol/vol). Samples were extracted by a modified Bligh and Dyer technique as previously reported (50). Samples were applied to heat-activated one-dimensional Silica Gel GF plates and run in hexane/ether/acetic acid (50:50:1, vol/vol/vol) (50). Lipids were visualized by exposure to iodine vapor, scraped off the plates and counted in a scintillation counter (LS7000; Beckman Instruments, Inc., Fullerton, CA) in 1% Na thiosulfate to bleach the iodine and 10 ml of Dimiscint (National Diagnostics, Inc., Manville, NJ). Diacylglycerol was identified in comparison to standards of diolein, 1,2-distearin, 1,2-distearoyl-*rac*-glycerol, di-eicosenoin, triarachidonin, triolein and mono-lein (all 97–99% pure). Acyl chain substitutions do not alter the mobility of DG but 1,3 DG runs separately from 1,2 DG.

To determine what proportion of label was in the 1,2-*sn*-diacylglycerol as compared with the 1-*O*-alkyl, 2-acylglycerol pool, cells labeled with [^{14}C]glycerol were treated with FMLP (0.1 μ M) and the diglyceride and monoglyceride fractions were isolated as above. An aliquot of each was

counted for radioactivity. The diglyceride was reextracted from the silica and treated with 0.5 N NaOH (30 min, 25°C). The mixture was brought to neutral pH, lipids were extracted for the third time, separated by TLC and radioactivity in di- and monoglyceride fractions were measured. Resting ^3H -a.a-DG: $2,095 \pm 536$ cpm SEM; resting D- ^{14}C -G: 461 ± 57 cpm SEM.

Phospholipid Determination

Cells were prepared, prelabeled, and extracted as above. Extracts were run using a two dimensional thin layer chromatography system as follows: silica Gel H plates with 7.5% Mg acetate were activated by heating at 100°C for 1 h. Sample were applied under nitrogen and the first dimension (65:35:5.5, $\text{CHCl}_3/\text{MeOH}/\text{NH}_4\text{OH}$, vol/vol/vol) were run for 1 h. The plate was dried under N_2 for 1 h, rotated 90°, and run in the second dimension (30:40:10:10:5, $\text{CHCl}_3/\text{acetone}/\text{MeOH}/\text{CH}_3\text{COOH}/\text{H}_2\text{O}$ by volume) for 1 h 30 min. Lipids were visualized by exposure to iodine vapor, scraped from the plates and counted as above. Standards: L- α -phosphatidylcholine, β -arachidonoyl, Δ -stearoyl; L- α -phosphatidylinositol; L- α -phosphatidylethanolamine; L- α -phosphatidylserine; L- α -phosphatidic acid (16). Resting ^3H -a.a.-PC: $42,403 \pm 3,226$ cpm SEM; resting ^{14}C -gly-PC: $4,802 \pm 322$ cpm SEM, resting ^3H -a.a.-PA: 750 ± 202 cpm SEM; resting ^{14}C -gly-PA: 288 ± 41 cpm SEM.

When levels of phosphatidate alone were of interest, resting PA and PE was measured as above and a second aliquot was run in the second dimension only. Changes in counts per minute were attributed to increments in PA as PE remains unchanged throughout the time course of the experiment.

^{32}P -Labeling of Intact Cells

Purified neutrophils (30×10^6) were suspended in HEPES buffer with [^{32}P]orthophosphate (0.2 mCi/ml) and incubated 60 min, 37°C. Labeled cells (2.5×10^6) were treated with the appropriate stimulus for varying time points. The reaction was terminated by the addition of iced TCA (10%) with EDTA (1 mM), PMSF (2 mM) leupeptin (2.5 mM), antipain (3.7 mM), pepstatin A (3.3 mM), aprotinin, (0.01 trypsin inhibitory units), chymostatin (2.5 mg/ml), and TLCK (6.5 mM). Samples were then washed with 95% EtOH, 0.05 M NaAc, followed by 100% EtOH, followed by acetone. They were dissolved in Laemmli sample buffer containing PMSF. Insoluble material was removed by centrifugation in a centrifuge (Microfuge B; Beckman Instruments) (8,750 g; 5 min). Phosphoproteins were separated on a 10% SDS-polyacrylamide slab gel using the buffer system of Laemmli (48) followed by autoradiography using Kodak XAR-2 film. Relative molecular weights of proteins were estimated by comparison with proteins of known molecular weight: myosin, *b*-galactosidase, phosphorylase *b*, bovine albumin, egg albumin, and carbonic anhydrase.

Phospholipase C Activity

Purified phospholipase C activity was measured according to the method of Bomalaski (14). Briefly, 1,2 [^{14}C]-dioleoyl-phosphatidylcholine (Dupont New England Nuclear, Boston, MA) ($10 \mu\text{M}$, specific activity 72 mCi/mmol) was suspended in deoxycholate (1 mg/ml HEPES buffer).

Propranolol (200 μM) was incubated with the lipid substrate (37°C, 5 min) and phosphatidylcholine phospholipase C (*Clostridium perfringens*, 1.25 U/ml, Sigma Chemical Co. [St. Louis, MO]) was added for various time points. The reaction was stopped with the addition of, in sequence, equal volumes of chloroform:methanol (1:2, vol/vol), chloroform, and 4 M KCl. Samples were kept on ice for 30 min and lipids extracted, isolated by thin-layer chromatography and quantified as above.

Results

Effect of Protein I on Diacylglycerol Metabolism in Neutrophils

Protein I (250 nM) was added to a suspension of neutrophils (17×10^6 cells/ml) which had been prelabeled with [^3H]arachidonic acid (^3H]a.a., 3 $\mu\text{Ci}/\text{ml}$) and [^{14}C]glycerol (^{14}C]Gly., 7 $\mu\text{Ci}/\text{ml}$). Cells were incubated for 1 min and FMLP (10^{-7}M) was added for various time points. The reaction was stopped by the addition of chloroform/methanol, 2:1.

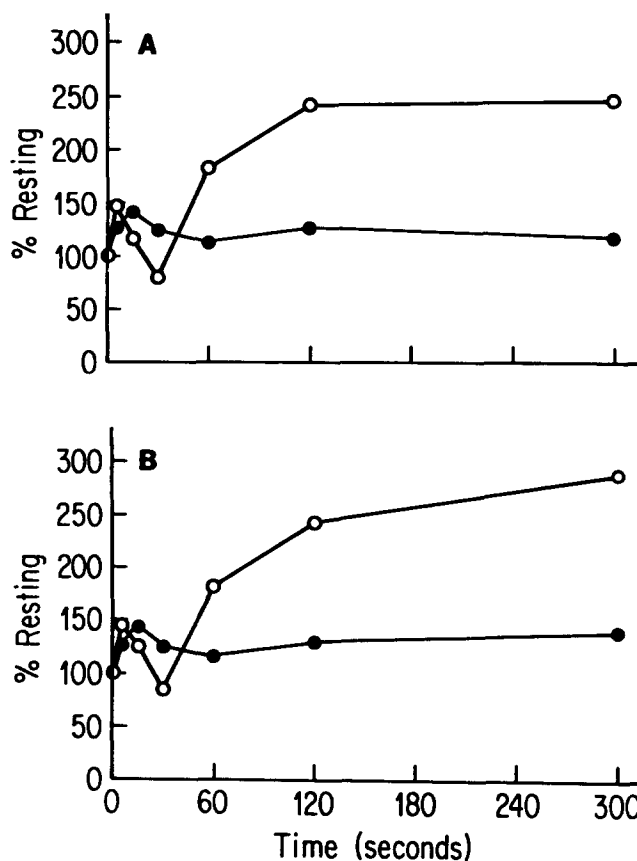


Figure 1. Effect of protein I on diacylglycerol production in neutrophils. Neutrophils ($75 \times 10^6/\text{ml}$) were incubated with [^3H]arachidonate (3 $\mu\text{Ci}/\text{ml}$) and [^{14}C]glycerol (7 $\mu\text{Ci}/\text{ml}$) for 30 min and washed twice in HEPES-BSA buffer. They were pretreated with protein I (250 nM) (●) or H-Z buffer (○) and treated with FMLP (0.1 μM) for various time points. The reaction was terminated by the addition of chloroform/methanol (2:5, vol/vol), samples were extracted and run on silica gel GF plates. DG was identified by comparison to known standards, scraped, and counted. Results are expressed as percent of resting levels and are a mean of four experiments. (A) [^3H]a.a.-DG production, resting: $2,095 \pm 536$ cpm SEM; (B) D [^{14}C]G production, resting: 461 ± 57 cpm SEM.

The lipids were extracted and analyzed for diacylglycerols by means of thin-layer chromatography. Treatment of cells with FMLP (Fig. 1, representative experiment) provoked the generation of diacylglycerol in a biphasic manner, as we have previously reported (29, 50). In accord with previously published data, the rapid wave peaked between 5 and 15 s after addition of FMLP, and returned toward baseline in both the ^3H -arachidonate and [^{14}C]glycerol-labeled pools of diacylglycerol. Between 30 and 60 s the generation of a slow, prolonged wave of diacylglycerol became apparent. Levels of diacylglycerol remained elevated for at least 300 s. In the presence of protein I, however, generation of both [^3H]a.a.-DG and diacyl- ^{14}C]glycerol was markedly altered. The rapid peak of [^3H]a.a.-diacylglycerol generation was somewhat diminished (142 ± 14 vs. $125 \pm 22\%$); the rapid peak of diacyl [^{14}C]glycerol was even more affected ($107 \pm 8.5\%$ vs. control = $125 \pm 10\%$). Most strikingly, the slow rise in both [^3H]a.a.-DG and diacyl- ^{14}C]glycerol was almost abolished.

Billah et al. (10) and Agwu et al. (1) have demonstrated

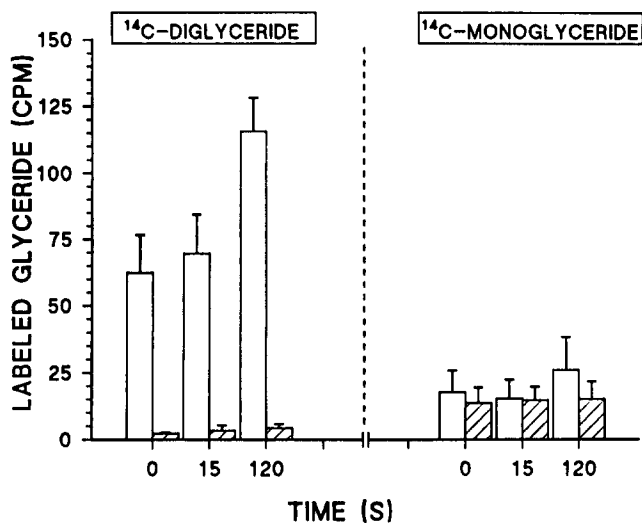


Figure 2. Lack of incorporation of ¹⁴C-glycerol into the ether-linked diglyceride pool in neutrophils. Neutrophils ($75 \times 10^6/\text{ml}$) were incubated with [¹⁴C]glycerol ($7 \mu\text{Ci}/\text{ml}$) for 30 min and washed twice in HEPES-BSA buffer. They were treated with FMLP and cellular diglycerides and monoglycerides were extracted and separated by TLC. The diglyceride and monoglyceride fractions were scraped and an aliquot counted for radioactivity. The diglyceride fraction was reextracted and subjected to alkali hydrolysis (0.5 N NaOH , 37°C , 15 min). Diglycerides and monoglycerides were quantitated as in Materials and Methods.

that a fraction of diglyceride generated by neutrophils treated with FMLP derives from 1-*O*-alkyl, 2-acyl glycerol (EAG). To determine what fraction of labeled diglyceride was EAG, neutrophils were pre-labeled with [¹⁴C]glycerol and treated with FMLP for various time points. Diglyceride and monoglyceride fractions were separated and an aliquot was counted for radioactivity. Diglyceride fractions were treated with 0.5 N NaOH to remove ester-linked side chains (10), reextracted and mono- and diglycerides were isolated and counted. Neutrophils treated with FMLP showed increasing amounts of [¹⁴C]diglyceride over time with only small amounts of monoglyceride detected (Fig. 2, *open bars*). When this [¹⁴C]-diglyceride was subjected to hydrolysis with NaOH, reextracted and re-separated by TLC, the diglyceride fraction was completely hydrolyzed (*right, hatched bars*). However, there was no parallel increase in the [¹⁴C]monoglycerides over time. This demonstrates that the [¹⁴C]glycerol is incorporated into the DAG rather than the EAG pool of diglyceride, in agreement with findings of others (19) and that our measurements of diglyceride derive solely from *sn*-1,2 diacyl-linked lipid precursors.

Effect of DIDS on Diacylglycerol Generation

DIDS, an inhibitor of the anion transporter, has been shown, both by morphological as well as enzymatic criteria, to inhibit degranulation from neutrophils without affecting superoxide anion generation (24, 32). We therefore determined whether DIDS affected diacylglycerol generation by neutrophils treated with FMLP. Neutrophils were pre-labeled with [³H]arachidonate and [¹⁴C]glycerol, treated with DIDS (10^{-4} M) for 5 min then treated with FMLP ($0.1 \mu\text{M}$) for various time points. Pretreatment of neutrophils with DIDS had no effect on generation of rapid ³H-aa-DG or D-¹⁴C-G

(Fig. 3); generation of both pools of slow DG was significantly inhibited (³H-aa-DG at 60 s after FMLP + $160 \pm 18\% \text{ SEM}$ vs. FMLP + DIDS = $123 \pm 5\%$ of resting values; D-¹⁴C-G at 60 s after FMLP = $148 \pm 5\% \text{ SEM}$ vs. FMLP + DIDS = $123 \pm 6\% \text{ SEM}$ of resting values).

Effect of Protein I on Kinase C Activation

Diacylglycerol is an intracellular messenger presumed to activate protein kinase C, the calcium, phospholipid-dependent protein kinase (39). Since treatment of neutrophils with protein I significantly inhibited the slow wave of diacylglycerol production, we determined whether PI also prevented phosphorylation of the 47_{phox} substrate of PKC in neutrophils. Neutrophils were pre-labeled with [³²P]orthophosphate, pre-treated with protein I ($0.25 \mu\text{M}$) for 1–2 min and then treated with FMLP ($0.1 \mu\text{M}$) for 5, 120, and 300 s. Total cell proteins were precipitated with 10% TCA, separated by PAGE and labeled proteins were identified by radioautography and quantified by densitometry (Fig. 4). Neutrophils treated with

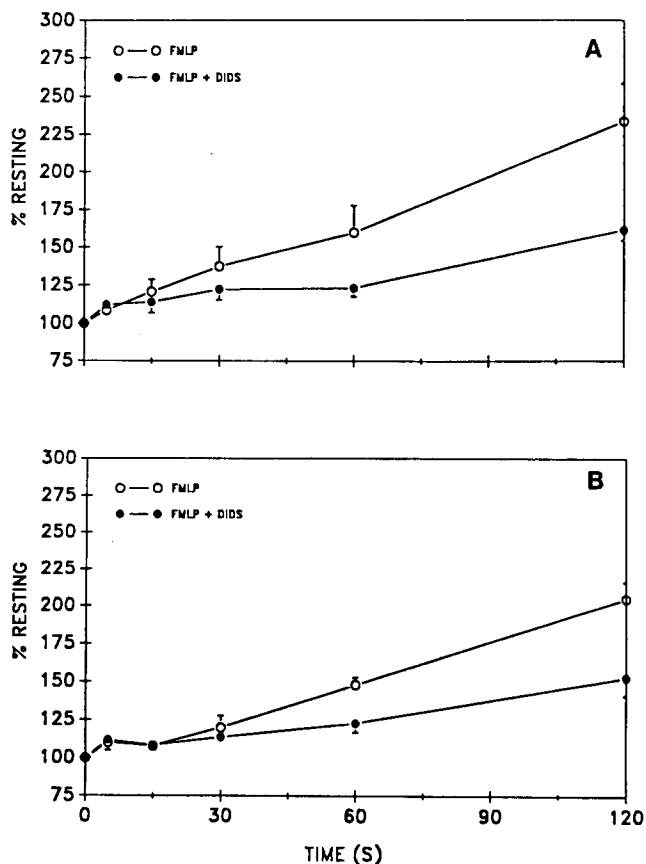


Figure 3. Effect of DIDS on diacylglycerol production in neutrophils. Neutrophils ($75 \times 10^6/\text{ml}$) were incubated with [³H]arachidonate ($3 \mu\text{Ci}/\text{ml}$) and [¹⁴C]glycerol ($7 \mu\text{Ci}/\text{ml}$) for 30 min and washed twice in HEPES-BSA buffer. They were pretreated with DIDS (10^{-4} M) (●) or buffer (○) and treated with FMLP ($0.1 \mu\text{M}$) for various time points. The reaction was terminated by the addition of chloroform/methanol (2:5, vol/vol), samples were extracted and run on silica gel GF plates. DG was identified by comparison to known standards, scraped and counted. Results are expressed as percent of resting levels, and are a mean of three experiments. (A) [³H]a.a.-DG production, resting: $2,335 \pm 329 \text{ cpm SEM}$; (B) D-[¹⁴C]G production, resting: $342 \pm 29 \text{ cpm SEM}$.

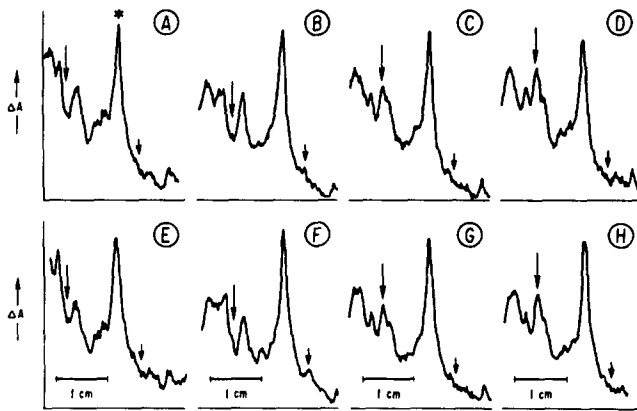


Figure 4. Effect of protein I on protein phosphorylation in neutrophils after FMLP. Purified neutrophils labeled with [³²P]orthophosphate (0.2 mCi/ml) were treated with the appropriate stimulus for varying time points. Proteins were precipitated with TCA (10%), washed, and separated on a 10% SDS-polyacrylamide slab gel followed by autoradiography and densitometry. (A) Buffer (H-Z) control; (B) H-Z + FMLP, 15 s; (C) H-Z + FMLP, 120 s; (D) H-Z + FMLP, 300 s; (E) Protein I (P.I.); (F) P.I. + FMLP, 15 s; (G) P.I. + FMLP, 120 s; (H) P.I. + FMLP, 300 s.

phorbol myristate acetate (1 μg/ml, 5 min) which directly activates protein kinase C were used as a positive control (not shown) and demonstrated phosphorylation of the 47,000 *M*_r protein component of the NADPH-oxidase as published previously by us (28, 50) and others (26, 47, 63). In addition, PMA provoked phosphorylation of a protein of 66,000 *M*_r (47, 50, 63), the function of which is as yet unknown, but which may be 66_{phox}. Resting neutrophils (Fig. 4 A) demonstrated phosphorylation of multiple proteins but showed no phosphorylation of 47_{phox} (short arrow) or 66_{phox} (long arrow). After treatment of neutrophils with FMLP, the 47-kD protein was phosphorylated by 15 s (Fig. 4 B) and became dephosphorylated by 120 s (Fig. 4 C). By 120 s, the 66-kD protein was phosphorylated and was even more prominent by 300 s (Fig. 4 D). Pretreatment with protein I alone for 60 s did not elicit phosphorylation of either of these proteins (Fig. 4 E); the pattern of protein phosphorylation was identical to that seen in untreated cells (Fig. 4 A vs. E). Moreover, treatment of cells with protein I did not interfere with the kinetics of the phosphorylation and dephosphorylation of the 47_{phox} and 66_{phox} proteins after treatment with FMLP (Fig. 4 F, G, and H). Confirming our previous results (50), slow diglycer-

ide cannot be required for activation of the protein kinase C, which phosphorylates 47_{phox} and 66_{phox}.

Effect of Protein I on Generation of Phospholipids

To determine the source of the slow diacylglycerol, metabolism of phospholipids by neutrophils treated with FMLP (0.1 μM) in the presence and absence of protein I was determined. Cells were prelabeled with [³H]a.a. and [¹⁴C]glycerol as before, pretreated with protein I (250 nM) and exposed to FMLP for various time points. The reaction was stopped with addition of chloroform/methanol 2:1 and extracted.

Treatment of neutrophils with FMLP had minimal effect on levels of either [³H]a.a.-phosphatidylinositol or [¹⁴C]phosphatidylinositol (Table I). At 5 s, [³H]a.a.-PI and [¹⁴C]PI were 112 ± 6 and 111 ± 2% of resting levels. By 60 s, [³H]a.a.-PI and [¹⁴C]PI were 91 ± 20 and 88 ± 17% of resting levels, respectively. Protein I pretreatment had no significant effect on the metabolism of phosphatidylinositol triggered by FMLP. These findings are incompatible with the hypothesis that slow DG is derived from breakdown of phosphoinositides and consistent with previous data that metabolism of the polyphosphoinositides is complete by 1–2 min (36).

Treatment of neutrophils with FMLP significantly altered the metabolism of both [³H]a.a.-phosphatidylcholine and [¹⁴C]-phosphatidylcholine (Fig. 5), results that parallel changes in lipid mass (55). At 5 s after treatment with secretagogue, levels of both [³H]a.a.-phosphatidylcholine and [¹⁴C]phosphatidylcholine remained close to resting (106 ± 1.2 and 102 ± 0.8%, respectively.) Treatment with protein I did not alter rapid levels of either [³H]a.a.-PC or [¹⁴C]PC (98 ± 2.3 and 90 ± 6%, respectively). However, by 60 s, treatment of neutrophils with FMLP resulted in significant metabolism of both [³H]a.a.-PC and [¹⁴C]PC. [³H]a.a.-PC had diminished to 58 ± 10% of resting levels and [¹⁴C]PC had diminished to 57 ± 6.5% of resting levels. In the presence of protein I, metabolism of PC after FMLP was inhibited: [³H]a.a.-PC remained at 95 ± 5% of resting values and [¹⁴C]PC diminished only to 86 ± 8% of resting values. We conclude that slow diacylglycerol is derived from metabolism of diacyl-linked phosphatidylcholine.

To determine whether metabolism of slow DG was via a phospholipase C or phospholipase D followed by activity of phosphatidate phosphohydrolase (PPH), we examined the effect of protein I on generation of phosphatidic acid. Were

Table I. Effect of Protein I on Phosphatidylinositol Metabolism in Human Neutrophils

Condition	Time			
	5 s		60 s	
	[³ H]aa-PI	[¹⁴ C]gly-PI	[³ H]aa-PI	[¹⁴ C]gly-PI
	<i>cpm</i>			
FMLP	78,713 ± 3,908	5,562 ± 497	64,522 ± 23,808	4,937 ± 815
Protein I plus FMLP	68,714 ± 1,924	4,580 ± 537	64,600 ± 6,290	4,530 ± 779

Prelabeled PMN (17 × 10⁶/ml) were incubated with cytochalasin B (5 μg/ml, 37°C, 5 min) and treated with H-Z buffer or protein I (250 nM, 37°C, 2 min) and treated with FMLP (0.1 μM, 37°C, 5 min). The reaction was stopped by the addition of chloroform:methanol, 2:5, vol/vol. Samples were extracted and lipids separated by TLC as in Materials and Methods. Results are given as counts per minute ± SEM of radiolabeled phospholipid. Resting levels were normalized to the mean resting counts per minute of three experiments: [³H]aa-PI = 70,000; [¹⁴C]gly-PI = 5,000.

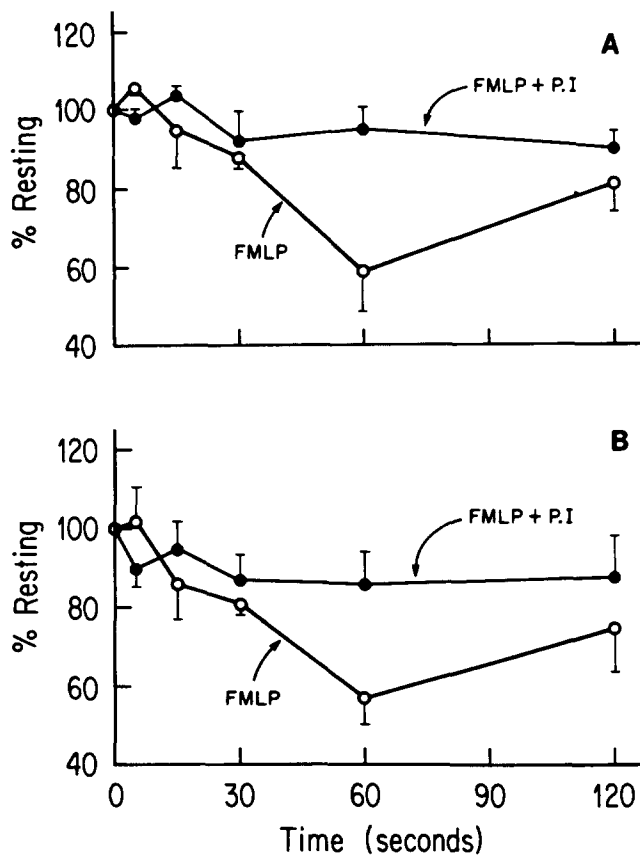


Figure 5. Effect of protein I on phosphatidylcholine metabolism by neutrophils. Neutrophils ($75 \times 10^6/\text{ml}$) were incubated with [^3H]arachidonate ($3 \mu\text{Ci}/\text{ml}$) and [^{14}C]glycerol ($7 \mu\text{Ci}/\text{ml}$) for 30 min and washed twice in HEPES-BSA buffer. They were pretreated with protein I (250 nM) (\bullet) or H-Z buffer (\circ) and treated with FMLP ($0.1 \mu\text{M}$) for various time points. The reaction was terminated by the addition of chloroform/methanol (2:5, vol/vol), samples were extracted and phospholipids isolated by two dimensional thin layer chromatography as in Materials and Methods. Phospholipids were identified by comparison to known standards, scraped, and counted. Results are expressed as percent of resting levels; figure shows mean of three experiments. (A) ^3H -a.a.-PC, resting: $42,403 \pm 3,226$ SEM; (B) [^{14}C]gly-PC, resting: $4,802 \pm 322$.

slow DG derived from a PC-specific PLC, we would predict protein I pretreatment would not affect changes in PA. Were slow DG derived from the sequential activity of a PC-specific PLD followed by that of a PA-phosphohydrolase, we would expect a parallel inhibition of phosphatidate production. Control neutrophils treated with FMLP demonstrated an increase in phosphatidate, beginning at the earliest time points, essentially reaching a plateau by 30 s and remaining at least until 120 s after activation (Fig. 6, representative experiment). By 5 s, ^3H -a.a.-PA had increased to $149 \pm 11\%$ SEM ($n = 3$) of resting levels, and to $341 \pm 99\%$ SEM by 60 s. By 15 s, [^{14}C]phosphatidate had increased to $221 \pm 85\%$ of resting values and remained at $210 \pm 73\%$ of resting levels at 60 s. Protein I pretreatment (Fig. 6) had no significant effect on increases in either [^3H]a.a.-PA (5 s = 147 ± 16 SEM% resting; 60 s = $358 \pm 42\%$ resting) or [^{14}C]PA (15 s = $151 \pm 10\%$ resting; 60 s = $187 \pm 20\%$ resting) seen after FMLP. The demonstration of a normal increment in both [^3H]a.a.-PA and diacyl[^{14}C]phosphatidate in the ab-

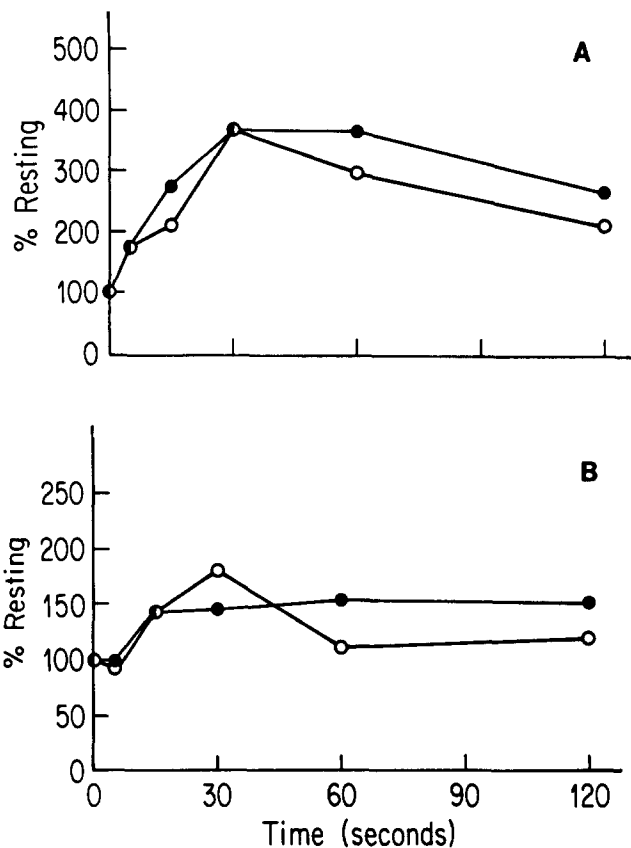


Figure 6. Effect of protein I on phosphatidate generation by neutrophils. Neutrophils ($75 \times 10^6/\text{ml}$) were incubated with [^3H]arachidonate ($3 \mu\text{Ci}/\text{ml}$) and [^{14}C]glycerol ($7 \mu\text{Ci}/\text{ml}$) for 30 min and washed twice in HEPES-BSA buffer. They were pretreated with protein I (250 nM) (\bullet) or H-Z buffer (\circ) and treated with FMLP ($0.1 \mu\text{M}$) for various time points. The reaction was terminated by the addition of chloroform/methanol (2:5, vol/vol), samples were extracted and phospholipids isolated by two dimensional thin layer chromatography as in Materials and Methods. Phospholipids were identified by comparison to known standards, scraped and counted. Results are expressed as percent of resting levels; figure is representative experiment of $n = 3$. (A) [^3H]a.a.-PA; resting: 750 ± 202 cpm SEM. (B) [^{14}C]gly-PA; resting: 288 ± 41 cpm SEM.

sence of consumption of [^3H]diacyl PC and/or diacyl[^{14}C]PC affirms that protein I did not inhibit PC-PLD. This same parallel increment in [^3H]diacyl-PA and diacyl[^{14}C]PA in the absence of PC-PLD suggests that protein I does not inhibit a PA-phosphohydrolase. Slow DG is therefore derived from activity of a phosphatidylcholine-specific PLC. Diacyl-PA is, most likely, synthesized de novo.

Effect of Ethanol and Propranolol on Generation of Phosphatidate and Diacylglycerol

Both ethanol and propranolol have been used as indicators of phospholipase D activity in neutrophils labeled with [^3H] and [^{32}P]l-O-alkyl lysophosphatidylcholine. We used these inhibitors as a second approach to determine the role of PLD in the generation of slow diacylglycerol after FMLP. In the presence of alcohols, PLD can catalyze a transphosphatidylation of these organic alcohols to phosphatidate (23, 68), thus depleting substrate for PPH and inhibiting generation of DG by this dual-enzyme pathway. We treated neutrophils

Table II. Lack of Effect of Ethanol (0.5%) on Generation of DAG and PA

	^3H Diacylglycerol (% Resting \pm SEM)		Diacyl ^{14}C glycerol (% Resting \pm SEM)	
	15 s	60 s	15 s	60 s
FMLP (0.1 μM)	112 \pm 8	147 \pm 15	112 \pm 6	140 \pm 10
FMLP plus EtOH (0.5%)	116 \pm 2	133 \pm 11	113 \pm 3	132 \pm 6

	^3H Phosphatidate (% Resting \pm SEM)		^{14}C Phosphatidate (% Resting \pm SEM)	
	15 s	60 s	15 s	60 s
FMLP	242 \pm 53	276 \pm 93	173 \pm 25	169 \pm 7
FMLP plus EtOH (0.5%)	196 \pm 76	194 \pm 61	132 \pm 10	140 \pm 2

Neutrophils ($17 \times 10^6/\text{ml}$) were labeled with ^3H arachidonate and ^{14}C glycerol as described. They were incubated with ethanol (0.5% by volume, 37°C , 5 min) and then treated with FMLP for 15 or 60 s. The reaction was stopped and the lipids were extracted as described. Extracted lipid was divided into two aliquots which were separately analyzed for diacylglycerol or phosphatidate. Results are expressed as % resting levels \pm SEM of three experiments done in duplicate. Resting levels ^3H aa-diacylglycerol = $1,971 \pm 171$ cpm; diacyl ^{14}C glycerol = 324 ± 27 cpm; ^3H phosphatidate = $2,019 \pm 508$ cpm; ^{14}C phosphatidate = 268 ± 17 cpm.

with 0.5% ethanol after labeling with ^3H -a.a. and ^{14}C glycerol and measured radiolabeled DG and PA at 15 and 60 s after treatment with FMLP. Incubating cells with high concentrations of ethanol before treatment with FMLP had no effect on changes in ^3H aa-DAG or D- ^{14}C -G (Table II). Pretreatment of neutrophils with EtOH had, at best, trivial effects on increments in phosphatidate (Table II, $p = \text{NS}$ for all comparisons). This confirms that radiolabeled diacylglycerol and PA are not generated by means of PLD activity and suggests that diacyl-PA is only minimally accessible to phospholipase D.

Propranolol has been used as an indicator of PLD activity in that it inhibits generation of diglyceride from phosphatidate via PPH (49). Propranolol is not a direct inhibitor of the PPH but rather binds to phosphatidate thereby blocking activity (49). To rule out a similar inhibition of PLC by pro-

pranolol, we examined the effect of propranolol on purified PC-PLC from *C. perfringens*. 1,2di-[^{14}C]oleyl-phosphatidylcholine was pretreated with 200 μM propranolol for 5 min. Purified PC-PLC was added and the reaction was stopped with chloroform at various time points. Lipids were extracted and generation of ^{14}C diacylglycerol was measured. As shown in Fig. 7, propranolol markedly inhibited purified PC-PLC activity at the earliest time points measured. In concordance with these data, propranolol markedly inhibited generation of both ^3H aa-DAG and D- ^{14}C G in neutrophils treated with FMLP for 60 s (FMLP = 158% resting vs. FMLP + Propranolol = 116% resting levels).

Discussion

Ligation of cell surface receptors by chemoattractants triggers a discrete series of intracellular signals. Engagement of chemoattractant receptors activates GTP-binding proteins which, in turn, activate a (poly)phosphoinositide-specific phospholipase C (45, 46, 62) to generate the second messengers, inositol trisphosphate and diacylglycerol. Inositol

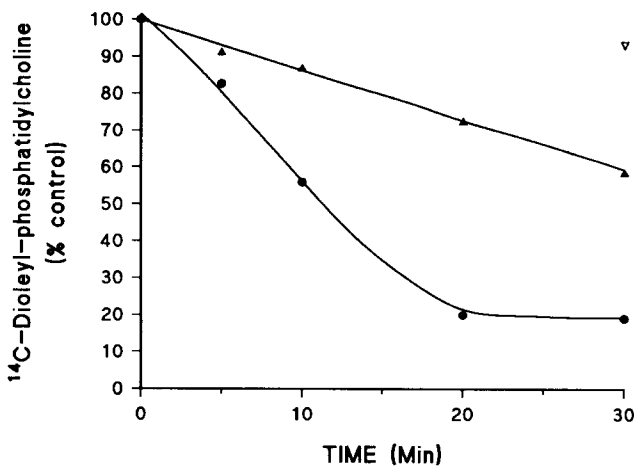


Figure 7. Effect of propranolol on phosphatidylcholine-phospholipase C activity. 1,2[^{14}C]dioleilphosphatidylcholine was suspended in deoxycholate (0.5 mg/ml HEPES buffer). Propranolol (200 μM) was incubated with the lipid substrate (37°C , 5 min) and phosphatidylcholine phospholipase C (*C. perfringens*, 1.25 U/ml) added for various time points. Lipids were extracted, isolated by thin layer chromatography, scraped and counted. (●) ^{14}C -dioleil-PC; (▲) ^{14}C -di-oleil-PC plus 200 μM propranolol, (▽) ^{14}C -dioleil-PC without enzyme.

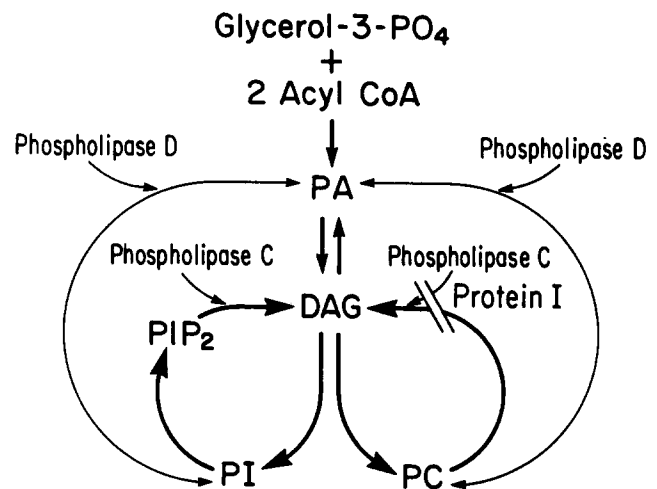


Figure 8. Effect of protein I on lipid metabolism in neutrophils triggered by FMLP.

trisphosphate provides the rapid rise of intracellular Ca^{2+} (9). Diacylglycerol is usually implicated in activation of PKC, resulting in phosphorylation of proteins necessary for activation of the superoxide generating system (17, 38, 44, 53, 64). Recently the kinetics of DG production have become clear. Although Cockcroft et al. (20) and Rider and Niedel (51), by measuring mass of DG, were unable to detect increments in DG production until after O_2^- generation had begun, studies with radiolabeled DG, (4, 6, 50, 59) have shown that DG is generated in a biphasic manner. When we compared the ratio of [^3H]a.a.-DG and diacyl[^{14}C]glycerol, it became obvious that the rapid and slow phases of DG production were derived from two distinct sources (50). Using protein I, the gonococcal porin which inhibits enzyme release from neutrophils (30), we have obtained further evidence that rapid and slow diacylglycerol derive from separate pools. Pretreatment of neutrophils with protein I before activation with the chemoattractant, FMLP, inhibited generation of slow diacylglycerol but had no significant effect on rapid DG. Moreover, since treatment of neutrophils with protein I has no effect on superoxide anion generation (30), the data demonstrate that slow DG (diacylglycerol) is not required for O_2^- production. If the paradigm is correct, that DG is the endogenous activator of PKC, the data are also consistent with our earlier contention that rapid DG is sufficient to trigger activation of kinase C and subsequent phosphorylation of 47_{phox} , a component of the NADPH oxidase system (44, 64). Generation of rapid, rather than slow DG is also sufficient to trigger the cascade of events resulting in phosphorylation of the 66-kD substrate of PKC; we assume that substrate is 66_{phox} . By 120 s, this protein is phosphorylated after either treatment of neutrophils with phorbol esters or engagement of FMLP receptors (2, 27, 50). We have previously demonstrated that leukotriene B_4 , a poor stimulus for generation of O_2^- , provokes formation of rapid DG and elicits slow DG only weakly (50), while nevertheless provoking phosphorylation of 47_{phox} .

Taken together, our results indicate that (a) rapid DG is sufficient to trigger phosphorylation of the 47_{phox} component of the NADPH oxidase; (b) generation of 47_{phox} alone is not sufficient to trigger superoxide anion generation; (c) an additional event, which we have previously termed "activation" is required. Generation of superoxide anion has been demonstrated by us (34) and others (56, 57) to require continuous occupancy of the triggering receptor, and it has been postulated that continued production of slow DG is required for O_2^- production (59). Our data demonstrate, however, that the "activation" step required for the generation of O_2^- cannot be slow DG, since superoxide anion is generated normally in its absence.

Production of slow DG has been documented in many cell types and a general consensus has been reached that the source of the slow DG is phosphatidylcholine (reviewed in reference 25). However, the metabolic pathways from whence slow DG is derived remain unresolved. Neither the enzymes responsible for the formation of slow DG nor the fatty acid linkage of substrate used has been defined. Diacylglycerol can be produced directly by the action of phospholipase C on several phospholipids; alternatively, diacylglycerol may be formed via phosphatidate, either by activation of a phospholipase D followed by that of a PA phosphohydrolase or by de novo synthesis of phosphatidate, followed by activity

of a phosphohydrolase. Evidence for each of these pathways has been demonstrated by various authors. Treatment of MDCK cells with PMA provokes metabolism of 1-*O*-alkyl-phosphatidylcholine to DG via a phospholipase C (22). Hepatocytes contain both phospholipase C (31) and phospholipase D (13) activity, as do endothelial cells (41, 42). Neutrophils metabolize 1-*O*-alkyl-phosphatidylcholine as a source of 1-*O*-alkyl, 2-acyl glycerol through activity of phospholipase D followed by that of phosphatidate phosphohydrolase (1, 10). The product appears as slow DG and the reaction depends upon "priming" by PMA or cytochalasin B (60). Similar to rapid DG derived from phosphoinositides, production of slow diglyceride by this pathway appears to be under the control of GTP-binding proteins which are sensitive to the actions of pertussis toxin (1, 31).

In this report, we have examined turnover of the diacylphospholipid pool and have determined that phosphatidylcholine is the source of slow diacylglycerol. However, in contradistinction from alkyl, acyl glycerol, diacylglycerol is generated by activity of a phosphatidylcholine-phospholipase C. We again present evidence for de novo synthesis of phosphatidate. In agreement with our previous work and that of others (1, 10, 55), we demonstrate that formation of phosphatidic acid is rapid, peaking by 60 s after treatment with chemoattractant, and remaining elevated for up to 300 s. Generation of diacylglycerol, on the other hand, is biphasic, with a rapid peak at 5 s, and a second peak which remains elevated for up to 300 s. This second phase of DG production can be completely inhibited by pretreatment of neutrophils with protein I. As has been demonstrated by this laboratory (55) and by others (1, 10) neutrophils treated with FMLP metabolize phosphatidylcholine, detectable by 30 s and continuing for up to 120 s. Pretreatment of neutrophils with protein I markedly inhibits metabolism of PC while at the same time inhibiting formation of DG. These results are in agreement with those of others that slow DG derives from phosphatidylcholine (1, 10, 13, 22, 41, 42). However, generation of phosphatidate is unchanged in the presence of protein I. Thus, it is unlikely that slow diacylglycerol derives from, or contributes to, the new pool of phosphatidic acid. Nor, finally, is phosphoinositide metabolism altered in the presence of protein I. We must therefore conclude that the rapid DAG most likely derives from metabolism of (poly)phosphoinositides and is important in activating protein kinase C. Moreover, our data show that slow DAG derives from phospholipase C activity on phosphatidylcholine (Fig. 7); phosphatidate is a product of de novo synthesis since we detected neither an increase nor decrease in phosphatidate. Studies with ethanol and propranolol confirm this data. Neither generation of DAG nor PA is altered in the presence of ethanol, at doses which will inhibit generation of PA via activity of phospholipase D. Thus phospholipase D does not significantly affect the diacyl-phospholipid pool. Moreover, we have demonstrated that propranolol, which inhibits both phosphatidate phosphohydrolase and PC-phospholipase C augments generation of PA in the absence of PLD activity, again indicating that PA is generated de novo.

Evidence for generation of diglyceride and phosphatidate by any of the synthetic pathways does not preclude the necessity for generation of lipid messengers by other enzymatic pathways. It is likely that the alkyl-acyl and diacyl-lipids each activate a specific kinase or phosphohydrolase critical to con-

trol cell activation. Evidence has long existed for the presence of multiple second messenger systems in the activation of neutrophils. Bass et al. (3) have demonstrated that 1-(5-isoquinolonesulfonyl)-piperazine, an inhibitor of phorbol-induced kinase activation, has no effect on activation of the NADPH oxidase system triggered by FMLP, and does not inhibit priming of the oxidase induced by 1-oleoyl-2-acetyl glycerol. Kramer et al. (37) has demonstrated that 1-O-alkyl-2-O-methylglycerol inhibits phosphorylation of 47_{phox} and the respiratory burst of neutrophils after FMLP, but recovery of oxygen consumption did not require a proportional recovery of phosphorylation of 47_{phox} . We have demonstrated that inhibition of the generation of slow diacylglycerol was associated with inhibition of exocytosis by neutrophils after treatment with FMLP, and Cox et al. (21) have demonstrated that treatment of neutrophils with didecanoylglycerol provoked secretion of specific granule enzymes without triggering O_2^- generation. Conversely, in accordance with our previous data (35), Rossi et al. (52) and Bauldry et al. (6) have demonstrated O_2^- generation by neutrophils in the absence of diglyceride generation. Whether these findings are causally or casually related cannot be determined with such data. However the direct relationships between phosphatidate formation and assembly of the NADPH oxidase, and between the slow DG wave and degranulation, suggest that both phosphatidate and DG are intracellular messengers in the response of neutrophils to chemoattractants.

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