Phosphatidic Acid Regulates Tyrosine Phosphorylating Activity in Human Neutrophils

ENHANCEMENT OF Fgr ACTIVITY*

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In human neutrophils, the activation of phospholipase D and the Tyr phosphorylation of proteins are early signaling events upon cell stimulation. We found that the pretreatment of neutrophils with ethanol (0.8%) or 1-butanol (0.3%), which results in the accumulation of phosphatidylalcohol at the expense of phosphatidic acid (PA), decreased the phorbol myristate acetate-stimulated Tyr phosphorylation of endogenous proteins (42, 115 kDa). When neutrophil cytosol was incubated in the presence or absence of PA, these and other endogenous proteins became Tyr-phosphorylated in a PA-dependent manner. In contrast, phosphatidylalcohols exhibited only 25% (phosphatidylethanol) or 5% (phosphatidylbutanol) of the ability of PA to stimulate Tyr phosphorylation in the cell-free assay. Similarly, other phospholipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylserine, phosphatidylinositol, polyphosphoinositides, and sphingosine 1-phosphate) showed little ability to stimulate Tyr phosphorylation. These data suggest that PA can function as an intracellular regulator of Tyr phosphorylating activity. Gel filtration chromatography of leukocyte cytosol revealed a peak of PA-dependent Tyr phosphorylating activity distinct from a previously described PA-dependent phosphorylating activity (Waite, K. A., Wallin, R., Qualliotine-Mann, D., and McPhail, L. C. (1997) J. Biol. Chem. 272, 15569–15578). Among the protein Tyr kinases expressed in neutrophils, only Fgr eluted exclusively in the peak of PA-dependent Tyr phosphorylating activity. Importantly, Fgr isolated from unstimulated neutrophil lysates showed increased activity in the presence of PA but not phosphatidylbutanol. Moreover, the pretreatment of neutrophils with 1-butanol decreased Fgr activity in cells stimulated with formyl-methionyl-leucyl phenylalanine plus dihydroxyocholesin B. Together, these results suggest a new second messenger role for PA in the regulation of Tyr phosphorylation.

Neutrophils are dynamic, short lived cells that play a vital role in the early phases of the immune response. They provide formidable killing mechanisms against invading organisms. Neutrophils sense their environment through a variety of cell surface receptors that allow the cells to respond to bacterial products, inflammatory cytokines, immunoglobulins, complement cleavage products, and adhesion molecules. Upon receptor ligation, numerous signaling events ensue within the cell that culminate in a repertoire of functional responses appropriate for a given stimulus. Among the many signaling events induced, increased Tyr phosphorylation of proteins is commonly observed for a wide variety of neutrophil stimuli (1, 2). Importantly, Tyr phosphorylation appears to play a central role in signaling pathways because treatment of neutrophils with inhibitors of protein Tyr kinases has been shown to interfere with most cellular functions (reviewed in Ref. 3).

Neutrophils express a large number of nonreceptor protein Tyr kinases (2–4) with representatives from eight of the nine known families (5). The functional roles of only a few of these enzymes in neutrophils have been clearly defined (reviewed in Ref. 2). Similarly, little is known about the regulation of protein Tyr kinases in neutrophils. The factors that influence Tyr phosphorylation in neutrophils are diverse and may reflect the variety of enzymes expressed in these cells. Examples of signaling events shown to alter protein Tyr kinase activity include ligand-induced dimerization of the Fc receptor (2), reactive oxygen intermediates (4, 6, 7), and G protein activation (8).

Recently, the enhancement of Tyr phosphorylation by exogenously added (9–11) or endogenously generated (9, 11) PA,1 the lipid product of phospholipase D, has been observed in neutrophils and other cell types. The mechanism by which PA stimulates Tyr phosphorylation has not been addressed.

Like Tyr phosphorylation, the activation of phospholipase D is an early cellular signaling event and has been linked to functional responses of the neutrophil (12, 13). This enzyme metabolizes membrane phospholipids, in a stimulus-dependent manner, yielding PA and the free head group (e.g. choline or ethanolamine). Although slow to gain acceptance as a lipid second messenger, PA has now been reported to interact with or activate a variety of signaling components including protein Ser/Thr kinases (14–17), protein Ser/Thr (18) and Tyr (19) phosphatases, a cyclic nucleotide phosphodiesterase (20), regulators of G proteins (21, 22), lipid kinases (23), and phospho-

1 The abbreviations used are: p47phox, 47 kDa phagocytic oxidase component; DFP, diisopropyl fluorophosphate, Me3SO, dimethyl sulf oxide; MMLP, formyl-methionyl-leucyl phenylalanine; GST, glutathione S-transferase; PA, phosphatidic acid; PMA, phorbol myristate acetate; PC, phosphatidylcholine; MAPK mitogen-activated protein kinase; PAGE, polyacrylamide gel electrophoresis.
lipases (24, 25). Thus it is possible that the PA-stimulated Tyr phosphorylation previously observed (9–11) could be due to the interaction of PA with any of these signaling molecules or perhaps a protein Tyr kinase. In this report, we examined the stimulatory role of PA on Tyr phosphorylating activity in neutrophils, characterized this activity, and identified at least one protein Tyr kinase that appears to be regulated by PA. Our findings suggest a novel facet of protein Tyr kinase regulation and a new signaling role for PA.

**EXPERIMENTAL PROCEDURES**

**Materials—**Phorbol myristate acetate (PMA), formyl-methionyl-leucyl phenylalanine (FMLP), dihydroxyethanolamine B, phosphatidylinositol 4- phosphate, and phosphatidylinositol 4,5-bisphosphate were obtained from Sigma. The anti-phospho-Tyr (clone 4G10) antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Fgr antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA; for immunoprecipitation) and Wako Bioproducts (Richmond, VA; for immunoblotting). Other protein Tyr kinase antibodies were obtained from Santa Cruz Biotechnology (Lyn, Hk, SY), Calbiochem (cAb) and Dr. Peter Greer (Queen’s University, Canada; Fer, Fes). Anti-p47phox (26) and Escherichia coli containing the plasmid encoding glutathione S-transferase (GST)-p47 fusion protein were gifts from Dr. Thomas Leto (National Institutes of Health). The GST-p47 fusion protein was prepared as described previously (27). Horseradish peroxidase-conjugated secondary antibodies were from Transduction Laboratories (Lexington, KY). Protein A-Sepharose beads were from Amersham Pharmacia Biotech. Enolase was from Roche Molecular Biochemicals. The following lipids were purchased from Avanti Polar Lipids (Alabaster, AL), the phosphatidic acid (PA) dioctanoyl (di8:0), dicapryl (di10:0), dilauroyl (di12:0), dipalmitoyl (di16:0), dicerotate (di18:0) and dioleoyl (di18:1); phosphatidic acids (PA) dioctanoyl (di8:0), dicapryl (di10:0), dilauroyl (di12:0), dipalmitoyl (di16:0), distearoyl (di18:0) and dioleoyl (di18:1); dioleoyl phosphatidylethanol; dioleoyl phosphatidylbutanol; dioleoyl phosphatidylglycerol; and sn-1 oleoyl lysophosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, phosphatidylycerine, and egg lecithin-derived PA were obtained from D. D. Dobberstein, Jr., and supplied by Dr. Thomas F. Mobley (National Institutes of Health). The GST-p47 fusion protein was prepared as described previously (27).

**Experimental Procedures**

**Neutrophil/Leukocyte Isolation, Stimulation, and Fractionation—**Neutrophils were isolated from heparinized venous blood obtained from healthy adult volunteers by dextran (T500; Amershams Pharmacia Biotech; for unit volumes of blood) or Isolymph (Gallard-Schlesinger Industries, Carls Place, NY; for small volumes of blood) sedimentation, centrifugation over Isolymph, and hypotonic lysis of contaminating erythrocytes as described previously (29). Pooled leukocytes were isolated from whole blood as described for neutrophils except that the Isolymph centrifugation step was omitted. All neutrophils (>95% purity) or leukocyte preparations were resuspended at 5 × 10⁶ cells/ml in cold Hank’s buffered salt solution (Life Technologies, Inc.) and treated with dioxoperpropyl fluoride (DFP) as described previously (30). For cell stimulation experiments, neutrophils (5 × 10⁶ cells/ml) were warmed for 5 min at 37 °C in the presence or absence of ethanol (0.8%, v/v), butanol (0.3%, v/v) or ethanol (0.8%, v/v) and butanol (0.3%, v/v) and then stimulated (10 mM PMA or 1 μM FMLP plus 10 μM dihydroxyethanolamine B) for various times. The incubation was terminated by collecting cells after a rapid centrifugation (50–40 000 × g, 9500 × g, 10 min). In experiments designed to assess whole cell Tyr phosphorylation (Fig. 1), cells were rapidly resuspended in ice-cold protection buffer (phosphate-buffered saline plus 10 mM EDTA, 5 mM EGTA, 25 mM NaF, 5 mM Na2VO4, 1 mM phenylmethylsulfonyl fluoride, 1 mM DFP, 10 μg/ml leupeptin, 10 μg/ml pepstatin and 1 μg/ml aprotinin) (31). An equal volume of 2× Laemmli sample buffer (32) was added, and the cell lysate was immediately boiled. (Chemicon, CA) and rabbit IgG (30 μg/ml) were added. The sample was boiled for 10 min. Aliquots (4.5 × 10⁶ cell equivalents) of lysates were subjected to SDS-PAGE and immunoblotting for phospho-Tyr detection, described below. In experiments designed to assess Fgr activation, pelleted cells were lysed in ice-cold solubilization buffer (10 μM Tris (pH 7.5), 100 mM NaCl, 2 mM EGTA, 2 mM EDTA, 50 mM NaF, 1 mM Na2VO4, 2 mM μg/ml pepstatin, 10 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM DFP, 1% Nonidet P-40) for 15 min on ice with 10 passages through a 25-gauge needle. The lysate was cleared by centrifugation and subjected to immunoprecipitation. Neutrophil cytosol was obtained from cells disrupted by sonication. The resultant lysate was subjected to ultracentrifugation over a discontinuous sucrose gradient (15/40%, w/v) as described previously (30, 33). The supernatant of pooled leukocyte fractions subjected to ultracentrifugation at 4 °C (0.2%, v/v, 150,000 × g) in the absence of the sucrose gradient was collected as the cytosol. All cytosolic fractions were stored at −70 °C. Protein concentrations were determined with the Coomassie-Plus Protein assay (Fierce) using bovine serum albumin as a standard.

**Preparation of Lipids—**Lipids were prepared daily by drying a known volume of chloroform solution under a stream of nitrogen and then sonicating in deionized water with a probe sonicator. The concentration of phospholipids was verified by lipid phosphorus analysis (34).

**Tyr Phosphorylation Assay—**The cell-free Tyr phosphorylation assay was carried out in kinase buffer (50 mM NaPO4 buffer (pH 7), 1 mM EGTA, 5 mM MgCl2, 0.1 mM Na2VO4 plus 100 μM lipid, 30 mM ATP, 6.7 μg/ml exogenous substrate (GST-p47), and cytosolic protein (0.1 mg/ml) in a final volume of 135 μl. The reaction mixture (minus cytosol) was warmed for 5 min at 25 °C, and cytosol was added and incubated for 45 min. The reaction was terminated by the addition of 15 μl of 10× dithiothreitol in Laemmli sample buffer. Samples were quickly boiled and subjected to SDS-PAGE. In preliminary experiments, phosphatidylcholine was found ineffective at stimulating Tyr phosphorylation, although it was shown to allow for exogenous substrate recovery on immunoblots to an extent equal to that of PA. Thus, phosphatidylcholine was routinely used in the minus PA control condition.

The phosphorylation assay was modified for the measurement of kinase activities in gel filtration column fractions (Fig. 5) to compare protein Tyr kinase (detected by anti-phospho-Tyr immunoblotting) and protein Ser/Thr kinase (detected by incorporation of radiolabel activity) in parallel. The ATP concentration was reduced to 8 μM, and gelatin (0.1 mg/ml), an inert bulk protein, was included to ensure equivalent recovery of exogenous substrate protein on immunoblots. The protein Tyr kinase activity was determined in the presence of Na2VO4 and unlabeled ATP, whereas protein Ser/Thr kinase activity was determined in the presence of Na2VO4 and radiolabeled ATP (10 Ci/mmol (16)). Preliminary experiments indicated that Tyr phosphorylation was not readily detected at the reduced ATP concentration (8 μM) in the absence of Na2VO4. In addition, at 8 μM ATP, there was a negligible increase in radiolabel incorporation into the exogenous substrate in the presence of Na2VO4 compared with its absence. This is probably due to the large number of Ser (compared with Tyr) phosphorylation sites in the exogenous substrate, the incorporation of radiolabel into the exogenous substrate in the absence of Na2VO4 was used to estimate PA-stimulated protein Ser/Thr kinase activity.

**Immunoprecipitation and Immune Complex Kinase Assay—**Neutrophil lysates were cleared with protein A-Sepharose and rabbit IgG (30 min, 4 °C). Fgr was isolated by immunoprecipitation by incubation with Fgr antibody and protein A-Sepharose for 2 h (4 °C). Fgr-bound beads were washed three times with solubilization buffer without DFP, EDTA, or Nonidet P-40 and then twice with kinase buffer. The washed beads were prepared for the immune complex kinase assay by the addition of 40 μl of kinase buffer and warmed for 5 min at 25 °C before the addition of 10 μl of ATP mix containing 10 μl of [32P]ATP (6000 Ci/mmol). Samples were incubated for 45 min at 25 °C, terminated by the addition of 12.5 μl of 5× dithiothreitol in Laemmli sample buffer, and prepared for SDS-PAGE.

**SDS-PAGE and Immunoblotting—**Protein samples were electrophoretically separated on 7 (phospho-Tyr) or 8% (Fgr activity) gels and transferred to nitrocellulose in a transfer buffer containing 25 mM Tris-HCl, 192 mM glycine, 20% methanol, supplemented with 0.1% SDS (36) to promote the efficient transfer of the hydrophobic GST fusion protein exogenous substrate. Blots were subjected to immunoblotting and visualized by enhanced chemiluminescence (Pierce) as described previously (30), except that blots probed for phospho-Tyr were blocked with 0.04% gelatin plus 0.5% goat serum in Tris-buffered saline with Tween 20 (Tris-buffered saline, 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1% Tween 20). To verify recovery of the exogenous substrate, blots were probed with anti-p47phox antibody. Tyr phosphorylation data for the exogenous substrate were routinely normalized to exogenous substrate protein to account for small differences in sample loading. Immunoblots were analyzed and quantified by densitometry (PDI, Huntington Station, NY); data from radiolabeled kinase assays were quantified by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA).

**Gel Filtration Chromatography—**Leukocyte cytosol (130 mg of pro-
Pretreatment of Neutrophils with Primary Alcohols Reduces PA-stimulated Tyr Phosphorylation

**RESULTS**

**Pretreatment of Neutrophils with Primary Alcohols Reduces PA-stimulated Tyr Phosphorylation**—PMA has been shown to stimulate both Tyr phosphorylation (37–39) and PA production (40) in neutrophils. To determine whether PA-stimulated Tyr phosphorylation is related to PA production, we pretreated neutrophils with ethanol or 1-butanol, both commonly used tools to study phospholipase D-derived PA. These primary alcohols act by competing with water as the hydroxyl donor in the hydrolysis of membrane phospholipids by phospholipase D (41). Thus, in the presence of primary alcohols, the phosphatidylylcholine is produced at the expense of PA (41, 42). Fig. 1A shows that PMA-stimulated Tyr phosphorylation (detected by immunoblotting) of many neutrophil proteins (compare lanes 1 versus 3 and 5 versus 6), whereas others were constitutively phosphorylated or dephosphorylated. Two proteins (~115 and 42 kDa) exhibited Tyr phosphorylation that was reduced by the pretreatment of cells with either ethanol (lane 4) or 1-butanol (lane 7). In contrast, the secondary alcohol, 2-butanol (lane 8), had little effect on the PMA-stimulated Tyr phosphorylation of these proteins. Since primary, but not secondary, alcohols can be utilized by phospholipase D, PA levels in 2-butanol-treated cells are not compromised.

When data from several experiments were pooled, we found that the PMA-stimulated Tyr phosphorylation of these proteins (~115 and 42 kDa) was transient but reached a maximum by 5 min (Fig. 1, B and C). The PMA stimulation of PA accumulation occurs over a similar time course (40). Moreover, the Tyr phosphorylation of these proteins at 5 min was reproducibly reduced by ethanol pretreatment of cells. Taken together, these findings suggest that PA may play a role in enhancing Tyr phosphorylation in stimulated neutrophils.

**Neutrophil Cytosol Contains PA-stimulated Tyr Phosphorylating Activity**—To characterize the PA-stimulated Tyr phosphorylating activity in neutrophils, we developed a cell-free assay to measure this activity. Fig. 2A shows a typical phospho-Tyr immunoblot (right panel) of neutrophil cytosol that was incubated with the exogenous substrate in the presence of 100 µM phosphatidylcholine (~PA) or dicapryl (di10:0) PA (+PA) for 45 min at 25 °C. The reaction was terminated by the addition of 10× Laemmli sample buffer. Proteins were separated by SDS-PAGE, and Tyr-phosphorylated proteins were detected by immunoblotting (right panel). The open arrowheads show endogenous cytosolic proteins that were Tyr-phosphorylated in a PA-dependent manner. The exogenous substrate was detected on the same blot after stripping and reprobing with anti-p42 (left panel). The closed arrowhead shows the exogenous substrate (GST-p47). The migration of molecular mass standards is indicated on the left. The results shown are representative of 7–8 neutrophil cytosol preparations derived from different donors. B, the time course of PA-dependent Tyr phosphorylation of the exogenous substrate (~PA) and p115 (~PA) in the cell-free assay was determined from anti-phospho-Tyr immunoblots by densitometric analysis. Data are the mean ± S.E. for cytosol preparations derived from three different donors.

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fining substrates of potential PA-regulated protein Tyr kinase as well as the PA target(s) responsible for the Tyr phosphorylation.

Fig. 2A also shows that an exogenous substrate, the GST fusion protein of p47phox (GST-p47, filled arrowhead), was Tyr-phosphorylated in a PA-dependent manner. The difference in the phosphorylation status of the exogenous substrate in the two incubation conditions was not due to differences in the amount of substrate protein. The phospho-Tyr blot was stripped and reprobed with an anti-p47phox antibody to confirm that the amount of substrate was the same in both incubation conditions (Fig. 2A, left panel). Employing this exogenous substrate facilitated the comparison of Tyr phosphorylating activity in different cytosol preparations and quantification of the data. An examination of other exogenous substrates showed that recombinant p47phox (produced in a baculovirus system (26)) and enolase, to a lesser extent, also became Tyr-phosphorylated in the presence of PA (not shown). In contrast, other substrates used to measure protein Tyr kinase activity (poly(Glu, Tyr)12, and poly(Glu, Tyr)12) were not Tyr-phosphorylated (not shown).

The Tyr phosphorylation of substrates occurred in a time-dependent fashion. When reactions were incubated at 25 °C, the PA-dependent Tyr phosphorylation of the exogenous substrate increased linearly for about 45 min and then plateaued (Fig. 2B). In contrast, the PA-dependent Tyr phosphorylation of p115 occurred more rapidly. In the absence of PA, Tyr phosphorylation of substrates was negligible (not shown).

To characterize further PA-stimulated Tyr phosphorylation, we assessed the pH and divalent cation requirements. The pH maximum for PA-dependent Tyr phosphorylation was between pH 6.5 and 7.0 for both the exogenous substrate and p115 (not shown). We chose to use pH 7.0 in our standard assay conditions. With respect to divalent cations, we found that the PA-dependent Tyr phosphorylation was not different in the presence of Mg2+ and Mn2+ (99 ± 3% of Mg2+ control; mean ± S.E., n = 3; not shown) in Tris-buffered reaction mixtures (not shown). Tyr phosphorylating activity could not be measured in NaPO4-buffered reaction mixtures, because a manganese salt precipitated. Finally, the addition of Ca2+ (1 mm) to either Mg2+ or Mn2+-containing reaction mixtures resulted in an approximate 50% decrease in the PA-dependent Tyr phosphorylation of the exogenous substrate, whereas the Tyr phosphorylation of endogenous proteins was almost completely blocked (not shown).

Lipid Specificity of Stimulated Tyr Phosphorylation—Many studies have shown that the treatment of neutrophils with either ethanol or 1-butanol results in the production of the corresponding phosphatidylalcohols in the stimulated cell (11, 12, 43–46). Since this treatment decreased PMA-stimulated Tyr phosphorylation in intact cells (Fig. 1), phosphatidylalcohols should be less efficient activators of Tyr phosphorylation than PA, if PA is involved in this response. To test this hypothesis, we assessed the ability of phosphatidylethanol and phosphatidylbutanol to mimic the ability of PA to stimulate Tyr phosphorylation in the cell-free assay. Fig. 3 shows that phosphatidylethanol induced the Tyr phosphorylation of the p115 to only 25% (p < 0.001) of the level induced by PA having the same acyl chain composition (dioleoyl, di18:1). This suggests that we probably underestimated the PMA-stimulated Tyr phosphorylation of endogenous proteins due to PA in intact cells pretreated with ethanol (Fig. 1, B and C). The inability of phosphatidylbutanol to stimulate the Tyr phosphorylation of endogenous protein was more striking. The presence of phosphatidylbutanol stimulated only 5% (p < 0.001) of the Tyr phosphorylation of p115 compared with the dioleoyl PA control conditions. In contrast to the endogenous protein, we did not observe a marked difference between the ability of phosphatidylethanol and phosphatidylbutanol to mimic the Tyr phosphorylation of the exogenous substrate, which exhibited only 34 and 42%, respectively, of the activity of PA.

As a positive control, we included dicapryl (di10:0) PA in the experiments shown in Fig. 3. The data suggest that PAs of different acyl chain length may vary in their ability to stimulate Tyr phosphorylation. We therefore examined the importance of acyl chain composition on PA-stimulated Tyr phosphorylation using synthetic and naturally occurring (derived from egg lecithin) PAs. Dioleoyl (di18:1) PA was as effective as the shorter chain dicapryl PA in stimulating the Tyr phosphorylation of p115 (Fig. 4A) and the exogenous substrate (Fig. 4B). The naturally occurring egg lecithin-derived PA (egg; at 100 μM) exhibited 53 and 75% of the Tyr phosphorylation stimulating activity of dicapryl PA for p115 (Fig. 4A) and the exogenous substrate (Fig. 4B), respectively.

PAs with short (di8:0), intermediate (di12:0 and di16:0), and long (di18:0) saturated acyl chains were further tested for their ability to stimulate the Tyr phosphorylation of the exogenous substrate (Fig. 4C). Like dicapryl PA, the short chain di-octanoyl (di8:0) PA was an effective activator of Tyr phosphorylation. However, as the saturated acyl chain length increased, the ability of PA to stimulate Tyr phosphorylation decreased. The presence of unsaturation in dioleoyl (di18:1) PA restored the ability of the lipid to stimulate Tyr phosphorylation. Whether the inability of the intermediate and long saturated acyl chain PAs to activate the Tyr phosphorylation response was due solely to chain length or to different physical properties of the vesicles formed upon preparation of these lipids is not clear. However, preparation of these PAs in phosphatidylcholine vesicles or in Triton micelles did not improve their ability to stimulate the PA response (not shown). Thus it appears as though PAs with at least one long, unsaturated acyl chain (dioleoyl) and egg lecithin-derived PA preferentially stimulate the Tyr phosphorylation of the exogenous substrate and endogenous proteins. The short chain (8:0 and 10:0) PAs may be of suitable chain length to mimic the proximal portion of the acyl chains in dioleoyl (di18:1) PA, which has a site of unsaturation at carbon 9 along the acyl chain. Together, these data suggest the acyl chain composition of PA is an important determinant for interaction with the lipid-sensitive target.
PA Regulates Protein Tyrosine Kinase Activity

PA-stimulated Tyr Phosphorylation Is Sensitive to Inhibitors of Protein Kinases—We next addressed the mode of action of PA. Dialysis of neutrophil cytosol failed to alter PA-stimulated Tyr phosphorylation compared with undialyzed cytosol (not shown). This suggests that small cytosolic molecules (<10,000 Da) are not involved in the lipid-stimulated response. Alternatively, the Tyr phosphorylation observed in the presence of PA may only be an apparent enhancement if ATP was consumed more rapidly in the absence of this lipid, due to a protective effect of PA on ATP. This was not the case. At the end of the cell-free incubation, the ATP concentration had decreased more in the presence of PA compared with its absence. Thus, at an initial concentration of 30 μM ATP (used in the cell-free kinase assay), there was a 30% decrease in the ATP concentration in the presence of PA compared with a 25% decrease in its absence (not shown). Similarly, there was a 50% decrease in the ATP concentration in the presence of PA compared with 30% in its absence when the initial concentration was 8 μM ATP (not shown; used in the modified cell-free and immune complex kinase assays below). This indicates that PA promotes ATP consumption.

The simplest explanation for PA-stimulated Tyr phosphorylation is either the activation of a protein kinase or inhibition of a protein phosphatase. The cell-free Tyr kinase assay was routinely performed in the presence of Na3VO4, a protein Tyr phosphatase inhibitor, and ATP was required to observe PA-stimulated Tyr phosphorylation (not shown). These observations argue against the involvement of protein Tyr phosphatase in the response. Since PA has been reported to activate protein Ser/Thr kinases (14–17), we screened a number of protein kinase inhibitors to determine what type(s) of enzymes was involved in the PA-stimulated response.

Neutrophil cytosol was pretreated for 5 min with inhibitors prior to the addition of lipid, ATP, and exogenous substrate. Table II indicates that inhibitors of both protein Tyr and protein Ser/Thr kinases impaired the PA-stimulated Tyr phosphorylation of the exogenous substrate. The results mirrored those observed for the Tyr phosphorylation of the endogenous substrates (not shown). Compounds reported to be selective for protein Tyr kinases caused varied degrees of inhibition of the Tyr phosphorylation response, with genistein (47), PP1 (48), and piceatannol (49) being the most effective (~30, 50, and 70% of control activity, respectively). Herbimycin A and erastatin analog, both commonly used protein kinase inhibitors (50, 51), had only modest inhibitory effects on the PA-dependent Tyr phosphorylation. Staurosporine, a potent nonselective protein kinase inhibitor (52, 53), was the most effective inhibitor of the PA-stimulated response.

Among the inhibitors of protein Ser/Thr kinases, the nonselective C-I (28) caused a modest decrease in the Tyr phosphorylation response. In contrast, GF109203X, a selective protein kinase C inhibitor (54), had only a slight inhibitory effect. Surprisingly, inhibitors of Ca2+/calmodulin kinase II (KN-62 (55) and p38 SB203580 (56)) reduced the PA-dependent Tyr phosphorylation of the exogenous substrate to 20 and 30%, respectively, of control activity. However, the calmodulin antagonist chlorpromazine (100 μM) had no effect on PA-stimulated Tyr phosphorylation (not shown), and the assay routinely contained EGTA. These later observations argue against a role for Ca2+/calmodulin kinase II in the PA-stimulated response. Although present in neutrophils, the p38 protein Ser/Thr kinase, a MAPK cascade component, was not activated in our assay conditions, as judged by the inability to detect the dual phosphorylated p38 by immunoblotting (not shown). Thus, the inhibitors directed against Ca2+/calmodulin kinase II and p38 appear to lack the selectivity originally attributed to them, as

Cell membranes are composed of several phospholipids, and the activation of neutrophils results in the generation of a variety of lipid second messengers. Thus, it was important to determine whether other lipids could mimic the ability of PA to stimulate Tyr phosphorylation. Phospholipids were prepared in a uniform manner and tested at a final concentration of 100 μM for their ability to stimulate the Tyr phosphorylation of the exogenous substrate (Table I). PA, either naturally occurring or synthetic, was clearly the most effective activator of exogenous substrate Tyr phosphorylation. The results mirrored those reported previously (di12:0), or long (di16:0, di18:0, di18:1) acyl chain length. To facilitate a comparison among lipids, data are presented as the percent-


**TABLE I**

Inability of phospholipids and PA metabolites to mimic PA stimulation of Tyr phosphorylation

Neutrophil cytosol (0.1 mg/ml) was incubated with the indicated lipid (100 μM) or H2O (none) and the exogenous substrate for 45 min at 25 °C. The reactions were stopped and processed as described under “Experimental Procedures.” Blots were analyzed by densitometry, and the data are presented as percent activity of dicapryl PA for the Tyr phosphorylation of the exogenous substrate. The data are the mean ± S.E. determined using three cytosol preparations derived from different donors. In the case of diacylglycerol, phosphatidylinositol 3,4,5-phosphate, and the free fatty acids (arachidonic and oleic), the data are the average of two determinations using different cytosol preparations. The predominant acyl chain compositions for naturally occurring lipids for the indicated sources are presented according to the suppliers.

| Lipid                  | Source         | Acyl chain composition          | Dicapryl PA activity % |
|------------------------|----------------|-------------------------------|------------------------|
| None                   |                |                               | 0                      |
| Phosphatidic acid      | Synthetic      | 1,2-Dicapryl (di10:0)         | 100                    |
|                        | Synthetic      | 1,2-Dioleoyl (di18:1)         | 134 ± 24               |
|                        | Egg            | Palmitoyl (16:0), oleoyl      | 67 ± 10                |
| PA precursors and metabolites |            |                               |                        |
| Phosphatidylcholine    | Synthetic      | 1,2-Dioleoyl                  | 4 ± 4                  |
|                        | Egg            | Palmitoyl, oleoyl             | 0                      |
|                        | Porcine liver  | Oleoyl, arachidonyl (20:4)    | 0                      |
| Lyso-phosphatidic acid | Synthetic      | 1-Oleoyl                      | 22 ± 5                 |
| Diacylglycerol         | Synthetic      | 1-Oleoyl, 2-acetyl            | 0                      |
| Glycerol phosphate     | Synthetic      | None                          | 3 ± 3                  |
| Membrane phospholipids |                |                               |                        |
| Phosphatidylethanolamine | Porcine liver   | Stearoyl (18:0), arachidonyl  | 0                      |
|                        | Egg            | Palmitoyl, oleoyl             | 11 ± 8                 |
|                        | Porcine brain  | Stearoyl, oleoyl              | 8 ± 6                  |
|                        | Bovine brain   | Stearoyl, oleoyl              | 6 ± 4                  |
| Signaling lipids       |                |                               |                        |
| Sphingosine 1-phosphate | Synthetic      | Oleoyl                        | 0                      |
| Phosphatidylinositol-4-Phosphate | Bovine brain | Stearoyl, arachidonyl         | 6 ± 3                  |
| 4,5-Phosphate          | Bovine brain   | Stearoyl, arachidonyl         | 3 ± 1                  |
| 3,4,5-Phosphate        | Synthetic      | Dipalmitoyl                   | 0                      |
| Arachidonic acid       |                |                               | 0                      |
| Oleic acid             |                |                               |                        |

**TABLE II**

Effect of protein kinase inhibitors on PA-dependent Tyr phosphorylation of the exogenous substrate by human neutrophil cytosol

Neutrophil cytosol (0.1 mg/ml) was pretreated for 5 min at 25 °C with the indicated concentration of the inhibitor or Me2SO, after which ATP, dicapryl (di10:0), PA and exogenous substrate were added. Incubations were terminated after 45 min at 25 °C, and samples were prepared for SDS-PAGE and immunoblotting as described under “Experimental Procedures.” PA-dependent activity was calculated as the difference in activities between the PA and PC incubation conditions. Results are presented as the percent of PA-dependent activity observed in the absence of inhibitor (Me2SO control). The data are the mean ± S.E. for the number of determinations indicated in parentheses. Cytosol preparations derived from six different donors were used for these experiments.

| Inhibitor          | PA-dependent activity | Inhibitor selectivitya |
|--------------------|-----------------------|------------------------|
|                    | General | Specific      |
| Genistein, 100 μM (4) | 30 ± 4 | PTK† None |
| Herbinycin A, 100 μM (4) | 73 ± 13 | PTK SrC |
| Erbstatin analog, 100 nM (4) | 78 ± 8 | PTK EGFR† |
| PPL1, 100 nM (5) | 49 ± 10 | PTK Lyn, Fgr, Hck |
| Piceatannol, 10 μM (4) | 68 ± 13 | PTK Syk |
| Staurosporine, 100 nM (4) | 2 ± 2 | PSK/PTK None |
| C-I, 300 μM (3) | 59 ± 19 | PSK ³ |
| GF109203X, 300 nM (4) | 89 ± 3 | PSK PKC⁵ |
| KN-62, 100 μM (4) | 19 ± 5 | PSK CAMKII⁶ |
| SB203580, 20 μM (8) | 27 ± 2 | PSK p38 |
| PD98059, 50 μM (8) | 77 ± 4 | DSK⁷ MEK |

a See text for references.

b PTK, protein Tyr kinase; PSK, protein Ser/Thr kinase; DSK, dual specific kinase; EGFR, epidermal growth factor receptor; PKC, protein kinase C; CAMKII, Ca²⁺/calmodulin kinase II; MEK, MAPK/extracellular signal-regulated kinase.

dNoted by others for p38 (57). Finally, the inhibitor of the dual specific protein kinase MAPK/extracellular signal-regulated kinase kinase (PD98059 (58)) showed only a slight inhibitory effect on PA-stimulated Tyr phosphorylation of the exogenous substrate, suggesting that this protein kinase is probably not a major upstream target of PA.

These data suggest that the PA-stimulated response is complex and may involve a protein Ser/Thr kinase upstream of a protein Tyr kinase. Alternatively, a lipid-activated protein Tyr kinase with unusual sensitivities to protein kinase inhibitors provides the simplest explanation to account for the observed results. Although the inhibitor screen provided useful information, it did not clearly indicate the nature of the PA target responsible for enhanced Tyr phosphorylation.

Identification of a PA-regulated Protein Tyr Kinase—We have previously shown that neutrophil cytosol contains a PA-activated protein kinase with an approximate molecular mass of 125 kDa, determined by gel filtration chromatography (16). The partially purified enzyme preparation phosphorylated GST-p47 (the exogenous substrate) on both Ser and Tyr residues, suggesting the presence of a dual specific kinase or multiple protein kinases sensitive to PA. To distinguish between these possibilities, we subjected leukocyte (neutrophil + monocyte + lymphocyte) cytosol to gel filtration chromatography and assayed the eluted fractions for both Tyr and Ser phosphorylating activity. PA-stimulated phosphorylating activity was comparable in cytosol from neutrophils, lymphocytes, and monocytes (16).² We therefore used leukocyte cytosol to obtain sufficient starting material for chromatographic studies. Fig. 5 shows that the PA-dependent Ser/Thr phosphorylation (open circle) of the exogenous substrate eluted in fractions corresponding to a molecular mass of 108 ± 12 kDa (mean ± S.E., n = 3). This agrees with our earlier molecular mass estimation for the PA-activated protein kinase (16). In contrast, the activity responsible for PA-dependent Tyr phosphorylation (closed circle) of the same substrate eluted with the void volume of the gel filtration column. These data clearly indicate that the PA-stimulated Tyr- and Ser/Thr-directed phosphorylation activi-
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Leukocyte cytosol contains at least two PA-stimulated phosphorylating activities. Leukocyte cytosol (~130 mg) was adjusted to 40% saturated ammonium sulfate (v/v). The precipitated proteins were redissolved and chromatographed on a Sephacryl S-200 gel filtration column. Aliquots (40 µl) of eluted fractions were incubated in the presence or absence of 100 µM dicapryl (di10:0) PA, the exogenous substrate, and either unlabeled ATP (Tyr phosphorylation) plus Na3VO4 or radiolabeled ATP (Ser/Thr phosphorylation) minus Na3VO4, according to the modified phosphorylation assay described under “Experimental Procedures.” Immunoblots (Tyr phosphorylating activity) and autoradiographs (Ser/Thr phosphorylating activity) were analyzed by densitometry and PhosphorImager analysis, respectively. PA-dependent protein kinase activity was calculated as the difference in activities between the PA and PC (~PA) incubation conditions and normalized to densitometric data for the exogenous substrate protein. The data show the PA-dependent Tyr (●) and Ser/Thr (○) phosphorylating activities. The dashed line represents the protein elution profile (A280). The arrows at the top of the graph indicate the elution of molecular mass standards. The Fgr elution profile was determined by preparing equal volumes of column fractions for SDS-PAGE followed by immunoblotting for Fgr (bottom panel). The horizontal bar marks fractions 22–24 in which Fgr eluted. The data shown are representative of three experiments using cytosol from different donors.

We next considered the possibility that PA could directly activate a protein Tyr kinase. The elution fractions of the gel filtration column were screened by immunoblotting for the presence of protein Tyr kinases expressed in leukocytes. Of the protein Tyr kinases examined (cAbl, Lyn, Hck, Fgr, Fes, Fer, and Syk), only Fgr was found to elute exclusively in the peak of PA-stimulated Tyr phosphorylating activity (Fig. 5, bottom panel). Although some of the other protein Tyr kinases examined (cAbl, Lyn, Fes, and Fer) did elute in the peak of PA-dependent Tyr phosphorylating activity, they were also present in fractions that exhibited low activity (~fraction 28; not shown). Hck and Syk eluted in fractions closely corresponding to their estimated molecular masses (fractions 30–35; not shown).

These findings suggested that PA might activate Fgr. To test this hypothesis, we took two approaches. First, we assessed the sensitivity of Tyr phosphorylating activity in gel filtration column fractions to PP1, the selective Src family inhibitor. We found that PP1 (100 nM) decreased PA-dependent Tyr phosphorylation in Fgr-containing fractions (fractions 22–24) by 28% (not shown). Next, we isolated Fgr from lysates of unstimulated neutrophils by immunoprecipitation, and we assessed its kinase activity in the presence of various lipids using an immune complex kinase assay. Fig. 6A shows that dioleoyl PA stimulated a 2-fold increase in Fgr autophosphorylation compared with the phosphatidylcholine (PC) control condition. This PA-induced increase in Fgr activity was highly significant (p < 0.007). The use of an irrelevant antibody during immunoprecipitation failed to isolate Fgr protein or a phosphorylated band comigrating with Fgr (not shown). As an additional control, we added PP1 (100 nM) to immunoprecipitated Fgr, and we observed a blockade of phosphorylating activity (not shown). In contrast to PA, the presence of phosphatidylbutanol failed to stimulate Fgr autophosphorylation. The autoradiographs below the graph show a representative experiment of Fgr autophosphorylation (top panel) and the corresponding Fgr protein (bottom) detected on the same blot. Thus, the differences in Fgr autophosphorylation were not due to differences in amounts of kinase protein. Taken together, these data provide evidence that PA can regulate the activity of a nonreceptor protein Tyr kinase.

To determine whether a lipid regulatory mechanism of Fgr
was operative in intact cells, we stimulated neutrophils in the presence or absence of butanol (0.3%), and the activity of immunoprecipitated Fgr was assessed. Although many neutrophil agonists elevate intracellular PA, we selected fMLP plus dihydrocystealin B as a stimulus since it is a potent activator of phospholipase D (12). Moreover, fMLP/dihydrocystealin B-stimulated functional responses have been shown to be sensitive to primary alcohols (12). When neutrophils were pre-treated with dihydrocystealin B and then stimulated with fMLP, Fgr autophosphorylating activity increased nearly 4-fold over that in unstimulated cells (Fig. 6B). This observation was confirmed by immunoprecipitating Fgr from lysates of unstimulated and stimulated neutrophils with an antibody directed against a different Fgr epitope (not shown). Pretreatment of cells with 1-butanol, a primary alcohol (0.3%), significantly (p < 0.007) inhibited fMLP/dihydrocystealin B-stimulated Fgr activity by 50%. In contrast, treatment of cells with the corresponding secondary alcohol (2-butanol), which can not be utilized by phospholipase D, had no inhibitory effect on stimulus-induced Fgr activity. These data clearly suggest that Fgr activity in the intact cell can be regulated in a phospholipase D-dependent manner.

**DISCUSSION**

Our understanding of the cellular roles of protein Tyr kinases is rapidly expanding because of molecular techniques that allow for the manipulation of protein expression. In contrast, our understanding of the regulatory events leading to the activation and inactivation of these signaling molecules in the intact cell lags behind. Our findings show that Tyr phosphorylating activity in human neutrophils can be regulated in a positive manner by a cell membrane-derived lipid. This represents a novel facet of protein Tyr kinase regulation. Moreover, it links protein Tyr kinase activation to stimulus-induced membrane lipid metabolism, both of which are early signaling events in most cell types.

The pattern of Tyr-phosphorylated proteins observed with PMA stimulation of neutrophils was similar to that described previously (11, 38, 39). Moreover, certain proteins appear to be Tyr-phosphorylated in a manner sensitive to primary alcohols (Fig. 1). Our observations in primary neutrophils are in agreement with those made in HL-60 cells, a line with the capability to differentiate into neutrophil-like cells (11). Together, these findings implicate phospholipase D and its product, PA, in the regulation of the Tyr phosphorylation response. In addition, exogenous PA has been shown to stimulate Tyr phosphorylation of neutrophil proteins by a mechanism that does not require its entry into cells (10). Thus, neutrophil function appears to be enhanced by PA, acting as either an intracellular messenger or an inflammatory mediator (59).

Our focus was on PA as an intracellular messenger. By using a cell-free system, we showed that PA stimulated Tyr phosphorylation (Fig. 2). However, PA metabolites, membrane phospholipids, and other signaling lipids failed to mimic this ability of PA (Table I). Importantly, phosphatidylalcohols, which are generated at the expense of PA upon incubation of cells with primary alcohols, also failed to mimic the ability of PA to stimulate Tyr phosphorylation (Fig. 3). Thus, the target(s) responsible for enhanced Tyr phosphorylation is (are) clearly able to discriminate among lipids (Table I and Figs. 3 and 4). This strengthens the link between the early signaling events of phospholipase D activation and Tyr phosphorylation.

The activation of a protein Tyr kinase or the inhibition of a protein Tyr phosphatase provides the most straightforward explanation for PA-stimulated Tyr phosphorylation. The latter has not been reported, but PA activation of a protein Tyr phosphatase (SHP-1) has been observed (19). Since protein Tyr kinases can be activated by Tyr dephosphorylation (60), this could explain our observations. However, reported properties of PA-stimulated SHP-1 activity differ significantly from those observed for PA-stimulated Tyr phosphorylation. SHP-1 activity was stimulated by PA as well as by phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, and arachidonic acid and was inhibited by Mn$^{2+}$ (19). In contrast, these phospholipids were poor activators of Tyr phosphorylation (Table I); arachidonic acid was inactive (Table I), and Mn$^{2+}$ easily replaced Mg$^{2+}$ in our assay conditions. Thus, it is unlikely that SHP-1 activation by PA is responsible for the observed lipid-stimulated increase in Tyr phosphorylation. In addition, PA-stimulated Tyr phosphorylation required the presence of ATP (not shown); a protein Tyr phosphatase inhibitor (Na$_3$VO$_4$) was routinely included in the Tyr phosphorylation assay mixture; and protein Tyr kinase inhibitors diminished PA-stimulated Tyr phosphorylation (Table II). These observations argue against the involvement of a protein Tyr phosphatase in the PA response. Neutrophils express numerous protein Tyr kinases (2–4). There is limited information on the interaction of nonreceptor protein Tyr kinases with lipids. However, some of these enzymes localize to membranes viz a myristoylated amino terminus (60), and Lyn and Fyn, both Src family protein Tyr kinases, have been reported to interact with glycolipids (61–63). The activity of purified Src was previously reported to be enhanced by acidic phospholipids (64). Indeed, our observation that PA-stimulated Tyr phosphorylation activity is sensitive to inhibitors of protein Tyr kinases (Table II) suggests that a protein Tyr kinase is involved in the response. Among the more selective inhibitors tested, PP1, a Src family protein Tyr kinase inhibitor (48), blocked the PA-stimulated response by nearly 50%. This suggests that a member(s) of this protein Tyr kinase family may participate in the PA-stimulated response. We observed that Fgr, a Src family enzyme, eluted exclusively in the peak of PA-dependent Tyr phosphorylation activity obtained from the gel filtration fractionation of leukocyte cytosol (Fig. 5). Moreover, PA-dependent Tyr phosphorylation activity in Fgr-containing fractions was decreased by PP1. Since no other protein Tyr kinase examined exhibited a comparable elution profile, we pursued Fgr as a candidate enzyme for regulation by PA. Fgr is primarily a membrane-associated protein (65, 66), yet we detected this protein Tyr kinase in the cytosolic fraction of cells disrupted by sonication. However, to maximize Fgr recovery from cells, we opted to isolate the protein by immunoprecipitation from whole cell lysates. We found that PA, but not phosphatidylbutanol, could activate Fgr (Fig. 6A). Furthermore, Fgr activation in the intact cell appears to be regulated in part by PA (Fig. 6B). A variety of stimuli (e.g., opsonized zymosan, fMLP, PMA, the calcium ionophore A23187) are known to both elevate PA levels (12, 40, 67) and activate Fgr (66, 68, 69) in suspended neutrophils. These observations suggest that stimulus-dependent Tyr phosphorylation of proteins is mediated, in part, by PA and Fgr.

The unique elution pattern of Fgr led us to discover its sensitivity to PA. However, we cannot rule out the possibility that another protein Tyr kinase(s) is regulated by PA. Several observations suggest that Fgr is not alone in its regulation by PA. These include the following: 1) PA-dependent Tyr phosphorylation activity was present in elution fractions in which Fgr was not present; 2) the selective Src family protein kinase inhibitor did not completely abolish PA-stimulated Tyr phosphorylation activity; and 3) other protein Tyr kinases coeluted with Fgr in the peak of PA-dependent Tyr phosphorylation activity.

The mechanism by which PA might regulate the activity of
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Fgr is not yet clear. Fgr is a 55–58-kDa protein, yet it eluted from the gel filtration column in the void volume (Fig. 5). This suggests that Fgr probably associates with a protein complex. Both cellular and viral Fgr have been reported to associate with cellular proteins (70–73). This raises the possibility of an indirect action of PA on the activation of Fgr, i.e., a known PA effector may be activated by PA, which in turn activates Fgr. The sensitivity of the PA-stimulated Tyr response to inhibitors of protein Ser/Thr kinases (Table II) suggests this possibility as well. Based on the data presented, we can rule out some of the known PA effectors that could potentially regulate Tyr phosphorylating activity. It is unlikely that protein kinase C is an upstream effector of a protein Tyr kinase in the cell-free system. The selective inhibitor of this protein Ser/Thr kinase had little effect on PA-stimulated Tyr phosphorylation (Table II), and the activators of protein kinase C (calcium, diacylglycerol ± PA, phosphatidylserine) were inhibitory or had no effect on PA-stimulated Tyr phosphorylation in the cell-free system. However, in the intact neutrophil, protein kinase C may play a role in the alcohol-sensitive Tyr phosphorylation due to its participation in the regulation of phospholipase D (74, 75).

Among the other PA effectors, our data clearly indicate that the novel PA-activated protein Ser/Thr kinase present in neutrophil cytosol (16) is not an upstream regulator of a protein Tyr kinase. Activity associated with this protein kinase eluted from the gel filtration column in fractions distinct from those exhibiting PA-stimulated Tyr phosphorylation activity (Fig. 5). Finally, PKN, which is activated by free fatty acids, can also be activated by PA (76). In our cell-free assay, neither arachidonic acid nor oleic acid, both potent PKN activators, had stimulated Fgr activity is more variable than that shown (Fig. 6).

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Addendum—We have now observed that the magnitude of PA-stimulated Fgr activity is more variable than that shown (Fig. 6A). The variability appears to result from as yet undefined differences in lots of tissue-derived phosphatidylcholine. After testing three lots of phosphatidylcholine, we find that PA-stimulated activity in the presence of PA is 1.45 ± 0.16 (mean ± S.E., n = 8, p = 0.015)-fold higher than that in the presence of phosphatidylcholine.

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