Diaclylglycerol Generation in Fluoride-Treated Neutrophils: Involvement of Phospholipase D

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Neutrophils exposed to fluoride ion (F\(^{-}\)) respond with a delayed and sustained burst of superoxide anion release that is both preceded by and dependent on the influx of Ca\(^{2+}\) from the extracellular medium. The results of this study demonstrate a similarly delayed and sustained generation of 1,2-diacylglyceride in F\(^{-}\)-treated neutrophils, over 90% of which was 1,2-diacylglycerol. Diacylglycerol generation was not dependent on the presence of extracellular Ca\(^{2+}\). Conversely, in contrast to results obtained with other agonists, removal of extracellular Ca\(^{2+}\) markedly potentiated synthesis of diacylglycerol in F\(^{-}\)-treated neutrophils. This effect was accompanied by a corresponding decrease in the recovery of phosphatidic acid. In either the presence or absence of extracellular Ca\(^{2+}\), phosphatidic acid accumulated before diacylglycerol in F\(^{-}\)-treated cells, suggesting the latter was derived from the former. Consistent with this hypothesis, the phosphatidic acid phosphohydrolase inhibitor, propranolol, suppressed generation of diacylglycerol as it potentiated the accumulation of phosphatidic acid in F\(^{-}\)-treated neutrophils. This effect was observed both in the presence and absence of extracellular Ca\(^{2+}\). Moreover, high levels of propranolol (160 \(\mu\)mol/L) effected complete inhibition of diacylglycerol generation in F\(^{-}\)-treated neutrophils with a corresponding increase in phosphatidic acid generation. Phosphatidylethanol accumulated in neutrophils stimulated with F\(^{-}\) in the presence of ethanol. The extent of phosphatidylethanol accumulation at all time points after addition of F\(^{-}\) corresponded to decreased levels of both phosphatidic acid and diacylglycerol, indicating that phosphatidylethanol was derived from the phospholipase D-catalysed transphosphatidylation reaction. The results indicate that F\(^{-}\) activates a Ca\(^{2+}\)-independent phospholipase D, which appears to be the major, if not sole, catalyst for both phosphatidic acid and diacylglycerol generation in F\(^{-}\)-treated neutrophils. Ca\(^{2+}\), mobilized as a result of F\(^{-}\) stimulation and possibly as a consequence of phospholipase D activation, exerts a profound effect on cellular second messenger levels by modulating the conversion of phosphatidic acid to diacylglycerol. © 1991 by The American Society of Hematology.
metabolism by F⁻, however, has facilitated identification and analysis of the temporal relationship of biochemical events that participate in the stimulus-response pathway leading to superoxide release. Superoxide release is preceded by the influx of extracellular Ca²⁺, which, in turn, follows phosphoinositide hydrolysis and release of inositol phosphates. The kinetics of Ca²⁺ influx in F⁻-treated neutrophils closely parallel the kinetics of PA generation, suggesting these responses may be related. Fluoride activates an inositol-specific phospholipase in isolated neutrophil plasma membranes. This response is inhibited by the nonhydrolysable GDP analog, GDP-β-S, suggesting the involvement of a guanine nucleotide regulatory protein. In intact cells, functional responses induced by F⁻, like those induced by FMLP, are dependent on guanine nucleotides while those induced by PMA are not, further implicating the involvement of guanine nucleotides in the neutrophils' response to F⁻. Originally used as an activator of the G proteins that regulate adenylate cyclase, F⁻ has become a valuable probe for investigating the role of G proteins in defined cellular responses as well as identifying novel G protein-effector enzyme systems in both intact cells and isolated cell membranes.

The present study was undertaken to define the pathways leading to diglyceride (DG) synthesis in F⁻-treated neutrophils. The results demonstrate that essentially all of the DG generated in F⁻-treated neutrophil is derived from the action of PA phosphohydrolase on PA. PA, which accumulates in F⁻-treated neutrophils when extracellular Ca²⁺ is available, appears to be an important second messenger in the neutrophil stimulus-response pathway; alterations in the rate of PA catabolism by depletion of extracellular Ca²⁺ may contribute to the role of Ca²⁺ in functional activation of cells stimulated with F⁻ as well as with other, physiologically relevant metabolic agonists.

MATERIALS AND METHODS

Lipid standards were purchased either from Avanti Polar Lipids (Birmingham, AL) (phosphatidylethanol [PEt]) or Sigma Chemical Co (St Louis, MO) (phosphoinositides, DG, phosphatidate, and other phospholipids). Diacylglycerol kinase was obtained from Lipidex (Westfield, NJ). Phospholipase A from Rhizopus arrhizus was purchased from Boehringer Mannheim (Indianapolis, IN). The radioisotopes used in this investigation, including [3H]glycerol (200 mCi/mmol), [3H]myristate (tetradecanoic acid; 10 Ci/mmol), ATP and ADP (3,000 Ci/mmol; 10 mCi/mL), and orthophosphoric acid ([32P]Pi, 25 mCi/mL) were purchased from New England Nuclear (Boston, MA). Sodium fluoride, PMA, FMLP, thin layer chromatography (TLC) plates, and other chemicals and reagents were obtained from Sigma Chemical Co. Propranolol-HCl was purchased from Sigma and dissolved in HEPES-buffered saline at a concentration of 1 mg/mL.

Neutrophil preparation and labeling. Blood was obtained from healthy individuals, heparinized, and processed by dextran sedimentation of erythrocytes followed by ficoll-Hypaque separation of polymorphonuclear and mononuclear leukocytes as previously described. All manipulations involving labeled cells, including preparation, washing, stimulation, and lipid extraction were performed in 16 × 125 mm disposable borosilicate glass culture tubes (Fisher Scientific, Pittsburgh, PA). Contaminating erythrocytes remaining in the neutrophil pellets were removed by lysis with isotonic ammonium chloride. Neutrophils were washed and resuspended in isotonic (0.15 mol/L) sodium chloride containing 1.0 mmol/L CaCl₂, 5.6 mmol/L glucose, and buffered to pH 7.4 with 10 mmol/L HEPES. Where indicated, Ca²⁺ was omitted and EGTA (0.1 mmol/L) was added to the final resuspension buffer.

For labeling experiments, cells were resuspended at a concentration of 1.5 × 10⁷ cells/mL and incubated with the indicated radiopharmaceutical for 45 minutes at 37°C. [3H]myristate was obtained in ethanol, which was removed by evaporation under nitrogen before cell labeling. Cells were incubated with approximately 100 µCi/mL of [3H]-choline, 10 µCi/mL of [3H]myristate, 100 µCi/mL of [3H]-glycerol, or 0.5 mCi/mL of [32P]Pi. Following the incubation period, the cells were washed twice and resuspended in HEPES-buffered saline containing 1.0 mmol/L Ca²⁺ and 5.6 mmol/L glucose, unless otherwise indicated. After the second centrifugation (800g, 2.5 minutes), the firmly packed cell pellets were gently but thoroughly rinsed with 3 to 5 mL of resuspension buffer continuously delivered with a Pasteur pipette to remove as much of the residual extracellular isotope as possible. Cells were then resuspended at a final concentration of 1.2 × 10⁷ cells/mL, unless stated otherwise.

Cell stimulation and lipid extraction. Phospholipid changes, inositol phosphate generation, DG synthesis, and phospholipase D-dependent PET generation were compared in unlabeled as well as in prelabeled cells stimulated for stated time intervals with nothing (control), 20.0 mmol/L NaF, 100 ng/mL PMA, or 10⁻⁷ mol/L FMLP. In experiments with FMLP, neutrophils were preincubated for 2 minutes at 37°C with 5 µg/mL of cytochalasin B (Sigma Chemical Co). Pharmaceuticals, cations, enzyme substrates, and other lipids were added before stimulation as indicated. For each determination, 1 mL of cells (1.2 × 10⁷ cells) was aliquoted into each 16 × 125 mm borosilicate glass culture tube and warmed to 37°C before agonist addition. After addition of the metabolic stimuli, reactions were stopped by addition of 5.0 mL of chloroform:methanol:HCl (30:60:1). Phases were then separated by the addition of 1 mL of water and 1 mL of chloroform, followed by vortexing and centrifugation. The aqueous phase was removed, re-extracted twice with chloroform, and the chloroform extracts combined. These were washed once with acidified (0.1 mol/L HCl) methanol, dried under nitrogen, and further processed as described below. Recoveries of DG, phosphatidate, phosphatidylcholine, and phosphatidylinositol bisphosphate were approximately 95%, 91%, 93%, and 64%, respectively, estimated using known amounts of exogenously supplied labeled standards.

Phospholipid analysis. The entire residue of each organic extract obtained from each extraction of 1.0 mL of cells was resuspended in 40 µL of chloroform:methanol (90:1) and spotted on 20 × 20 cm oxalate-coated, polyester-backed silica gel 60 TLC plates. After each spot was dried, residual lipids in each tube were collected in a second 40-µL aliquot of chloroform and carefully applied to the former spot. This procedure resulted in excellent consistency by eliminating variability associated with rapid evaporation of the organic solvents because the entire extract obtained from each aliquot of stimulated cells was applied to one individual lane of the TLC plate. Up to nine spots of approximately 1.0 × 0.2 cm were applied to each plate, approximately 2 cm from the base of the plate. Variability was further reduced by using the polyester-rather than glass-backed silica gel plates, allowing the resolved lipids to be removed by excision rather than by scraping. Cutting the lipid spots from the polyester-backed plates also greatly reduced processing time. Deviations in phospholipid recoveries were consistently less than 5.0% (coefficient of variation) for three to five individual samples processed from a single cell preparation. Much greater variations were observed between different cell preparations processed on separate days. Therefore, data reported...
reflect results from individual experiments, each of which was reproduced at least three times for verification. For kinetic analyses of lipid changes, each data point reflects results obtained after TLC of one cellular extract, unless otherwise indicated. Other experiments were performed in triplicate, with mean ± SD given.

Phospholipids were resolved in a solvent system consisting of chloroform:methanol:20% aqueous methylamine (60:35:10, solvent system I) or in a solvent system consisting of chloroform:acetone:methanol:glacial acetic acid:water (80:30:26:24:14, solvent system II) at 4.0°C. For experiments with 32P-labeled cells, plates were thoroughly dried after chromatography and exposed to Kodak XAR-5 film overnight to localize radioactive lipid spots. For experiments with 1H-labeled cells, unlabeled phospholipid standards (20 μg) were added to the dried residue before spotting to facilitate identification of the phospholipid of interest after chromatography. The position of the phospholipid standards was determined by spraying plates with molybdenum blue (0.65% molybdenum oxide in 4.2 mol/L sulfuric acid) or by exposure of plates to iodine vapors. Resolved spots, as well as spots corresponding to the position of radioactive lipids localized by autoradiography, were carefully excised, placed into glass scintillation vials containing 10 mL of liquid scintillation fluid (Beckman Bionalytic Systems Group, Fullerton, CA), and assayed 24 hours later for radioactivity by liquid scintillation counting.

DG analysis. Two methods were used to assay DG levels in organic extracts from stimulated cells. In the first method, referred to hereafter as the "radiometric method," lipids in extracts and cell membranes prelabeled with either 1H-glycerol or with 1H-myristate were resolved in a solvent system consisting of hexane:diethylether:90% formic acid (90:60:6, solvent system III). Before chromatography on 20 x 20 cm polyester-backed silica gel-60 plates at 4°C, each sample was "spiked" by the addition of 20 μg of a mixture of nonradioactive 1,2-diacylglycerol (DAG) and 1,3-diacylglycerol (diolein, mixed isomers; Sigma Chemical Co) dissolved in chloroform. The resolved bands were therefore easily localized after chromatography by exposure of the dried plates to iodine vapor. The bands were excised, placed into vials containing 10 mL of liquid scintillation fluid, and assayed for radioactivity by liquid scintillation counting.

In the second method, referred to as the "mass assay," lipids were extracted from cells that were not prelabeled. Mass levels of 1,2-DG in these extracts were quantitated after conversion of the glycerides to 32P-labeled PA with diacylglycerol kinase and ATP (32P) using the adaptations of the method of Preiss et al 4 indicated in the legend of Fig 1. Values of DAG, calculated from the conversion rate and specific activity of ATP-32P, are expressed as pmoles/10^6 cells.

The portion of the total diglyceride that was attached to the C-1

**Fig 1.** Generation of DAG in fluoride-treated neutrophils. Unlabeled neutrophils were incubated for 60 minutes at 37°C in the presence of 1.0 mmol/L Ca(2+) either without stimulus or with 20.0 mmol/L NaF as indicated. Reactions were stopped with chloroform:methanol:HCl (30:60:1); lipids were extracted and dried under nitrogen. Extracts were solubilized in chloroform and separated by TLC using solvent system III to isolate DG from phospholipids, neutral lipids, fatty acids, and other components within the organic extracts. Spots from the TLC plates corresponding to the migration position of DG were excised. DG were then eluted from the silica gel with chloroform:methanol (1:1). The eluted DG were dried under nitrogen, solubilized in a buffer consisting of Triton X-100, Mg(2+), phosphatidylethanolamine, and cardiolipin, and converted to radioactive PA with DG kinase and ATP-γ-32P, according to the method of Preiss et al. 4 After conversion, phospholipase A, from Rhizopus arrhizus was added to some samples to selectively degrade PA possessing fatty acids acylated at the C-1 position, leaving C-1 alkyl-PA (derived from EAG) intact. 4 The action of this lipase thereby results in the accumulation of LPA as diacylphosphatidic acid disappears. Figures are autoradiographs of TLC of lipase treated (+) and untreated (-) reaction products using solvent system I. Radioactive bands located between PA and LPA have not been identified, but are not due to phosphorylation of lipids in the neutrophil extracts because identical bands were observed in autoradiographs obtained when the buffers used (containing cardiolipin and phosphatidylethanolamine) were exposed to the commercial preparation of DG kinase and ATP-γ-32P in the absence of additional unlabeled DG and cellular extracts. The results demonstrate the accumulation of DG in F -treated neutrophils, the majority of which was DAG. However, a significant amount of lipase-resistant PA accumulated when the DG was exposed to kinase and ATP, as shown by the radioactivity in the PA band of the extracts of fluoride-stimulated cells subsequently exposed to Rhizopus lipase (see also Fig 2). In this experiment, the radioactivity recovered in excised PA bands increased from 190,419 (±446; mean and range of duplicate determinations) in unstimulated cells to 358,774 (±10,929) CPM in extracts of F -stimulated cells, an increase of 166,355 CPM. These values decreased to 20,237 (±140) CPM and 66,464 (±7.183) CPM in extracts of unstimulated and stimulated cells treated with lipase, respectively. Concurrently, radioactivity recovered with LPA increased from 30,447 (±7.263) CPM to 171,549 (±23,420) on addition of lipase to the extract from unstimulated cells. Lipase treatment increased radioactivity recovered with LPA from 32,565 (±1,089) CPM in unstimulated cells to 295,718 (±10,809) CPM in F -stimulated cells.
glycerol backbone with an ether linkage (1-0-alkyl-2-acyl-glycerol) was distinguished from the 1,2-diacyl species (DAG) by the method of Tyagi et al.2 After phosphorylation of the DG in cellular extracts to the corresponding 32P-PA as described above, lipase from Rhizopus arrhizus was added to selectively degrade the C-1-acyl-containing phosphatidate, leaving 1-0-alkyl-2-acyl-PA intact (see Fig 1). The C-1 phosphatidic acid (LPA) migrated slower than the parent diacyl species or the alkyl-acyl species of PA on TLC developed with solvent system I (Fig 1). The appearance of 32P-LPA and decrease in 32P-PA were used to calculate the relative amounts of alkyl-acyl and diacyl PA, reflecting the quantities of alkyl-acyl and diacylglycerol originally present in the cell extracts.

**Generation of PEt** Phospholipase D-catalysed transphosphatidylolation of labelled phospholipids to ethanol in intact cells was measured by a modification of previously described methods. Briefly, 1H-glycerol or 1H-myristate-labeled cells in a final volume of 1.0 mL were preincubated at 37°C for 5 minutes with 0.8% ethanol before stimulation. At various times after stimulation, reactions were terminated and lipids were extracted as described above. Dried extracts were spiked with 20 µg of both nonradioactive PA and PEt before spotting on 20 x 20 cm silica gel-60 plates for resolution by TLC. Samples were resolved in a solvent system consisting of chloroform:methanol:glacial acetic acid (50:25:8, solvent system IV) that completely separated PA (Rf 0.25), other phospholipids (Rf < 0.25), and PEt (Rf 0.85). After resolution, PA and PEt were localized by exposure of the plates to iodine vapor. The spots were excised and assayed for radioactivity as described above.

For kinetic analysis of simultaneous changes in PA, PEt, and 1,2-DG levels at defined intervals after cell stimulation, extracts were prepared from 1-mL aliquots of labeled cells as described above. Unlabeled PA, PEt, and mixed DG isomers were added to the dried extracts, which were then dissolved in chloroform: methanol:conc. HCl (90:10:0.1) and spotted 2 cm above the base of the 20 x 20 cm oxalate-impregnated, polyester-backed silica gel-60 TLC plates. Plates were developed half way to the top with solvent system IV to resolve PA and PEt from other phospholipids. Neutral lipids, including mono-, di-, and triglycerides migrated to the solvent front. Plates were removed, dried throughly, and developed from the base to the top with solvent system III. This solvent did not alter the position of the previously resolved phospholipids but resolved monoglycerides, DG, triglycerides, as well as free fatty acids in the upper half of the plate. Exposure of the developed plates to iodine vapor allowed definition of the phosphatidate, PEt, 1,2-, and 1,3-DG spots, which were excised, placed into vials containing liquid scintillation fluid, and assayed for radioactivity by liquid scintillation counting.

**Determination of intracellular free Ca2+.** Neutrophils were loaded with the Fura-2 by incubation with Fura-2 acetoxymethyl ester as described above. The cation was monitored using the formula of Grynkiewicz et al. The above experiments indicate that changes in intracellular free Ca2+ exert a profound influence on either the early synthesis or catabolism of DAG in F'--stimulated neutrophils. Adequate interpretation of these results, however, requires direct comparison of the changes in intracellular free Ca2+ levels in neutrophils resuspended in media with or without EGTA. Table 1 demonstrates the changes of F'--stimulated Ca2+ levels in neutrophils resuspended in media with or without Ca2+ under the conditions described above. When cells were exposed to NaF in the presence of extracellular Ca2+, intracellular Ca2+ levels began to increase approximately 1.5 minutes after stimulation, as indicated by an increase in FURA-2 fluorescence at 340 nm excitation and a decrease in fluorescence at 380 nm excitation. Ca2+ continued to increase slowly over the next 10 minutes of stimulation. This response was markedly attenuated in the absence of Ca2+ and was almost abrogated when intracellular Ca2+ was depleted by resuspending cells in media containing EGTA. The intracellular free
Ca\(^{2+}\) level of cells stimulated with F\(^{-}\) in the presence of extracellular Ca\(^{2+}\) increased 143 nmol/L over the resting cell level of 69 nmol/L within 10 minutes. When cells were resuspended in Ca\(^{2+}\)-free media (that did not contain EGTA), the F\(^{-}\)-stimulated increase was reduced to 90 nmol/L. Buffering extracellular Ca\(^{2+}\) with EGTA under the conditions used further reduced the F\(^{-}\)-stimulated Ca\(^{2+}\) elevation to an increase of only 11 nmol/L over levels observed in resting cells. Thus, buffering extracellular Ca\(^{2+}\) with EGTA decreased F\(^{-}\)-stimulated Ca\(^{2+}\) mobilization by over 90%. It is also noteworthy that depletion of extracellular Ca\(^{2+}\) with EGTA caused a substantial decrease in the intracellular free Ca\(^{2+}\) levels of resting cells.

The enhancement of DG synthesis observed in the absence of extracellular Ca\(^{2+}\) was not observed with neutrophil agonists other than F\(^{-}\). On the contrary, DG synthesis induced by PMA was only slightly inhibited by removal of extracellular Ca\(^{2+}\), while synthesis induced by FMLP in neutrophils pretreated with cytochalasin B was strongly inhibited by removal of extracellular Ca\(^{2+}\) (Table 2). Removal of Ca\(^{2+}\) had little influence on DG synthesis induced by PMA (Table 2). The effects of Ca\(^{2+}\) depletion on FMLP and PMA-induced DG synthesis were observed when DG was quantitated either by mass assay or by following the incorporation of either \(^{3}H\)-myristate or \(^{3}H\)-glycerol into material migrating with DAG during TLC of organic extracts of prelabeled cells.

PA. The results of the above experiments indicate that Ca\(^{2+}\) influenced either the generation or the catabolism of DAG in F\(^{-}\)-treated neutrophils. The decrease in recovery of diacylglycerol observed by addition of Ca\(^{2+}\) to the media of F\(^{-}\)-treated neutrophils, moreover, was consistently accompanied by a corresponding increase in recovery of PA. Figure 3 demonstrates that in either the presence or absence of Ca\(^{2+}\), the amount of diacylglycerol and PA generated, taken together, remained constant, although the

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**Table 1. Changes in Intracellular Free Ca Levels in F\(^{-}\)-Treated Neutrophils**

<table>
<thead>
<tr>
<th>Preincubation Conditions</th>
<th>Intracellular Free [Ca(^{2+})] (nmol/L)</th>
<th>F(^{-})-Stimulated Increase in Intracellular Free Ca(^{2+}) (nmol/L)</th>
<th>% Inhibition</th>
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<tbody>
<tr>
<td>1.0 mmol/L Ca(^{2+})</td>
<td>69.1 ± 5.1</td>
<td>212.5 ± 9.1</td>
<td>143.4</td>
</tr>
<tr>
<td>No added Ca(^{2+}) or cation chelator</td>
<td>46.2 ± 1.6</td>
<td>136.8 ± 4.9</td>
<td>90.6</td>
</tr>
<tr>
<td>1.0 mmol/L EGTA</td>
<td>24.4 ± 0.7</td>
<td>35.4 ± 1.0</td>
<td>11.0</td>
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Intracellular free Ca\(^{2+}\) levels were measured from the ratio of fluorescence at 340 and 380 nm excitation of neutrophils loaded with FURA-2. Resting levels were determined before addition of 20.0 mmol/L NaF; levels in F\(^{-}\)-stimulated cells were determined 10 minutes later, a time at which maximal, stimulated fluorescence levels were observed for all three conditions of incubation.\(^2\)
Neutrophils (1.0 mL, 1.2 x 10⁶ cells) were incubated for 10 minutes without stimulus or with 10⁻⁵ mol/L FMLP or 100 ng/mL PMA before extraction of lipids. In experiments with FMLP, cells were preincubated for 2 minutes with 5 μg/mL of cytochalasin B.

*Cells were stimulated in the presence or absence of 1.0 mmol/L extracellular Ca²⁺ as indicated.

†DG synthesis was assayed radiometrically, using cells prelabeled with ³H-glycerol or ³H-myristate or by the mass assay method described above (Fig 1). Results (mean ± SD; n = 3) reflect the percent increase in DG levels over values recovered in unstimulated cells under otherwise identical conditions.

relative amounts contributed by each lipid changed dramatically. This result is consistent with the hypothesis that Ca²⁺, mobilized as a result of exposure of cells to F⁻, influences the diacylglycerol-PA partitioning, possibly by activating diacylglycerol kinase or by inhibiting PA phosphohydrolase. In this respect, it is of interest that Ca²⁺ has been demonstrated to markedly inhibit PA phosphohydrolase in other systems.⁴⁺

To further explore the influence of PA phosphohydrolase on levels of diacylglycerol generated in F⁻-treated neutrophils, we preincubated neutrophils with the PA phosphohydrolase inhibitor, propranolol.⁵⁺,⁶⁺ Recent studies with stimulated cells confirm that relatively high levels of propranolol effectively inhibit PA phosphohydrolase.⁶⁺,¹⁺,¹⁺ In the present study, propranolol caused a strong, dose-dependent inhibition of F⁻-dependent diacylglycerol generation in intact cells (Fig 4). High levels of propranolol (160 μmol/L) almost completely inhibited generation of diglyceride induced by F⁻. Inhibition of DG synthesis by propranolol was observed in the absence of extracellular Ca²⁺ (Fig 4), although removal of Ca²⁺ decreased the extent of inhibition obtained with levels of propranolol above 50 μmol/L. Propranolol inhibition of DAG generation in cells preincubated with either ³H-glycerol or ³H-myristate was accompanied by a corresponding increase in the recovery of labeled PA (Fig 5), indicating that propranolol, like Ca²⁺, shifted the balance of PA-diacylglycerol in favor of PA. As shown in Fig 5, propranolol did not inhibit F⁻-stimulated hydrolysis of phosphatidylinositol bisphosphate. Furthermore, propranolol had no influence on alterations in the levels of phosphatidylcholine effected by F⁻ in neutrophils prelabeled with either ³H-myristate or ³P, or on inositol phosphate release in cells preincubated with ³H-inositol (data not shown), indicating that the pharmacologic agent
did not directly inhibit the activity of inositol-specific phospholipase C or choline-specific phospholipase D.

**Phospholipase D.** The above results indicate that the major portion of the DG that accumulates in F−-treated neutrophils is derived from PA. We investigated the source of PA in F−-treated neutrophils. Recent reports have demonstrated the activation of phospholipase D in mammalian cells. Phospholipase D catalyses a unique transphosphatidylation reaction with aliphatic alcohols to generate the corresponding phosphatidylalcohols. This reaction is highly specific for phospholipase D, and is not catalysed by phospholipid base exchange enzymes, and thereby provides compelling evidence for the agonist-induced activation of phospholipase D in responsive cells. Before 1987, PEt formation by phospholipase D had only been reported to occur in plant tissue. Since the initial report by Gustavsson and Alling of PEt formation catalysed by rat brain phospholipase D in the presence of ethanol, several reports have appeared wherein generation of PEt by cells or cell-free systems stimulated in the presence of ethanol provide evidence for the involvement of phospholipase D in signal transduction.

In the present study, when neutrophils were incubated with 0.8% ethanol in the absence of stimulus, no PEt was generated as assessed by TLC of extracts of 3H-myristate- or 3H-glycerol-labeled cells. When neutrophils prelabeled with 3H-myristate were exposed to F− for 15 minutes in the presence of ethanol, substantial generation of PEt was observed (Fig 6). Similar results were obtained with 3H-glycerol-labeled neutrophils (data not shown). When extracellular Ca2+ was available to decrease conversion of PA to diacylglycerol, the generation of PEt was accompanied by a corresponding decrease in accumulation of labeled PA. Thus, within the first 15 minutes of stimulation, the total radioactivity recovered with PA and PEt together in cells...

Fig 5. Effect of propranolol on F−-mediated changes in 1,2-diacylglyceride, PA, and phosphatidylinositol bisphosphate (PIP2). Neutrophils, prelabeled with either (A) 3H-myristate or (B) 3H-glycerol, were stimulated with 20.0 mmol/L NaF in the presence of 1.0 mmol/L extracellular Ca2+. Where indicated (P), propranolol (160 μmol/L) was added before addition of F−. The percent change in radioactivity from values recovered in extracts of unstimulated cells is illustrated. Values are mean ± SD of three determinations from one cell preparation. F− effected hydrolysis of PIP2 as indicated by the (−) sign during the incubation period. Increases in DG levels were diminished by propranolol to an extent that corresponded with propranolol-induced increases in recovery of PA.

Fig 6. PA and PEt generation in neutrophils stimulated with F−. Neutrophils were prelabeled with 3H-myristate and stimulated for 15 minutes in the presence or absence of 0.8% ethanol. After stimulation, lipids were separated by TLC. PA and PEt levels were determined radiometrically. Under the conditions used, the amount of radioactivity recovered with PA and PEt together in cells stimulated with F− in the presence of ethanol was equivalent to that recovered with PA alone in cells stimulated with F− in the absence of ethanol. Results reflect the mean and standard deviation of three determinations within one experiment that was separated twice for confirmation.
stimulated with F– in the presence of ethanol was only slightly less than the radioactivity recovered with PA alone in neutrophils stimulated with F– in the absence of exogeneous ethanol (Fig 6). The difference in the two values presumably resulted from the low but significant amount of diacylglycerol derived from PA at these early time periods in the presence of Ca++. These conclusions are supported by the comprehensive kinetic analysis of PA, PEt, and DAG generation illustrated in Fig 7. Removal of extracellular Ca++ enhanced synthesis of diacylglycerol and diminished production of PA (Fig 7A and B). In the presence of ethanol, PEt accumulated at the expense of both PA and diacylglycerol (Fig 7C). Propranolol markedly inhibited the accumulation of diacylglycerol and correspondingly potentiated the recovery of PA in F–-treated neutrophils (Fig 7D). Under each experimental condition shown in Fig 7, the total radioactivity (CPM) recovered with the individual lipids at each time point after stimulation remained constant. For example, 20 minutes after exposure of cells to F– in the presence of ethanol, the radioactivity associated with DAG, PA, and PEt, taken together, was similar to the radioactivity recovered with diacylglycerol and PA together after 20 minutes after stimulation of cells with F– in the absence of ethanol. This value was similar to that recovered with PA alone in cells stimulated in the presence of Ca++ and propranolol and in the absence of ethanol. Taken together, the results support the conclusion that, in F–-treated neutrophils, the major portion of diacylglycerol is derived by the action of PA phosphohydrolase on PA, generated by the activation of phospholipase D.

**DISCUSSION**

Emerging data indicate that several phospholipases D with divergent activation requirements, optimal reaction conditions, and substrate specificities exist in different types of mammalian cells. Moreover, the characteristics of the enzyme or enzymes responsible for this activity within any one cell type may change depending on the route of activation as well as the availability of cations, cofactors, and potential substrates. In both hepatocytes and endothelial cells, for example, Ca++-mobilizing hormones and purinergic receptor agonists activate a choline-specific phospholipase C that is regulated by a guanine nucleotide binding protein.23 While the activity of this enzyme can be stimulated by the nonhydrolysable GTP analog, GTPyS, in a cell-free system in the virtual absence of Ca++, stimulation of choline-specific phospholipase D in homogenates of HL-60 cells by GTPyS is Ca++-dependent.28,35 Endothelial cell phospholipase D is dependent on detergent (Triton X-100) for activity in cell-free systems,6 but similar detergent requirements have not been demonstrated for phospholipase D activity of other cell types. While 1-0-alkyl-phosphatidylcholine is a poor substrate for endothelial cell phospholipase D,6 ether-linked phospholipids are readily hydrolysed by the enzyme in homogenates of HL-60 cells in the presence of Ca++.6,35 Balsinde et al identified an inositol-specific phospholipase D in isolated neutrophil plasma membranes,8 but phospholipase D present in lysates of endothelial cells do not display activity against phosphoinositides.6 Earlier studies by Cockcroft et al53,54 postulated the existence of an inositol-specific phospholipase D activated by FMLP in neutrophils. Reinhold et al27 postulated that three separable but interacting routes lead to the activation of phospholipase D in human neutrophils: (1) direct activation by protein kinase C in cells stimulated with PMA; (2) Ca++-dependent activation in cells exposed to Ca++ ionophore; and (3) a currently undefined route stimulated by the receptor-dependent agonist, FMLP. Our data

![Fig 7. Effect of propranolol and ethanol on the kinetics of DG, PA, and PEt generation in F–-treated neutrophils. Cells were prelabeled with 3H-myristate and exposed to 20.0 mmol/L NaF in the presence (A, C, and D) or absence (B) of extracellular Ca++. (C) illustrates the kinetics of PA, PEt, and DG generation in the presence of 0.8% ethanol. (D) 160 μmol/L propranolol was added. Each data point reflects results obtained from a single determination.](from-www.bloodjournal.org-by-guest-on-september-14,-2017.-for-personal-use-only.)
suggest an additional pathway of neutrophil phospholipase D activation that displays markedly different characteristics than the pathway activated by FMLP, PMA, or Ca\(^{2+}\) ionophore.

Our results demonstrate that diacylglycerol is derived from PA in F\(^{-}\)-treated neutrophils and indicate that Ca\(^{2+}\) may be a physiologically relevant regulator of the activity of PA phosphohydrolase, which itself may be a crucial regulatory enzyme. We examined the influence of extracellular Ca\(^{2+}\) on DG generation because the cation is required for optimal functional activation (superoxide release) induced by fluoride, because Ca\(^{2+}\) is required for optimal neutrophil diacylglycerol generation induced by FMLP and other metabolic agonists, and because many phospholipases are known to be Ca\(^{2+}\) dependent. We therefore examined the possibility that diacylglycerol formed when neutrophils were exposed to F\(^{-}\) was a consequence of Ca\(^{2+}\) mobilization, rather than an essential intermediate in the signal-transduction pathway leading to Ca\(^{2+}\) influx and functional activation. In marked contrast to the results obtained here and by others\(^{29,30,35,36}\) with FMLP and certain other stimuli, removal of extracellular Ca\(^{2+}\) markedly potentiated the synthesis of diacylglycerol in F\(^{-}\)-stimulated neutrophils. This result was observed when diacylglycerol levels were quantitated either with the use of radioactive glycerol, labeled fatty acids, or by mass assay (Fig 2). The potentiality of diacylglycerol levels by removal of Ca\(^{2+}\) could be attributed either to enhanced generation or decreased catabolism of the lipid. Consistent with the former possibility is the observation that PA phosphohydrolase in other cell types is inhibited by Ca\(^{2+}\).\(^{45,50}\) That Ca\(^{2+}\) alters the conversion of PA to diacylglycerol is also consistent with the observation that the radioactivity recovered with PA and diacylglycerol together was constant in either the presence or absence of Ca\(^{2+}\), although the amounts individually contributed by each of the two lipids varied (Figs 3 and 7). An alternative hypothesis is that Ca\(^{2+}\) activates a DG kinase that generates PA. Comparison of the kinetics of PA and diacylglycerol generation in the presence and absence of Ca\(^{2+}\) argues against this possibility. Furthermore, the results with propranolol, a potent inhibitor of PA phosphohydrolase,\(^{29,30,35,36}\) confirm the conclusion that the major portion of the diacylglycerol generated in F\(^{-}\)-treated neutrophils is derived from PA, therefore supporting the hypothesis that Ca\(^{2+}\) shifts the diacylglycerol-PA partition by inhibiting PA catabolism. Thus, high levels of propranolol effected almost complete inhibition of diacylglycerol generation in F\(^{-}\)-treated neutrophils (Fig 4). This effect was not due to nonspecific toxicity, because PA levels were potentiated when diacylglycerol synthesis was inhibited by propranolol (Fig 7). The levels of PA recovered in neutrophils stimulated with F\(^{-}\) in the presence of propranolol were equivalent to levels of PA plus diacylglycerol together recovered in cells stimulated in the absence of propranolol. The results indicate that PA and diacylglycerol are maintained in a dynamic balance that can be altered either by pharmacologic modulators or by physiologically relevant messengers, including intracellular cations.

In future studies it will be important to determine if the transient increase in intracellular Ca\(^{2+}\) effectuated by various stimuli is sufficient to inhibit the conversion of PA to DAG and thus contribute to PA accumulation. Further experiments will also be required to confirm that Ca\(^{2+}\) depletion caused the observed effects by influencing PA phosphohydrolase rather than DG kinase. In recent work, we have demonstrated that Ca\(^{2+}\) effectively inhibits the activity of neutrophil PA phosphohydrolase in a cell-free system (Taylor GS, Greene B, and English D, manuscript in preparation). While this result supports our hypothesis that Ca\(^{2+}\) exerts its influence in intact cells by inhibiting PA phosphohydrolase (rather, for example, than by activating DG kinase) it does not explain the remarkable difference in the influence of Ca\(^{2+}\) on DG accumulation in cells treated with FMLP and other stimuli in comparison with F\(^{-}\). There are two other differences in DG generation that may directly relate to this observation. First, DG generation in neutrophils treated with many stimuli shows a strong dependency on the presence of extracellular Ca\(^{2+}\).\(^{28,34,45,50}\) (Table 1). Second, while F\(^{-}\)-treated neutrophils generated very little EAG, appreciable amounts of this species are generated in neutrophils challenged with ionophore, FMLP, PMA, and in primed neutrophils treated with FMLP.\(^{41}\) We have evaluated our methods for detecting 1-O-alkylglycerols by exposing cytochalasin B-primed neutrophils to FMLP and were able to recover appreciable levels of EAG (data not shown). A possible explanation is that the Ca\(^{2+}\)-independent phospholipase D activated by F\(^{-}\) is a different enzyme and displays a different substrate specificity than the Ca\(^{2+}\)-dependent enzyme activated by FMLP and other agonists. The latter enzyme may also be activated by PMA and by stimulation of PMA-primed cells with other agonists because the phorbol ester may, by directly activating protein kinase C, eliminate the Ca\(^{2+}\) requirement for phospholipase activation.\(^{23}\) In any event, the significance of Ca\(^{2+}\)-independent phospholipase D activation in the early events of the neutrophil signal-transduction pathway is not clear. Products of these enzymes presumably exert their influence on processes activated after Ca\(^{2+}\) is mobilized.

PA is derived from the activation of phospholipase D in fluoride-treated neutrophils. This conclusion is supported by the kinetics of PA and diacylglycerol generation as well as by the generation of PEt at the expense of both PA and diacylglycerol when neutrophils were stimulated with F\(^{-}\) in the presence of ethanol (Figs 6 and 7). PEt generation results from the transphosphatidylation activity of phospholipase D.\(^{44-45}\) That PEt generation resulted exclusively from diversion of the phospholipase D catalysed pathway from PA to PEt is evidenced by the fact that the extent of synthesis of labeled PEt at all time points after addition of fluoride mirrored the extent of inhibition of generation of labeled PA plus diacylglycerol from their substrate(s). Thus, radioactivity recovered with PA plus diacylglycerol in cells stimulated in the absence of ethanol was equivalent to that recovered with PEt plus PA plus diacylglycerol in cells stimulated in the presence of ethanol at all time points after addition of F\(^{-}\).

The results of our study demonstrate that F\(^{-}\) activates Ca\(^{2+}\)-independent neutrophil phospholipase D. This en-
zyme appears to be responsible for most, if not all, of the PA and diacylglycerol generated in F- treated neutrophils. Because F- induces neutrophil oxidative metabolism, activation of phospholipase D therefore is sufficient to initiate the sequence of metabolic events that result in activation of cellular function. Furthermore, the ability of F- to activate guanine nucleotide regulatory proteins to the dependence of F--stimulated neutrophil activation on guanine nucleotides strongly suggest that F- initially exerts its influence on neutrophils by activating or modulating the activity of a unique G-protein that regulates the activity of this phospholipase D. Because neutrophil oxidative activation induced by F- is dependent on the influx of extracellular Ca2+ while activation of phospholipase D by F- is not, our results are consistent with the hypothesis that phospholipase D activation facilitates the transport of Ca2+ across the plasma membrane. Ca2+ influx may result from structural alterations secondary to phospholipid hydrolysis, the ionophoretic properties of PA, or from other, as yet unidentified processes.

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Diacylglycerol generation in fluoride-treated neutrophils: involvement of phospholipase D

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