Phosphatidic Acid-mediated Phosphorylation of the NADPH Oxidase Component p47-phox

EVIDENCE THAT PHOSPHATIDIC ACID MAY ACTIVATE A NOVEL PROTEIN KINASE*

(Received for publication, September 6, 1996, and in revised form, March 21, 1997)

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Phosphatidic acid (PA), generated by phospholipase D activation, has been linked to the activation of the neutrophil respiratory burst enzyme, NADPH oxidase; however, the intracellular enzyme targets for PA remain unclear. We have recently shown (McPhail, L. C., Qualliotine-Mann, D., and Waite, K. A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7931–7935) that a PA-activated protein kinase is involved in the activation of NADPH oxidase in a cell-free system. This protein kinase phosphorylates numerous endogenous proteins, including p47-phox, a component of the NADPH oxidase complex. Phospholipids other than PA were less effective at inducing endogenous protein phosphorylation. Several of these endogenous substrates were also phosphorylated by this kinase during stimulation of intact cells by opsonized zymosan, an agonist that induces phospholipase D activation. We sought to identify the PA-activated protein kinase that phosphorylates p47-phox. The PA-dependent protein kinase was shown to be cytosolic. cis-Unsaturated fatty acids were poor inducers of protein kinase activity, suggesting that the PA-activated protein kinase is not a fatty acid-regulated protein kinase (e.g. protein kinase N). Chromatographic techniques separated the PA-activated protein kinase from a number of other protein kinases known to be activated by PA or to phosphorylate p47-phox. These included isoforms of protein kinase C, p21 (Cdc42/Rac)-activated protein kinase, and mitogen-activated protein kinase. Gel filtration chromatography indicated that the protein kinase has an apparent molecular size of 125 kDa. Screening of cytosolic fractions from several cell types and rat brain suggested the enzyme has widespread cell and tissue distribution. The partially purified protein kinase was sensitive to the same protein kinase inhibitors that diminished NADPH oxidase activation and was independent of guanosine 5′-3-O-(thio)triphosphate and Ca2+. Phosphoamino acid analysis showed that serine and tyrosine residues were phosphorylated on p47-phox by this kinase(s). These data indicate that one or more potentially novel protein kinases are targets for PA in neutrophils and other cell types. Furthermore, a PA-activated protein kinase is likely to be an important regulator of the neutrophil respiratory burst by phosphorylation of the NADPH oxidase component p47-phox.

Phospholipase D (PLD) is activated in a variety of cells by hormones and growth factors (1–3). This activation results in the generation of phosphatidic acid (PA), which can be further metabolized by PA phosphohydrolase to diacylglycerol (DG). The generation of PA by PLD in neutrophils has been linked, by us and others, to the activation of the respiratory burst enzyme, NADPH oxidase (2–7). The enzymes involved in the activation of the oxidase, which are downstream of the generation of PA, have not been identified. We have developed a cell-free activation system for NADPH oxidase, which utilizes PA and is phosphorylation-dependent (7, 8). The system requires the presence of both cellular membranes and cytosol, as well as the presence of DG. Since a variety of potential enzyme targets for PA are present, this system provides a means to identify these enzymes and their role in activation of the NADPH oxidase. We have recently shown that, during phosphorylation-dependent oxidase activation, PA, but not DG, induces phosphorylation of a wide range of proteins, in which the most prominent protein phosphorylated is the NADPH oxidase component p47-phox (phagocyte oxidase component) (8). This indicates that potential targets for PA, involved in NADPH oxidase activation, are protein kinases.

Several protein kinases have been demonstrated to be activated by PA in vitro. These include isoforms of protein kinase C (PKC) (9–13) as well as the recently described fatty acid-activated protein kinases (14–20). In addition, Raf-1 (a mitogen-activated kinase kinase kinase) has been shown to bind anionic phospholipids, including PA, and to translocate from cytosol to membrane in Madin-Darby canine kidney cells in response to PLD activation (21). In vitro studies have shown that PKC, mitogen-activated protein kinase (MAPK) and isoforms of the newly identified p21 (Cdc42/Rac)-activated protein kinase (PAK) are able to phosphorylate the NADPH oxidase component p47-phox (22–25). Neutrophils are known to contain p42 and p44 MAPK isoforms (26, 27) and PAK-1 and PAK-2 (24, 28, 29). Potentially, p42 and p44 MAPK isoforms could be activated as a consequence of Raf-1 activation by PA (21). PA is known to be generated in neutrophils under conditions in which these protein kinases are activated (5, 6, 30). PAK activation could be a consequence of PA-mediated activation of the small GTPase Rac (24, 28, 29, 31), through the inhibition of

* This work was supported by National Institutes of Health (NIH) Grant 2RO1AI-22564. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported through a training grant in Signal Transduction Mechanisms and Cell Function (NIH Grant T32CA8422).

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1 The abbreviations used are: PLD, phospholipase D; AA, arachidonic acid; CL, cardiolipin; DG, diacylglycerol; GTP, guanosine 5′-triphosphate; MAPK, mitogen-activated protein kinase; p47-phox, 47-kDa phagocyte oxidase component; PA, phosphatidic acid; PAK, p21 (Cdc42/Rac)-activated protein kinase; PC, phosphatidylycerol; PE, phosphatidylethanolamine; PKC, protein kinase C; PKN, novel protein kinase; PRR, protein kinase C-related protein kinase; PS, phosphatidylserine; PAGE, polyacrylamide gel electrophoresis; GTP-S, guanosine 5′-3-O-(thio)triphosphate; Pipes, 1,4-piperazinediethanesulfonic acid.
Rho-GDP dissociation inhibitor (32, 33). In fact, the production of PA from PLD activation has been linked to the activation of PAK isoforms in neutrophils (34, 35). Thus, while MAPK and PAK isoforms are not known to be activated directly by PA, they could be activated downstream of other protein targets of PA and ultimately be responsible for the phosphorylation of p47- phox.

We examined whether the PA-dependent protein kinase activity in human neutrophils was due to the activation of one of these known protein kinases. Our results indicate that this is not the case and that a potentially novel protein kinase, which phosphorylates p47-phox, may be the target for PA in this system. This protein kinase may therefore play a role in the activation of the NADPH oxidase.

EXPERIMENTAL PROCEDURES

Materials

Sf9-expressed recombinant p47-phox (36) and Escherichia coli containing the plasmid encoding glutathione S-transferase (GST)-p47-phox fusion protein were generous gifts from Dr. Tom Leto (National Institutes of Health). The GST-p47-phox fusion protein was prepared as described previously (37). The PA used was 1,2-diacyl-sn-glycero-3-phosphate and was obtained from Avanti Polar Lipids (Alabaster, AL). All other phospholipids were from Serdary Research Laboratories (Port Huron, MI). Lipids were prepared for use by sonication (7). Arachidonic acid (AA) and oleic acid were from Nu-Chek Prep Inc. (Elysian, MN) and were dissolved in ethanol (7). [γ-32P]ATP (1 mCi/ml) and [32P]PiPO4 (10 mCi/ml) were from Du Pont NEN. Phosphoserine, phosphothreonine, and phosphotyrosine standards were from Sigma. Phosphoellulose paper (P-81) was from Whatman (Hillsboro, OR), and ECL reagents for Western blot analysis were obtained from Amersham (Arlington Heights, IL). Rabbit IgG was from Sigma. Enhanced chemiluminescence albumin was used as a standard.

Phosphorylation Assays

Intact Cell Assay—Isolated neutrophils were suspended to 1 × 10^8 cells/ml in loading buffer (10 mM Hepes (pH 7.2), 137 mM NaCl, 0.8 mM MgCl2, and 5.4 mM KCl). [32P]PiPO4 (1 mCi/ml) was added, and the mixture was incubated at room temperature for 90 min (45). Labeled neutrophils were centrifuged and resuspended at 1.5 × 10^8 cells/ml in stimulation buffer (10 mM Hepes (pH 7.2), 137 mM NaCl, 1.8 mM MgCl2, 5.4 mM KCl, and 0.5 mM CaCl2) (45) and stimulated with 10 mg/ml opsonized zymosan for 5 min (46). Stimulation reactions were terminated by the addition of a 10-fold excess of stimulation buffer and immediate centrifugation. Stimulated neutrophils were then resuspended in a diisopropylfluorophosphate treatment buffer (stimulation buffer plus 0.5 mM phenylmethylsulfonyl fluoride, pH 7.2, 50 μM leupeptin, 5.4 mM Na3VO4, and 25 mM NaF) and incubated with 1 mM diisopropylfluorophosphate for 5 min (47) on ice, at which time a 10-fold excess of stimulation buffer was added and the cells were centrifuged. Cells were then resuspended in a modified sonication buffer (10 mM Pipes, 1 mM EGTA, 103 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, pH 7.2, 50 μM leupeptin, 5.4 mM Na3VO4, 25 mM NaF, and 11% sucrose) (45) and sonicated to ~90% breakage as described above. Unbroken cells and nuclei were removed by low speed centrifugation, and subcellular fractions were obtained as described above. Proteins were separated by 8–15% SDS-PAGE (40, 48), silver-stained (49), dried, and analyzed via autoradiography.

Cell-free Assay: Analysis by SDS-PAGE—Reaction mixtures (150 μl total volume) contained 50 mM NaH2PO4, 1 mM MgCl2 (7, 8) plus one of the following: a combination of the cytosolic and membrane fractions (25:1 protein:protein ratio), the cytosolic fraction only, the membrane fraction only, partially purified enzyme, or column fractions. GST-p47-phox (1 μg/reaction) or recombinant p47-phox (Rp47, 1 μg/reaction) were added to the mixtures as indicated in the figure legends. Six μl of [γ-32P]ATP were added, followed by the immediate addition ofactivator, as indicated in the figure legends. The reaction mixture was allowed to incubate at 25 °C for 45 min. The reaction was then terminated by the addition of Laemmli sample buffer and prepared for SDS-PAGE analysis (48). Proteins were separated by 8–15% SDS-PAGE (40), silver-stained (49), dried, and analyzed via autoradiography. Protein kinase inhibitors were added 5 min prior to the addition of [γ-32P]ATP. For samples that were analyzed by Western blotting, reactions were performed as above with the following modifications. Cold ATP (10 μM) was used in place of radiolabeled ATP. Proteins were separated by 9% SDS-PAGE and then transferred to polyvinylidene difluoride (50).

Cell-free Assay: Analysis by Phosphocellulose Binding—Reaction mixtures were the same as above, except that [γ-32P]ATP, diluted to yield 2 × 10^6 cpm/reaction, was used (final ATP concentration is indicated in figure legends), and samples were allowed to preincubate for 5 min at 25 °C before the addition of sample material to be tested (51). After 30 min, 75% (112.5 μl) of the reaction mixture was transferred to Whatman P-81 phosphocellulose squares; the squares were washed with 75 mM phosphoric acid and quantitated by Cerenkov counting (52, 53). 1 μl GTPγS or 0.6 mM CaCl2 were added to samples as indicated in figure legends. For the labeling of neutrophils with [32P]PiPO4, 10 μM PA, PS, PE, and PC were prepared separately by sonication (7). PE, PC, and PS were then mixed together (final assay concentration: 25, 25, and 10 μM, respectively) with PA (final assay concentration: 0–300 μM). These mixtures were then used in the place of PA in the reaction mixtures. All samples were assayed in duplicate. Conditions used were linear with respect to protein concentration and time of incubation.

Isolation of Blood Cells and Subcellular Fractionation

Platelets were derived from heparinized venous blood by centrifugation (300 × g, 20 min, 25 °C) (39). The supernatant, containing platelet-rich plasma, was then centrifuged (2500 × g, 15 min, 4 °C) (39), and the pellet was resuspended in Buffer A (50 mM NaPO4, pH 7.0, 110 mM NaCl, 1 mM EGTA, and 0.5 mM phenylmethylsulfonyl fluoride) and processed as described below. Neutrophils, monocytes, and lymphocytes were isolated from heparinized venous blood obtained from consenting human donors by dextran sedimentation followed by hypotonic lysis (42, 43). Isolated neutrophils were suspended to a concentration of 1 × 10^8 cells/ml in Buffer A. All cells to be analyzed were sonicated, as described, to homogenate the membrane fraction, and were dissolved in ethanol (7). [32P]ATP, diluted to 10^-4 discontinuous sucrose gradient at a 2:1.1 (w/v) ratio and centrifuged at 150,000 × g (SW50 rotor, 30 min) (40). Cytosolic fractions were collected from the top layer down to the 15% interface, and membrane fractions were collected from the 15–40% interface and the 40% sucrose layer. To prepare cytosolic fractions only, sonicates were centrifuged at 150,000 × g (Type 50 rotor, 90 min), and the resulting supernatant was collected. Fractions were stored at −70 °C. Protein was determined using the Coomassie Plus Protein protocol from Pierce, which is based on the Bradford method (44). Bovine serum albumin was used as a standard.
Phosphatidic Acid May Activate a Novel Protein Kinase

Cytosol (2 mg of protein) was cleared by centrifugation at 10,000 × g and then adjusted with a solution of saturated ammonium sulfate at 4 °C to yield 40% ammonium sulfate saturation. This mixture was kept on ice for 10 min and was centrifuged (10,000 × g, 4 °C, 10 min) to collect precipitated protein. The resulting supernatant was adjusted with saturated ammonium sulfate to yield 70% saturation and was kept on ice for 10 min. The precipitate was collected by centrifugation. Both the 40 and 70% protein precipitates were resuspended in 500 μl of Buffer B (Buffer A plus 2 ml dithiothreitol). These samples, plus the resulting 70% supernatant, were cleared of residual ammonium sulfate by passage over a Sephadex G-25 column (90 × 15 mm) in Buffer B. The void volume fraction was collected for each sample and assayed for PA-dependent protein kinase activity using the phosphocellulose binding assay. The ammonium sulfate-free samples were also concentrated by centrifugation with Centricon-30 tubes (Amicon; Beverly, MA) and stored at −70 °C.

Hydrophobic Interaction Chromatography

The 40% ammonium sulfate protein precipitate obtained from 25 mg of cytosolic protein (containing 85% of recovered activity) was resuspended on ice in Buffer B containing 20% ammonium sulfate. After 10 min, the solution was cleared by centrifugation and applied to a Ranin Hydropore column (Woburn, MA), equilibrated with Buffer B containing 10 μg/ml leupeptin, 1 μg/ml pepstatin A, and 20% ammonium sulfate (Buffer C). The unretained proteins were collected, and the retained proteins were then eluted with Buffer C containing no ammonium sulfate. Fractions containing the retained and unretained proteins were concentrated via centrifugation using Centricon 30 tubes and were assayed for PA-dependent protein kinase activity, using the phosphocellulose binding assay, and for the presence of various protein kinases by Western blot analysis.

Gel Filtration Chromatography

Neutrophil cytosol (~120 mg of protein) was adjusted with a solution of saturated ammonium sulfate at 4 °C to yield 40% ammonium sulfate. This mixture was kept on ice for 10 min and was centrifuged (10,000 × g, 4 °C, 10 min) to pellet precipitated protein. The precipitate was resuspended in Buffer B containing 20% ammonium sulfate. After 10 min on ice, the solution was cleared by centrifugation and applied to a Pharmacia Sephacryl S-200 column equilibrated with Buffer C containing no ammonium sulfate. Fractions (4.2 ml) were collected and assayed for PA-dependent protein kinase activity by Western blot analysis. The unretained proteins were collected, and the retained proteins were then eluted with Buffer C containing no ammonium sulfate. Fractions containing the retained and unretained proteins were concentrated via centrifugation using Centricon 30 tubes and were assayed for PA-dependent protein kinase activity, using the phosphocellulose binding assay, and for the presence of various protein kinases by Western blot analysis.

Phosphoamino Acid Analysis

GST-p47-phox was phosphorylated in the presence of γ-[32P]ATP, separated by SDS-PAGE as described above, and transferred to polyvinylidene difluoride (50). The polyvinylidene difluoride membrane was then washed with H2O and subjected to autoradiography, and the phosphorylated GST-p47-phox band was excised from the membrane. The polyvinylidene difluoride membrane was then subjected to hydrolysis with 5.7 N HCl, at 110 °C for 1 h as described previously (54). Hydrolysis was stopped by the addition of 200 μl of H2O, and samples were centrifuged. The supernatant was then lyophilized, suspended in phosphoamino acid thin layer chromatography analysis buffer (62.5% isobutyric acid, 1.9% n-butyl alcohol, 4.8% pyridine, 2.9% glacial acetic acid) containing 1 mg/ml phosphoamino acid standards (55), and spoted on cellulose plates. The plates were then subjected to thin layer chromatography using the phosphoamino acid buffer (55). The plates were allowed to air dry, standards were visualized with ninhydrin (55), and radiolabeled phosphoamino acids were detected by autoradiography.

Immunodepletion of Raf-1 or B-Raf from Neutrophil Cytosol

Cytosol (250 μl) was diluted to 1 ml with 50 mM Tris (pH 7.0), 150 mM NaCl, 2 mM EDTA, and 1 mM EGTA, and incubated with 100 μl of Protein A-Sepharose beads for 10 min, followed by removal of the beads. 10 μg/ml of either anti-Raf-1 antibody, anti B-Raf antibody, or control IgG was added to 330 μl of diluted, precleared cytosol, and the mixture was incubated at 4 °C for 1 h. The beads were pelleted by centrifugation, and the supernatants were collected and assayed for PA-dependent protein kinase activity by the phosphocellulose binding assay. Immunoprecipitates and the resulting supernatants were analyzed by SDS-PAGE, followed by Western blotting with antibodies to Raf-1 or B-Raf.

Western Blot Analysis

Samples were prepared and separated by 9% SDS-PAGE (48) and transferred to nitrocellulose (50). The nitrocellulose blots were blocked by incubation for 1 h in 5% nonfat milk in TBS-T (10 mM Tris, 150 mM NaCl, and 0.1% Tween 20). Blots were then incubated for 2 h with the appropriate primary antibody at the following dilutions/concentrations: anti-FRα-α and anti-FRC-γ at 2 μg/ml; anti-PKC-p56 at 1:100; anti-PKC-α at 1:2000, anti-MAPK at 1:1000, anti-PAK-1 and anti-PAK-2 at 1:1000, anti-phosphotyrosine (4G10) at 1 ng/ml, anti-Raf-1 at 1:1000 and anti-B-Raf at 1:500. Blots were subjected to six 5-min washes. The blots were then incubated for 1 h with the appropriate horseradish peroxidase-conjugated secondary antibody at a 1:5000 dilution and washed again with TBS-T. Blots were visualized by enhanced chemiluminescence according to manufacturer’s recommendations.

Preparation of Opsonized Zymosan

Zymosan was suspended in 154 mM NaCl, boiled for 15 min, centrifuged (10 min, 300 × g, 4 °C), washed once, and resuspended in phosphate-buffered saline. Two volumes of pooled human serum, pooled from four or five healthy donors, and one volume of zymosan suspension were incubated for 30 min at 37 °C (56). The opsonized zymosan was then centrifuged, washed once in stimulation buffer, and resuspended in stimulation buffer for use.

RESULTS

Characterization of PA-induced Protein Phosphorylation—We have shown previously that the addition of PA to a mixture of cytosolic and membrane fractions from neutrophils results in the phosphorylation of a wide range of neutrophil proteins, including p47-phox (58). We investigated the ability of other phospholipids to induce protein phosphorylation in this system. Results are shown in Fig. 1. PC, PE, or cardiolipin (CL) did not induce protein phosphorylation above basal levels (H2O). Since CL was reported to activate a PA-sensitive protein kinase (19), we also examined a range of concentrations of this lipid (3 μM to 1 mM). No significant phosphorylation occurred at any of the CL concentrations tested (data not shown). PS and phosphatidylinositol each induced a moderate level of protein phosphorylation. While phosphatidylycerolipid induced protein phosphorylation to a higher degree than PS or phosphatidylinositol, the best activator of protein phosphorylation was PA. Indeed, the addition of PA resulted in several proteins being phosphorylated that were not phosphorylated in the presence of the other phospholipids (Fig. 1, arrows). This suggests that PA activates a protein kinase(s) not responsive to other phospholipids.

We next compared the phosphorylation pattern of cytosol stimulated with PA in vitro with cytosol isolated from opsonized zymosan-stimulated neutrophils. Opsonized zymosan stimulation has been shown to induce activation of PLD, and this activation has been linked to the activation of NADPH oxidase (46, 57). Increases in protein phosphorylation were harder to detect in the in vivo system due to high levels of labeled phosphate incorporated in nonstimulated cells. However, increased protein phosphorylation in stimulated samples could be observed upon longer autoradiographic exposures (Fig. 2). This indicates that a protein kinase is probably acti-
Phosphatidic Acid May Activate a Novel Protein Kinase

**FIG. 1.** Ability of various phospholipids to induce cell-free protein phosphorylation. Neutrophil cytosol (83 μg/ml) and membrane (3.3 μg/ml) were incubated in the presence of [γ-32P]ATP (7–10 μCi), H2O, or the indicated phospholipid (100 μM) for 45 min. Reactions were analyzed by SDS-PAGE (4 μg of protein/lane) and autoradiography as described under “Experimental Procedures.” Shown is a scan of an autoradiograph (8-h exposure), representative of three experiments. PG, phosphatidylglycerol; PI, phosphatidylinositol. The migration of molecular weight standards is indicated on the left. The arrows indicate proteins that are phosphorylated only in the presence of PA.

*Phosphatidic Acid May Activate a Novel Protein Kinase*—To determine the subcellular location of the protein kinase activated by PA, we used recombinant p47-phox (Rp47) as an exogenous substrate for the enzyme. Rp47 was added in excess to phosphorylation reaction mixtures that contained either cytosol and membranes, cytosol alone, or membranes alone. Phosphorylation of Rp47 was dependent upon the presence of PA (Fig. 3). Reaction mixtures that lacked cytosol did not result in PA-dependent phosphorylation of Rp47. In contrast, reaction mixtures that lacked the membrane fraction supported PA-dependent phosphorylation. Shown in Fig. 3 is a 5-min autoradiograph. In lanes with cytosol present, protein phosphorylation patterns similar to those seen in Fig. 1 (PA lane) were observed when longer exposures were viewed. Minimal protein phosphorylation was observed in longer exposures when reaction mixtures contained only membrane fractions (data not shown). The identical pattern was observed when the GST-p47-phox fusion protein was used as the exogenous substrate (data not shown). These data indicate that the PA-activated protein kinase is cytosolic and does not require membrane components for activation.

**Comparison of Protein Kinase Activation by PA and cis-Unsaturated Fatty Acids**—Rat liver (19, 20) and platelets (18) contain cytosolic PA-responsive protein kinases, which were identified as cardiopin-activated and cis-ununsaturated fatty acid-activated protein kinases, respectively. Recently, the hepatocyte protein kinase has been determined to be a novel protein kinase (PKN) (16), which is also the PKC-related protein kinase 1 (PRK1) (14, 17, 58). We tested the possibility that the protein kinase activity we observe in the neutrophil is the same or similar to these PA-responsive protein kinases. The results shown in Fig. 1 suggest the neutrophil enzyme is not a cardiopin-activated protein kinase. We tested whether the cis-unsaturated fatty acid AA could activate a protein kinase in neutrophil cytosol. Fig. 4 shows protein kinase activation as a function of PA and AA concentration, using the phosphocellulose assay. AA was a poor inducer of protein kinase activity with maximal activity of 40 ± 5 pmol of PO4/min/mg (25 μM AA, Fig. 4). In contrast, PA activated a protein kinase(s) in a dose-dependent manner with a 6-fold higher maximal activity (246 ± 44 pmol of PO4/min/mg, 30 μM PA, Fig. 4). Another cis-unsaturated fatty acid, oleic acid, also elicited little protein kinase activation (35 pmol of PO4/min/mg, 25 μM oleic acid, n = 2, data not shown). Thus, the PA-responsive protein kinase found in neutrophil cytosol is poorly activated by cis-unsaturated fatty acids or cardiopin and does not appear to be a fatty acid-activated protein kinase described by others (14, 16–20).

**Separation of the PA-activated Protein Kinase from PKC Isoforms**—PA has been shown, in vitro, to activate several isoforms of PKC (9–13), raising the possibility that the protein kinase target of PA in neutrophil cytosol is one or more of these enzymes. To test this possibility, we determined whether the PKC isoforms present in neutrophil cytosol (59–64) co-purified with the PA-activated protein kinase. We first subjected neutrophil cytosol to sequential ammonium sulfate precipitation, as described under “Experimental Procedures.” The fractions obtained were tested for the location of the PA-activated protein kinase, using the phosphocellulose protein kinase assay. The PA-activated protein kinase was recovered primarily in the 40% ammonium sulfate precipitate, which contained 85% of the total recovered PA-activated protein kinase activity (Fig. 5A). Much less activity was observed in the 70% precipitate and the resulting supernatant (13 and 2% of the total recovered activity, respectively). The same fractions were then evaluated for the presence of various PKC isoforms by Western blotting (Fig. 5B). The PKC isoforms α, βII, δ, and ζ were present in unfrac-

**FIG. 2.** Comparison of in vitro and in vivo phosphorylation. Neutrophil cytosol (83 μg/ml) (in vitro) was incubated in the presence of [γ-32P]ATP (7–10 μCi), in the presence (+) or absence (−) of 100 μM PA for 45 min. Reactions were analyzed by SDS-PAGE (4 μg of protein/lane) and autoradiography as described under “Experimental Procedures.” Intact neutrophils (in vivo) were labeled with [32P]H3PO4 and stimulated with opsonized zymosan (+) or stimulation buffer (−) for 5 min as described under “Experimental Procedures.” Cytosolic protein was obtained as described under “Experimental Procedures” and analyzed by SDS-PAGE (50 μg/lane) and autoradiography as described. Stimulus, 100 μM PA for in vitro lanes and 10 mg/ml opsonized zymosan for in vivo lanes. Migration of molecular mass standards is indicated on the left. The arrows indicate proteins that are phosphorylated in both the in vitro and in vivo systems. Shown are representative autoradiographs of seven experiments in vitro and four experiments in vivo.
tionated cytosol that had been passed over a Sephadex G-25 column as a control for the ammonium sulfate-treated fractions (G25). Following ammonium sulfate fractionation of the cytosol and Sephadex G-25 chromatography of the fractions, the calcium-dependent PKC isoforms, α and β₁₁, were recovered in the 70% ammonium sulfate precipitate. Similarly, the calcium-independent isoforms, δ and ζ, were recovered predominantly in the 70% ammonium sulfate precipitate, although small amounts of PKC-δ and ζ were present in the 40% ammonium sulfate precipitate.

Since PKC-δ and ζ remained in the 40% ammonium sulfate precipitate, we subjected the PA-activated protein kinase to an additional step of purification. The 40% ammonium sulfate pellet was resuspended in buffer containing 20% ammonium sulfate and subjected to hydrophobic interaction chromatography using a Rainin Hydropore column. The unretained proteins (Void) and the retained proteins (Bound) were collected as described under “Experimental Procedures.” Activity measurements (Fig. 6A) showed that the PA-activated protein kinase was predominantly present in the retained protein fraction (Bound, 78% of the recovered PA-activated protein kinase activity). The unretained protein and the retained protein fractions were then subjected to Western blot analysis for the presence of PKC-δ and ζ. Results (Fig. 6B) show that neither PKC-δ nor ζ were retained on the column; these PKC isoforms were observed solely in the unretained protein fraction (Void). The retained protein fraction (Bound) was also analyzed, by Western blotting, for the presence of PKC isoforms not known to be present in the neutrophil (γ, θ, ε, ι), to determine if one of these isoforms was present. No immunoreactive bands were observed (data not shown). These data indicate that the PA-activated protein kinase is not likely to be a PKC isoform.

Separation of the PA-activated Protein Kinase from PAK and MAPK Isoforms—Neutrophil cytosol contains MAPK and PAK isoforms, two classes of protein kinases recently shown to phosphorylate p47-phox, the in vitro substrate for the PA-activated protein kinase (24, 25, 29, 31). While neither of these protein kinases are known to be directly activated by PA, it was possible that these protein kinases are targets for PA in our system or are activated downstream of the PA-activated protein kinase. Neutrophil cytosol depleted of Raf-1 or B-Raf by nondenaturing immunoprecipitation still maintained PA-dependent protein kinase activity (data not shown), suggesting that neither Raf-1 nor B-Raf is the target for PA in this system. This also suggests that MAPK isoforms are not activated via PA-mediated activation of Raf-1. However, it was possible that MAPK isoforms may be activated directly by PA. Fractions obtained by ammonium sulfate precipitation and hydrophobic interaction chromatography, during the partial purification of the PA-activated protein kinase, were analyzed for the presence of MAPK or PAK by Western blotting. As presented in Figs. 5A and 6A, the PA-activated protein kinase was precipitated from a 40% saturated ammonium sulfate solution (40% cut = 85% of recovered activity) and was retained by the column resin during hydrophobic interaction chromatography (Bound = 75% of recovered activity). Fig. 7 shows that both PAK-2 and p42 and p44 MAPK isoforms are present in neutrophil cytosol. Upon 40% saturated ammonium sulfate precipitation, both kinase families distributed between the 40% cut and the resulting supernatant, although MAPK was predominantly in the 40% cut. In contrast to the PA-dependent protein kinase, both PAK and MAPK isoforms were found only in the unretained protein fraction eluting from the hydrophore column. Similar results to those obtained with PAK-2 antibodies were obtained with antibodies recognizing PAK-1 (data not shown). Thus, the PA-activated protein kinase co-purifies with neither PAK-1 nor PAK-2 nor p42 or p44 MAPK isoforms.

Molecular Weight Determination of the PA-activated Protein Kinase—The apparent molecular weight of the PA-dependent protein kinase was determined by gel filtration chromatography. Neutrophil cytosol was first subjected to ammonium sulfate precipitation and then gel filtration chromatography, as described under “Experimental Procedures.” The peak of PA-dependent protein kinase activity eluted at fraction 28 (Fig. 8A), which corresponds to a molecular mass of 125 kDa. We confirmed these results with more purified material obtained by subjecting neutrophil cytosol to both ammonium sulfate precipitation and hydrophobic interaction chromatography prior to gel filtration chromatography. Fractions were analyzed by the SDS-PAGE phosphorylation assay. Fig. 8B shows that, like the activity seen in Fig. 8A, the peak of GST-p47-phox phosphorylation occurred at fraction 28. These data indicate that the PA-responsive protein kinase has a maximum size of 125 kDa.

Characterization of the PA-activated Protein Kinase—Due to the low yield (4%) after gel filtration, we used the hydrophore-bound material to characterize the PA-activated protein kinase. We first analyzed the effect of calcium and GTPγS on the activation of the PA-activated protein kinase. We found that calcium slightly enhanced the PA-activated protein kinase (1571 pmol of PO₄/min/mg in the absence of calcium, 1780 pmol PO₄/min/mg in the presence of 0.6 mM calcium, n = 2). The addition of 1 μM GTPγS, sufficient to activate Rac in the

![Fig. 3. Subcellular localization of the PA-activated protein kinase.](image)

Reaction mixtures contained either the combination of neutrophil cytosol (Cyto., 83 μg/ml) and membrane (Membr., 3.3 μg/ml), cytosol alone, or membrane alone, as indicated. Recombinant p47-phox (Rp47, 6.7 μg/ml) was added to the indicated mixtures. Reactions were started by the addition of 100 μM PA (+) or H₂O (−) and continued for 30 min. Equal volumes (60 μl) of each sample were separated by SDS-PAGE, and phosphorylation was detected by autoradiography. This scan of an autoradiograph (5-min exposure) is representative of three separate experiments.

![Fig. 4. Comparison of PA and AA as cell-free protein kinase activators.](image)

Reaction mixtures contained 83 μg/ml neutrophil cytosol, 50 μM ATP, 6.7 μg/ml GST-p47-phox fusion protein, and the indicated concentrations of PA (closed squares) or AA (closed circles). Reactions were incubated for 30 min and then spotted on phosphocellulose squares as described under “Experimental Procedures.” Results are the mean ± S.E. of three experiments with cytosol from different donors.
from 30

 maximal protein kinase activation shifted, only slightly, inner membrane lipid layer (67). The concentration of PA yield mixed vesicles, containing PC, PS, and PE, which mimicked the PA-activated protein kinase was also active in the presence of using SDS-PAGE and autoradiography (data not shown). The protein kinase phosphorylated recombinant p47-phox fusion protein 6.7 μg/ml, 10 μM ATP, and 100 μM PA. Results are expressed as a percentage of total recovered activity and are the average of two experiments using cytosol from separate donors. B, Western blot. Fractions from the ammonium sulfate precipitation were assessed for the presence of PKC isoforms. 33% of the volume recovered from the Sephadex G-25 column for the 40% precipitate (40%), the 70% precipitate (70%), the resulting 70% supernatant (Supn.), and 1% of the unfractionated cytosol passed over a Sephadex G-25 column (G25), were analyzed by Western blotting as described under “Experimental Procedures.” Std., baculovirus-expressed standards. Shown are scans of autoradiographs from one experiment, representative of two performed with cytosols from different donors.

NADPH oxidase system (7, 8, 65, 66), had little effect on activation (1680 pmol of PO₄/min/mg activity in the absence of GTPyS, 1613 pmol PO₄/min/mg in the presence of GTPyS, n = 2). We also verified that the partially purified PA-activated protein kinase phosphorylated recombinant p47-phox (Rp47), using SDS-PAGE and autoradiography (data not shown). The PA-activated protein kinase was also active in the presence of mixed vesicles, containing PC, PS, and PE, which mimicked the inner membrane lipid layer (67). The concentration of PA yielding maximal protein kinase activation shifted, only slightly, from 30 μM PA (3830 pmol PO₄/min/mg, n = 2) in the absence of mixed vesicles to 100 μM PA (3634 pmol PO₄/min/mg, n = 2) in the presence of mixed vesicles. This suggests that activation of the PA-activated protein kinase can occur in a physiologically relevant manner.

We have previously shown that NADPH oxidase activation and endogenous protein phosphorylation induced by PA plus DG is sensitive to several protein kinase inhibitors (8). Fig. 9 shows that the partially purified PA-activated protein kinase is sensitive to the same inhibitors. In the absence of inhibitors, the GST-p47-phox fusion protein was phosphorylated by the protein kinase only when PA was present. Staurosporine and C-1 each completely inhibited the phosphorylation of GST-p47-phox, while GF-109203X was less effective. The autoradiograph shown in Fig. 9 is a long exposure to show that little protein phosphorylation occurred in the presence of these protein kinase inhibitors. Staurosporine is known to inhibit both protein serine/threonine kinases and protein-tyrosine kinases (51). This suggests that the PA-activated protein kinase is a protein serine/threonine kinase.
Phosphatidic Acid May Activate a Novel Protein Kinase

To further evaluate the amino acid specificity of the protein kinase, we performed phosphoamino acid analysis on phosphorylated GST-p47-phox. Fig. 10A clearly shows that serine residues are phosphorylated and that threonine residues are not. However, this analysis did not clearly separate phosphorylated and dephosphorylated tyrosine residues due to inorganic phosphate. To determine if tyrosine residues were phosphorylated, we extracted each of the phosphoamino acids from the cell line plate with methanol. This treatment did not elute inorganic phosphate (data not shown). The methanol extracts were then spotted onto cellulose plates and subjected to autoradiography. After extraction, only the phosphoserine and phosphorylserine extracts resulted in spots, as assessed by autoradiography (data not shown). This confirmed that serine residues were phosphorylated and that tyrosine residues were also phosphorylated. We confirmed tyrosine phosphorylation by Western blot analysis. The GST-p47-phox fusion protein became phosphorylated on tyrosine residues only when PA was present (Fig. 10B). It was possible that the GST portion of the fusion protein was phosphorylated by a protein kinase in the hydropore-bound material. However, we found that hydropore-bound material did not phosphorylate the cleaved GST portion of the fusion protein in the presence of PA (data not shown). In addition, recombinant p47-phox was phosphorylated on tyrosine residues, in a PA-dependent manner, using cytosol as a source for protein kinases (data not shown). Taken together, these data suggest that the hydropore-bound material contains one or more protein kinases that phosphorylate serine and tyrosine residues of p47-phox in a PA-dependent manner. Since no mammalian dual specificity protein kinases that phosphorylate both serine and tyrosine residues have been identified, we speculate that the hydropore-bound material contains at least two PA-responsive protein kinases.

Cell and Tissue Distribution of PA-activated Protein Kinase—Since PA is generated in many cell types upon stimulation of PLD (2), we investigated whether cytosol from other cell types contained a protein kinase responsive to PA. Cytosols were isolated as described under “Experimental Procedures,” and protein kinase activity was determined in the presence and absence of PA. Several cell types were analyzed. PA-dependent protein kinase activity (defined here as >2-fold stimulation upon PA addition) was present in all except three cell lines (HepG2, McA-RH7777, and IMR-32). The highest levels of PA-dependent protein kinase activity were present in neutrophil (305 ± 57 pmol of PO 4/min/mg, n = 6), lymphocyte (492 ± 55 pmol of PO 4/min/mg, n = 4), HL-60 (598 pmol of PO 4/min/mg, n = 2), THP-1 (346 ± 92 pmol of PO 4/min/mg, n = 3), and rat brain (528 ± 66 pmol of PO 4/min/mg, n = 4) cytosols. Platelet (132 ± 53 pmol of PO 4/min/mg, n = 5), CFTL-15 (153 pmol of PO 4/min/mg, n = 2), RAW 267.4 (241 pmol of PO 4/min/mg, n = 2), K562 (130 pmol of PO 4/min/mg, n = 1), and Madin-Darby canine kidney cell (104 ± 46 pmol of PO 4/min/mg, n = 3) cytosols had intermediate levels of activity. Thus, PA-responsive protein kinases exist in cells other than neutrophils.

We further investigated the possibility that the same enzyme present in neutrophil cytosol might be present in rat brain cytosol. Rat brain cytosol was subjected to 40% saturated ammonium sulfate precipitation followed by hydrophobic interaction chromatography. The purification profile obtained was...
similar to that obtained with neutrophil cytosol (see Figs. 4 and 5). A PA-dependent protein kinase was precipitated by 40% saturated ammonium sulfate (980 pmol of PO₄/min/mg, n = 1) and was retained during hydrophobic interaction chromatography (1590 pmol of PO₄/min/mg, n = 1). This suggests that the same or a similar enzyme was present in these two sources of material. However, other PA-responsive protein kinases appear to be more prevalent in the rat brain cytosol than in neutrophil cytosol, since the supernatant from the 40% ammonium sulfate cut contained 57% of the recovered activity, compared with 15% using neutrophil cytosol. This may account for the smaller increase in specific activity observed in rat brain material (1.8-fold increase) compared with neutrophil material (5-fold increase) after elution from the Hydrophore column.

**DISCUSSION**

We have recently developed a phosphorylation-dependent cell-free system for the activation of the neutrophil NADPH oxidase (7, 8). In this system, PA activates one or more protein kinases that phosphorylate the NADPH oxidase component p47-phox. Here, we have eliminated known protein kinase targets for PA in this system. Our results suggest that PA activates potentially two previously unidentified cytosolic protein kinases, which phosphorylate p47-phox.

We first characterized the lipid specificity for the activation of the PA-dependent protein kinase. Previous studies indicated that DG has only minor enhancing effects on protein phosphorylation induced by PA (8), suggesting that DG-regulated PKC isoforms were unlikely to be targets for PA in this system. Fig. 1 demonstrates that PA induced a high level of protein phosphorylation and activates a protein kinase(s) that is not a target for other phospholipids, supporting the concept that PA activates a novel protein kinase. Bocckino et al. (69) first demonstrated that PA could induce protein phosphorylation in various rat tissues, and our results confirm this. Like them, we found that PC and PE induced minimal protein phosphorylation and that phosphatidylinositol and PS induce moderate levels (Fig. 1). We show here that phosphatidylglycerol can also induce protein phosphorylation (Fig. 1). In the neutrophil system, more proteins appear to be substrates for a PA-dependent protein kinase(s) than in rat tissues.

We considered the possibility that the protein kinase target for PA was one of the recently described fatty acid-activated protein kinases (18–20), one of which has been shown to be PKN (16), known to be activated by cis-unsaturated fatty acids as well as CL (17) and identical to PRK1 (14, 17). The predicted molecular size of PRK1/PKN is 120 kDa (14, 15, 17, 58, 70), similar to the apparent size of the predominant protein kinase described here (Fig. 8). PA was much more effective than unsaturated fatty acids at inducing protein kinase activity (Fig. 4 and text). In addition, CL (3 μmol/l. 1 mm), was unable to induce protein phosphorylation in neutrophil cytosol (Fig. 1 and text). Furthermore, while PKN is regulated by the GTPase Rho, the PA-dependent protein kinase activity described here was independent of GTPγS. Thus, it is unlikely that Rho could activate PKN under these conditions. These data strongly suggest that PRK1/PKN (14, 15, 17, 58, 70) and/or the fatty acid-activated protein kinases previously described (18–20) are not responsible for the PA-dependent protein kinase activity in neutrophils. It is possible that the neutrophil PA-activated protein kinase is related to these enzymes and is an isozyme poorly responsive to cis-unsaturated fatty acids and highly responsive to PA. Such a determination will require identification of the neutrophil enzyme at the molecular level.

Our second approach was to purify the PA-activated protein kinase present in neutrophil cytosol to a stage where we could distinguish the PA-activated protein kinase from other protein kinase targets. The partial purification protocol successfully separated the PA-activated protein kinase(s) from a number of other protein kinases in neutrophils known to phosphorylate p47-phox, including PKC, MAPK, and PAK isoforms (Figs. 5, 6, and 7). We did not examine fractions for the presence of PKC-μ, which has not been reported to be in neutrophils. Since PKC-μ has a predicted molecular size of 115 kDa (73), it is possible that this isoform is responsible for some of the PA-dependent protein kinase activity in neutrophils. However, PKC-μ has been reported to have a putative transmembrane domain (73) and has been isolated in the particulate material after subcellular localization (74). In contrast, the PA-activated protein kinase is cytosolic. Also, PA has been reported to have little effect on activation of PKC-μ (75). Taken together, these observations strongly suggest that PKC-μ is not a PA-activated protein kinase in neutrophil cytosol.

MAPK and PAK isoforms were also candidates for the protein kinase(s) responsible for phosphorylation of p47-phox (24, 25). However, neither p42 or p44 MAPK isoforms nor PAK-1 or PAK-2 isoforms co-purified with the PA-activated protein kinase (Fig. 7 and text). In addition, depletion of cytosolic Raf-1 or B-Raf, which could lead to MAPK activation, by immunoprecipitation had little or no effect on PA-dependent protein kinase.
activity (see text). Finally, the PA-dependent protein kinase activity was GTPγS-independent, which suggests Rac was not participating in protein kinase activation in our system. We did not examine other protein kinases in these families (24, 28–31, 76).

These results strongly suggest that PA activates a novel protein kinase in neutrophil cytosol, which phosphorylates the NADPH oxidase component p47-phox. The PA-activated protein kinase was sensitive to the same protein kinase inhibitors that inhibited cell-free NADPH oxidase activation by PA plus DG (Fig. 9) (8). GF-109203X has been described as a selective PKC inhibitor (77), but it was partially effective against the hydropore-purified PA-activated protein kinase. This may indicate that a PA-activated protein kinase is similar to PKC; however, this remains to be determined. The PA-activated protein kinase phosphorylates p47-phox on serine residues (Fig. 10A), which has been shown to occur in vivo (25, 78, 79). The inhibitor results suggested strongly that the PA-activated enzyme is a protein serine/threonine kinase. However, the phosphoamino acid analysis revealed that PA also activates a protein tyrosine kinase, which phosphorylates p47-phox (Fig. 10, A and B). Thus, the hydropore-bound material either contains at least two PA-responsive protein kinases, or one of the kinases is activated downstream of the other. It is possible that the known lability of the phosphotyrosine bond (54, 55) accounts for the apparent serine/threonine specificity observed during SDS-PAGE analysis. This also may explain the appearance of only one peak of PA-dependent protein kinase activity during gel filtration (Fig. 8). Further purification of these enzymes will be necessary to address these issues and is beyond the scope of this paper.

Tyrosine phosphorylation of p47-phox has not been reported until now. Previous studies have analyzed the phosphorylation of p47-phox after stimulation of neutrophils with phorbol myristate acetate (25, 78, 79). Further work needs to be done to examine if other NADPH oxidase agonists stimulate tyrosine phosphorylation of p47-phox, in vivo. In these studies, it will be important to monitor the hydrolysis of p47-phox to maximize the ability to observe potential tyrosine phosphorylation, since phosphotyrosine is more labile than phosphoserine and phosphothreonine (54, 55). It is also likely that the phosphorylation of p47-phox on tyrosine residues occurs to a lesser extent than serine phosphorylation. Thus, the amount of p47-phox recovered prior to phosphoamino acid analysis and the assay conditions used are likely to be critical to observe tyrosine phosphorylation in vivo. P47-phox contains several tyrosine residues that may be a target for a protein-tyrosine kinase (80–82). Further work is necessary to determine which tyrosine residues are phosphorylated in response to PA. Once these sites are determined, the role of such phosphorylation can be examined more closely. It remains to be determined what role, if any, tyrosine phosphorylation of p47-phox has in NADPH oxidase activation.

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A primary significance of this study lies in the possibility that the PA-activated protein kinases are new and selective targets for intracellular PA generated by PLD activation. In neutrophils, the PA-activated protein kinase(s) could participate in the regulation of a variety of cell functions (1, 2, 83), such as activation of the NADPH oxidase. Activation of PLD and the resulting production of PA have been closely linked to the activation of the oxidase (4–8, 84). Our previous data clearly indicate that a PA-activated protein kinase partakes in the PA- and DG-dependent cell-free activation of NADPH oxidase (8). A PA-activated protein kinase retained the ability to phosphorylate p47-phox, even after three steps of purification (Fig. 8B), providing additional evidence indicating that the NADPH oxidase component p47-phox is a substrate for the enzyme. However, it remains to be determined if phosphorylation of p47-phox by a PA-activated protein kinase regulates the assembly and activation of the NADPH oxidase enzyme.

It is notable that we found PA-dependent protein kinase activity in a wide range of blood cells and hematopoietic cell lines and in rat brain cytosol, suggesting that the enzyme may be widely distributed. Activity was not apparent in the hepatoma and neuroblastoma cell lines, but the significance of these preliminary observations is not known. It is not yet clear if the same enzymes are responsible for the activity in all of these cells and in rat brain. However, like the enzyme in neutrophils, a PA-activated protein kinase in rat brain cytosol was precipitated at 40% ammonium sulfate saturation and retained on the Hydripose column. Once a PA-activated protein kinase has been purified and sequence information is available, it will be possible to use molecular and immunological approaches to definitively address the cell and tissue distribution of the enzyme.

In conclusion, we have shown that neutrophil cytosol contains potentially two novel PA-responsive protein kinases. The molecular weight of one of these protein kinases is 125 kDa. We have differentiated these enzymes from other known protein kinases that could be targeted by PA in our system and have evidence that the enzymes may be widely distributed in cells and tissues. These protein kinases phosphorylate p47-phox, a necessary component of the NADPH oxidase. Future work will focus on purifying and sequencing the PA-responsive protein kinases to identify them as either novel enzymes or known protein kinases with a new function. In addition, we plan to define the role of PA-activated protein kinases in the phosphorylation-dependent activation of the NADPH oxidase.

Acknowledgments—We thank the following people at Wake Forest University Medical Center: Dr. Susan Hutson for the use of fast protein liquid chromatography equipment; Drs. B. Moseley Waite, Larry Daniel, Mary Beth Fasano, Charles McCall, Greg Shellness, and Osvaldo Delbono for cell lines; Drs. Carol Cunningham and Susan Hutson for rat brain tissue; Mary Ellenburg for help with Western blot analyses, and Rose Waite for graphics. We also thank Drs. Arieh Abe and Gary Bokoch for antibodies to PAK. We are grateful to Dr. Tom Leto for purified recombinant p47-phox and E. coli containing cDNA for GST-p47-phox and to Drs. Sujoy Ghosh and Jay Strum for sharing results prior to publication.

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