A Phosphatidic Acid-activated Protein Kinase and Conventional Protein Kinase C Isoforms Phosphorylate p22\(^{phox}\), an NADPH Oxidase Component*  

(Received for publication, August 13, 1999, and in revised form, September 17, 1999)  

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Using a phosphorylation-dependent cell-free system to study NADPH oxidase activation (McPhail, L. C., Qualliotine-Mann, D., and Waite, K. A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7931–7935), we previously showed that p47\(^{phox}\), a cytosolic NADPH oxidase component, is phosphorylated. Now, we show that p22\(^{phox}\), a subunit of the NADPH oxidase component flavocytochrome b\(_{558}\) also is phosphorylated. Phosphorylation is selectively activated by phosphatidic acid (PA) versus other lipids and occurs on a threonine residue in p22\(^{phox}\). We identified two protein kinase families capable of phosphorylating p22\(^{phox}\): 1) a potentially novel, partially purified PA-activated protein kinase(s) known to phosphorylate p47\(^{phox}\) and postulated to mediate the phosphorylation-dependent activation of NADPH oxidase by PA and 2) conventional, but not novel or atypical, isoforms of protein kinase C (PKC). In contrast, all classes of PKC isoforms could phosphorylate p47\(^{phox}\). In a gel retardation assay both the phosphatidic acid-dependent kinase and conventional PKC isoforms phosphorylated all molecules of p22\(^{phox}\). These findings suggest that phosphorylation of p22\(^{phox}\) by conventional PKC and/or a novel PA-activated protein kinase regulates the activation/assembly of NADPH oxidase.

Phagocytic cells are the first line of defense against invading microorganisms (reviewed in Ref. 1). This defense is achieved, in part, by the respiratory burst, leading to the production of superoxide anion, which along with its metabolic products are toxic to invading microorganisms. The respiratory burst is mediated by the multicomponent enzyme complex, the NADPH oxidase. The two membrane-bound components (p22\(^{phox}\) and gp91\(^{phox}\)) form the heterodimeric flavocytochrome b\(_{558}\) (2). The flavocytochrome contains a putative NADPH binding site, FAD, and two hemes; thus, it possesses all of the electron machinery required to transfer two electrons from NADPH to molecular oxygen (3–10). The three required cytosolic components are Rac-GTP and the phosphoproteins p47\(^{phox}\) and p67\(^{phox}\) (11) (reviewed in Ref. 12). Activation of phagocytic cells leads to the translocation of the cytosolic components to the membrane, where they interact with flavocytochrome b\(_{558}\) (reviewed in Ref. 12). Assembly of the NADPH oxidase components is required for activation of electron flow, possibly by inducing a conformational change within the flavocytochrome b\(_{558}\) component.

The signaling mechanisms leading to assembly and activation of NADPH oxidase are not well understood. When a ligand binds its receptor on the neutrophil membrane, a cascade of events is initiated (reviewed in Ref. 1) that includes activation of phospholipases, generation of lipid second messengers, and the activation of protein kinases. One of the phospholipases activated in response to many physiological agonists of neutrophils is phospholipase D (13–27). Phospholipase D cleaves neutrophil phospholipids to form phosphatidic acid (PA),1 which can then be converted to diacylglycerol by PA phosphohydrolase. Numerous studies have correlated phospholipase D activation/PA production and NADPH oxidase activation (19, 28–34). Thus, it has been hypothesized that phospholipase D and its product, PA, play important roles in the signal transduction mechanisms leading to superoxide anion production.

Recently, our laboratory developed and characterized a cell-free system for NADPH oxidase activation, which was synergistically activated by PA + diacylglycerol (35, 36). NADPH oxidase activation was enhanced by ATP and reduced by protein kinase inhibitors. Furthermore, PA induced the phosphorylation of several neutrophil proteins. These data suggest that phosphorylation-dependent mechanisms are involved in the activation of NADPH oxidase in this cell-free system. We have reported that p47\(^{phox}\) is phosphorylated in this system by a novel, cytosolic PA-activated protein kinase (36, 37). Now we report that p22\(^{phox}\) also is phosphorylated in the system in a PA-dependent manner. Phosphorylation of p22\(^{phox}\) was first observed by Garcia and Segal (38) in intact cells. We have now characterized the phosphorylation of p22\(^{phox}\) \textit{in vitro} and show that a potentially novel, PA-activated protein kinase and conventional but not protein kinase C (PKC) isoforms are able to phosphorylate this protein.

EXPERIMENTAL PROCEDURES

Materials—

\textit{Escherichia coli}, containing a plasmid encoding GST-p22 (amino acids 127–195) or GST-p47, p22\(^{phox}\), and p47\(^{phox}\) antibodies were kindly provided by Dr. Tom Leto (National Institutes of Health). The fusion proteins were prepared as described previously (39). Purified, relipidated flavocytochrome b\(_{558}\) was a generous gift of Dr. Michael Kleinberg (University of Maryland) (3, 40). The mAb 44.1 to p22\(^{phox}\) (41)  

* This work was supported in part by National Institutes of Health Grant RO1-A122564 and March of Dimes Birth Defects Foundation Grants FY97-0443, FY98-0638, and FY99-0561. 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 by the Signal Transduction Mechanisms and Cell Function Training Program Grant CA-09422 from the National Institutes of Health.

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1 The abbreviations used are: PA, phosphatidic acid; CGD, chronic granulomatous disease; di10:0PA, 1,2-dicapryl-sn-glycero-3-phosphate; GST, glutathione S-transferase; PKC, protein kinase C; PS, phosphatidylserine; PAG, polyacylamlide gel electrophoresis; mAb, monoclonal antibody.
was the kind gift of Dr. Algirdas J. Jesuitis (Montana State University). Anti-mouse IgG and anti-rabbit IgG were from Transduction Labs (Lexington, KY). Phosphatidylinositol (pig liver), phosphatidycholine (pig liver), phosphatidyl ethanolamine (pig liver), phosphatidylserine (PS, bovine brain), cardiolipin (heart), 1,2-di(oxy)sn-glycerol-3-phosphate (DSPG). Reagents lecithin-derived PA, 1,2-dicapryl-sn-glycerol-3-phosphate (dil0:0PA), 1,2-di(oxy)sn-glycerol-3-phosphate, and 1,2-di-stearoyl-sn-glycerol-3-phosphate were obtained from Avanti Polar Lipids (Alabaster, AL). The 1-oleoyl-sn-glycerol-3-phosphate and 1-oleoyl-2-acetyl-glycerol-3-phosphate were from Serdy Research Laboratories (London, Canada). All lipids were prepared by sonication in water as described previously (35). Other reagents used for solubilization and subsequent immunoprecipitation (see below). The 1-(5-isoquinolinesulfonyl)piperazine was synthesized by Dr. Michael J. Thomas (Wake Forest University School of Medicine) (42). All other reagents were from Sigma.

Isolation and Subcellular Fractionation of Neutrophils—Neutrophils were purified as described, previously (35). The cells were then treated with diisopropyl fluorophosphate (1.71 mM), resuspended (2 x 10^9 cells/ml) in sonication buffer (150 mM NaCl, 5 mM benzamidine, 10 mM leupeptin, 10 mM pepstatin, 1 µg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride) and sonicated to 10% destruction of cytoplasmic activities. Membrane and cytosolic fractions were separated using a 15% (w/v) sucrose gradient, as described previously (44, 45). Protein concentrations were determined using the biocinchoninic acid protocol with bovine serum albumin as a standard.

Neutrophils from patients with chronic granulomatous disease (CGD) were prepared in a similar fashion; however, the sonication buffer contained 11% sucrose, 130 mM NaCl, 5 mM EGTA, and 1% methylthionylsulfonyl fluoride. Individuals from two families (four patients) were obtained. Two male patients, one X-linked patient, and one female from a second family (JB and EB) have >10% of normal cytochrome b_{558} levels (46, 47); whereas, one male and one female from a second family had no detectable cytochrome b_{558}.

In Vitro PA-dependent Protein Phosphorylation—Reactions mixtures (150 µl) consisted of 50 mM NaPO_4 pH 7.0, 1 mM EGTA, 5 mM MgCl_2, either neutrophil membrane fractions (3.1–6.25 µg of protein) or another substrate, and a lipid activator (10 µM di10:0PA (SR-37). 10 µM [γ-32P]ATP (10 µCi) and neuphil cytosol (12.5–25 µg of protein) or another source of protein kinase were added, and the reaction mixture was allowed to incubate for the times indicated in figure legends. The reaction was stopped by the addition of Laemmli sample buffer (48) for analysis by SDS-PAGE, autoradiography, and densitometry. 5% NaCl Solution (5 mM NaCl, 25 mM EDTA, 25 mM EGTA, 5 µM staurosporine, 25 mM sodium orthovanadate, 5 µM microcystin, 125 mM NaF, 5 mM p-nitrophenylphosphate, 50 µM benzamidine, 50 µM/ml leupeptin, 50 µM pepstatin, 5 µg/ml aprotinin, and 5 mM phenylmethylsulfonyl fluoride) was used to quench reactions that were used for solubilization and subsequent immunoprecipitation (see below).

In Vitro Protein Kinase C-mediated Protein Phosphorylation—For phosphorylation by conventional and novel PKC isoforms, previously described conditions were used (42, 44, 49). For PKC_i experiments, 100-µl reaction mixtures contained 25 mM Tris, pH 7.5, 5 mM MgCl_2, 0.5 mM EGTA, and 1 mM dithiothreitol and with or without the addition of 100 µM/ml PS. For PKC reaction mixtures containing GST-p47, 25 µg/ reaction whale myoglobin was added. Proteins were analyzed by separation on SDS-PAGE, transfer to nitrocellulose, and analyzed by autoradiography/densitometry or PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA). Western blotting was performed to confirm p22^{phox} and GST-p47 protein levels (described below).

Immunoprecipitation—Membrane fractions were solubilized as described previously (50) except that additional protease and phosphatase inhibitors (25 mM NaF, 1 mM p-nitrophenylphosphate, 5 mM Na_3VO_4, 1 mM microcystin, 125 mM NaF, 0.5 mM leupeptin, 10 µM pepstatin, 1 µg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride) were included in the buffers used.

Two methods for immunoprecipitation were used. In the first method, CNBr-activated Sepharose beads were conjugated to either mAb 44.1 or the isotype control, according to manufacturer’s directions (Pierce Chemical Co., Rockford, IL). Solubilized membrane was precleared with isotype control antibodies-conjugated beads and then incubated with the mAb 44.1-conjugated (~150 µg of solubilized membrane protein/15 µl antibody-conjugated beads) at 4 °C for 16 h. Alternatively, protein A-Sepharose and either the isotype control antibody or mAb 44.1 were incubated with the solubilized membrane (500 µg solubilized membrane/20 µg antibody) at 4 °C for 16 h. The beads were washed, and the immunoprecipitated proteins were analyzed by 14 or 8–15% SDS-PAGE.

SDS-PAGE, Autoradiography, and Densitometry—Protein samples were prepared for analysis by SDS-PAGE using Laemmli sample buffer (48) and separated on 8–15, 7, or 14% SDS-PAGE. Gels were stained with either Coomassie Brilliant Blue R-250 or GelCode Blue Stain (Pierce Chemical Co., Rockford, IL). Blots were prepared for analysis by SDS-PAGE using Laemmli sample buffer (48) and separated on 8–15, 7, or 14% SDS-PAGE. Gels were stained with either Coomassie Brilliant Blue R-250 or GelCode Blue Stain (Pierce Chemical Co., Rockford, IL). Blots were scanned by densitometry using NIH Image software. In some experiments, proteins were transferred electrophoretically (51) to nitrocellulose for autoradiography and Western blot analysis. Autoradiographs were analyzed by scanning densitometry (PDI, Huntington Station, NY).

Western Blot Analysis—Western blot analysis for p47^{phox} was performed as described previously (37). For p22^{phox} Western blotting, a transfer buffer optimized for flavocytochrome b_{558} (192 mM glycine, 25 mM Tris, 20% methanol, 0.1% SDS) was used (51, 52). Blots were blocked for 1 h with 5% milk in TBS-T (10 mM Tris, 100 mM NaCl, and 0.1% Tween-20), incubated with mAb 44.1 (1:1000 dilution) for 4 h at room temperature, and washed for 2 h. The blot was incubated with anti-mouse horseradish peroxidase-conjugated secondary antibody for 1 h followed by six 10-min washes. Proteins were visualized using SuperSignalECL Enhanced Chemiluminescence (Pierce).

Lipid Phosphorysor Assay—Lipid phosphorysor assays were performed as described previously (53), using NaH_2PO_4 (5–50 nmol) for the standard curve.

Phosphoamino Acid Analysis—Phosphorylated p22^{phox} was excised from polyvinylidene difluoride membranes and subjected to hydrolysis with 6 M HCl for 4 h at 110 °C (54). Phosphoamino acids were separated by thin layer electrophoresis at 1100 V for 45 min in water:acetic acid:pyridine (189:10:1, pH 3.5) (11, 54). H_3[32P]PO_4 was used as a standard for the migration of inorganic phosphate.

RESULTS

Identification of p22^{phox} as a Substrate for a Neutrophil PA-Activated Protein Kinase—As shown in Fig. 1A (left two lanes), when neutrophil membrane and cytosolic fractions were incubated with di10:0PA, a 22-kDa protein was phosphorylated. Because this phosphoprotein was the appropriate size for the light chain of the flavocytochrome, we hypothesized it could be p22^{phox}. Neutrophil membrane fractions from patients with flavocytochrome b_{558}-deficient CGD were substituted for normal membrane fractions in the reaction mixture. Membrane fractions from a total of four patients, two with the X-linked form of CGD and two with the autosomal recessive form, were tested. The two patients with X-linked CGD were previously characterized as having >10% of the normal levels of flavocytochrome b_{558}.2 whereas the sibling pair with the autosomal recessive form had no detectable flavocytochrome b_{558}.2 As shown in Fig. 1A (right two lanes), the substitution of neutrophil membrane fractions from a patient having >10% of normal levels of flavocytochrome b_{558} resulted in markedly reduced levels of the phosphorylated 22-kDa protein. Similar results were obtained with membrane fractions from the other X-linked patient, and no PA-dependent phosphorylation of a 22-kDa band was observed using membrane fractions from the two patients totally deficient in flavocytochrome b_{558} (data not shown).

2 S. Strum and L. C. McPhail, unpublished observation.

3 Whale myoglobin was added to reaction mixtures containing low total protein to prevent loss of GST-p47^{phox} during processing (unpublished observation).
To further confirm that p22<sub>phox</sub> was phosphorylated, we performed immunoprecipitation experiments using a monoclonal antibody to the protein (41). Following phosphorylation in the presence of cytosol, membrane fractions were reisolated, and solubilized proteins were subjected to immunoprecipitation with the p22<sub>phox</sub>-specific antibody. As shown in Fig. 1B (left two lanes), a phosphorylated 22-kDa protein was immunoprecipitated from membrane fractions incubated with neutrophil cytosol in the presence of di10:0PA. Very little phosphorylation was observed in immunoprecipitates from membrane fractions incubated in the presence of neutrophil cytosol without the addition of di10:0PA. In contrast, no phosphorylated proteins were immunoprecipitated with the isotype control antibody (Fig. 1B, right two lanes).

We performed two additional experiments to verify that p22<sub>phox</sub> is phosphorylated by a PA-dependent protein kinase present in neutrophil cytosol. First, purified, relipidated flavocytochrome b<sub>558</sub> was mixed with neutrophil cytosolic fractions (12.5 μg of protein) and [γ<sup>32</sup>P]ATP at 25 °C for 60 min. Neutrophil membrane fractions were collected by ultracentrifugation (150,000 × g, 90 min, 4 °C), and subjected to immunoprecipitation with either a p22<sub>phox</sub>-specific antibody (α p22, left two lanes) or an isotype control antibody (IgG2B, right two lanes), as described under "Experimental Procedures." Immunoprecipitates were separated by 14% SDS-PAGE and analyzed by autoradiography and subsequent densitometry. Shown is a scan of one experiment representative of two experiments with IgG2B controls and four experiments with the p22<sub>phox</sub>-specific antibody. C, PA-dependent phosphorylation of purified, relipidated flavocytochrome b<sub>558</sub>. Reaction mixtures contained either no flavocytochrome b<sub>558</sub> (H<sub>2</sub>O), phosphatidylcholine vesicles (vehicle), or purified flavocytochrome b<sub>558</sub> relipidated in phosphatidylinositol or phosphatidylglycerol vesicles (cyto b 0.72 pmol/reaction). Reactions were incubated with neutrophil cytosol (12.5 μg of protein) and [γ<sup>32</sup>P]ATP in the presence (−) or absence (+) of 10 μM di10:0PA, as noted, for 45 min at 25 °C. Proteins were separated on 8–15% SDS-PAGE and analyzed by autoradiography. Shown is a scan of one experiment representative of two experiments per condition. D, PA-dependent phosphorylation of a recombinant fragment of p22<sub>phox</sub>. Recombinant GST-p22 (amino acids 127–195) bound to glutathione-Sepharose beads was treated with thrombin (30 units, 1 h). The released peptide (−1 μg) was mixed with neutrophil cytosol (12.5 μg of protein) and [γ<sup>32</sup>P]ATP for 60 min at 25 °C in the presence (−) or presence (+) of 10 μM PA. Proteins were separated on 14% SDS-PAGE and analyzed by autoradiography. Shown is a scan of one experiment representative of two performed.

To characterize the PA-dependent Phosphorylation of p22<sub>phox</sub>—To characterize the phosphorylation of p22<sub>phox</sub> by a PA-activated protein kinase, we varied the concentration of di10:0PA from 0 to 300 μM, and the time of incubation from 0 to 120 min. Optimal phosphorylation of p22<sub>phox</sub> was obtained with incubation of neutrophil cytosol and membrane fractions in the presence of 10 μM di10:0PA for 60 min (data not shown). This concentration of PA is within the range of that measured in stimulated neutrophils (56), thus indicating that phosphorylation is mediated by a physiological concentration of PA.

We next determined the lipid specificity for the induction of p22<sub>phox</sub> phosphorylation. At 10 μM, only di10:0 PA (PA) clearly induced p22<sub>phox</sub> phosphorylation. However, the addition of 10 μM PS or phosphatidylinositol (PI) caused a faint darkening over background in the 22 kDa range (Fig. 2A, top panel). A concentration curve with PS (0–300 μM) revealed a low level of p22<sub>phox</sub> phosphorylation with a maximum at 100 μM (data not shown). We then screened various lipids at 100 μM for their ability to induce p22<sub>phox</sub> phosphorylation. At this concentration, PS, phosphatidylinositol (PI), phosphatidylglycerol (PG), and 1-octyl-sn-glycero-3-phosphate (LPA) induced low levels of p22<sub>phox</sub> phosphorylation (Fig. 2A). However, di10:0PA clearly induced the highest response.

We examined the ability of PA species with different acyl chain compositions to induce p22<sub>phox</sub> phosphorylation. As shown in Fig. 2B, di10:0PA (10:0), at either 10 or 100 μM, induced the highest level of p22<sub>phox</sub> phosphorylation; however, both 1,2-diacyl-sn-glycero-3-phosphates (18:1) and the physiological egg lecithin-derived PA (egg) also had activity. In contrast, 1,2-distearoyl-sn-glycero-3-phosphate (18:0) was unable to induce the phosphorylation of p22<sub>phox</sub> Thus, PA-induced phosphorylation of p22<sub>phox</sub> shows selectivity for short or unsaturated acyl chains over long, saturated acyl chains.

Identification of Protein Kinases That Phosphorylate p22<sub>phox</sub>—To identify the type of protein kinase that mediates

![Figure 1](image-url)
p22\textsuperscript{phox} phosphorylation, we tested the effect of several protein kinase inhibitors. 1-(5-isoquinolinesulfonyl)piperazine, an H-7 analog, selectively blocks protein Ser/Thr kinases (42). Stauroporine inhibits both protein Ser/Thr and tyrosine kinases (57, 58). GF109203X is a PKC-selective inhibitor (59). Each of these three inhibitors blocked p22\textsuperscript{phox} phosphorylation by 70–85\% (Fig. 3). In contrast, genistein, a protein tyrosine kinase inhibitor (60), had no effect on the level of p22\textsuperscript{phox} phosphorylation (Fig. 3). These data indicate that p22\textsuperscript{phox} is likely phosphorylated by a protein Ser/Thr kinase rather than a tyrosine kinase. Furthermore, the same Ser/Thr protein kinase inhibitors decrease NADPH oxidase activation by 70–80\% in the phosphorylation-dependent cell-free system (36).

A similar profile of inhibition was observed for the partially purified, cytosolic PA-activated protein kinase (37), suggesting it might be responsible for the phosphorylation of p22\textsuperscript{phox}. As shown in Fig. 4A, no phosphorylation of p22\textsuperscript{phox} was observed without the addition of neutrophil cytosol, thus indicating that the PA-activated protein kinase responsible for p22\textsuperscript{phox} phosphorylation is cytosolic. Next, we examined the ability of the partially purified PA-activated protein kinase to phosphorylate p22\textsuperscript{phox}. The protein kinase was partially purified by precipitation with 40\% saturated ammonium sulfate followed by passage over a hydrophobic interaction column, as described previously (37). As shown in Fig. 4B (right two lanes), when the partially purified protein kinase replaced cytosol in the reaction mixture, PA-dependent phosphorylation of p22\textsuperscript{phox} was observed. Thus, at least one protein kinase that can phosphorylate p22\textsuperscript{phox} is the apparently novel PA-activated protein kinase.

We next tested the ability of PKC to phosphorylate p22\textsuperscript{phox}, based on the following rationale. Many groups, including ours, have correlated PKC activation with the activation/assemby of NADPH oxidase (Refs. 44 and 61; reviewed in Ref. 1). Sequence analysis of p22\textsuperscript{phox} revealed a PKC phosphorylation motif (I/R/K/KS/T or S/T/X/R/K) at Thr\textsuperscript{47} (Wisconsin Package version 9.0, Genetics Computing Group). This region is in the C-terminal, putative cytosolic tail of p22\textsuperscript{phox} (55). GF109203X, a PKC-selective inhibitor, diminished the phosphorylation of p22\textsuperscript{phox} by 80\% (Fig. 3). Garcia and Segal (38) reported in 1988 that p22\textsuperscript{phox} is phosphorylated in whole neutrophils stimulated with phorbol myristate acetate, a potent PKC activator. Therefore, we tested whether a purified preparation of PKC (rat brain) was able to induce the phosphorylation of p22\textsuperscript{phox}. According to this method, we found that PKC was capable of phosphorylating p22\textsuperscript{phox} (Fig. 5A).
the manufacturer (Calbiochem), the rat brain PKC preparation consisted primarily of conventional isoforms. Under conditions optimal for PKC activation, neutrophil membrane fractions were incubated in either the absence or presence of PKC and in the absence or presence of PKC activators (PS, 1,2-dioleoyl-rac-glycerol, and Ca²⁺). As shown in Fig. 4C (top panel), the phosphorylation of p22phox was readily apparent in the presence of PKC and its activators. A low level of p22phox phosphorylation was observed in the absence of exogenous PKC, possibly because of slight contamination of the membrane fraction with cytosol containing endogenous PKC isoforms (44). To verify that PKC was active under these conditions, we observed increased autophosphorylation of the purified PKC (45). In addition, p22phox, a known substrate for rat brain PKC (62), underwent activator-dependent phosphorylation by PKC (middle panel). Thus, PKC is capable of phosphorylating p22phox.

To further characterize the phosphorylation of p22phox by PKC, we obtained individual human, recombinant PKC isoforms known to be expressed in human neutrophils (α, β, βII, δ, and ζ) (63–66) and tested each for the ability to phosphorylate p22phox. Neutrophil membrane fractions were incubated with each PKC isoform under optimal assay conditions (44, 62). As shown in Fig. 5A (top panel), the conventional PKC isoforms (α, β, and βII), but not the novel PKC δ, were able to phosphorylate p22phox. In contrast, all of the PKC isoforms tested were able to phosphorylate p47phox (Fig. 5B, bottom panel).

PKC ζ was tested under slightly different conditions (see “Experimental Procedures”), which were recommended by the manufacturer. PKC ζ was able to phosphorylate p47phox (Fig. 5B) but not p22phox (data not shown). The phosphorylation of p47phox induced by PKC ζ was slightly greater than that induced by PA, using cytosol as a protein kinase source. To
summarize, these data show differences in the ability of classes of PKC isoforms to phosphorylate p22phox but not p47phox.

The assays used for PKC activation differ in several respects from the assays used for phosphorylation of p22phox by the PA-dependent protein kinase(s) in neutrophil cytosol (see "Experimental Procedures"). Therefore, we tested whether purified PKC could substitute for cytosol and mediate PA-dependent phosphorylation of p22phox, under conditions optimal for the PA-dependent protein kinase. Little, if any, PA-dependent p22phox phosphorylation was observed when cytosol was replaced with the purified rat brain PKC, a source of conventional PKC isoforms, or PKCζ (Fig. 6). However, rat brain PKC and PKCζ were able to mediate low levels of phosphorylation of another endogenous membrane protein, indicating that the PKC isoforms were activated (Fig. 6). Western blotting demonstrated that the phosphorylation patterns observed were not caused by variations in p22phox protein levels (data not shown). Based on these results, it is unlikely that PKC isoforms present in neutrophil cytosol are responsible for the phosphorylation of p22phox observed under our PA-dependent assay conditions (used in Figs. 1, 2, 3, and 4A).

We next developed an assay to determine the proportion of phosphorylated to unphosphorylated p22phox. Using 8–15% SDS-PAGE followed by transfer of the proteins to nitrocellulose and Western blot analysis, we observed a retardation in the migration of phosphorylated versus nonphosphorylated p22phox (Fig. 7A). Using this assay, we have shown that all of the p22phox becomes phosphorylated under conditions optimal for the PA-dependent protein kinase (Fig. 7A) and for PKC (Fig. 7B). Furthermore, retardation of p22phox migration was observed when p22phox was phosphorylated with the human, recombinant, conventional PKC isoforms (data not shown). The retardation in migration was not due to lipid/p22phox interactions because the retardation was prevented when reactions were performed in the absence of neutrophil cytosol or in the presence of protein Ser/Thr kinase inhibitors (data not shown).

Phosphoamino acid analysis also was performed on p22phox phosphorylated by either the PA-activated protein kinase or rat brain PKC (Fig. 8). Under both conditions, only threonine residues became phosphorylated. This result along with the gel retardation data above (Fig. 7), suggest that both protein kinases may phosphorylate the same site in p22phox.

**Fig. 6.** Protein kinase C cannot replace neutrophil cytosol for the phosphorylation of p22phox in the PA-dependent protein kinase assay. Neutrophil membrane fractions (6.25 μg of protein) were incubated with either buffer, 25 μg of neutrophil cytosol protein, rat brain PKC (RBPKC, 100 ng/reaction, ~100 pmol phosphate transferred/min), or PKCζ (200 ng/reaction, ~50 pmol phosphate transferred/min), in the absence (−) or presence (+) of 10 μM di10:0PA for 60 min at 25 °C. Proteins were separated by 14% SDS-PAGE, transferred to nitrocellulose, and analyzed by autoradiography. Equal protein levels were confirmed using Western blot analysis for p22phox (data not shown). Scans of autoradiographs are representative of 4–5 experiments/condition. Arrows indicate the migration of p22phox. Arrowheads show an endogenous membrane protein that was phosphorylated upon addition of PA to reaction mixtures in the presence of PKC isoforms.

**Fig. 7.** Both neutrophil cytosol and conventional PKC isoforms cause complete retardation of p22phox protein migration because of phosphorylation. 6.25 μg of neutrophil membrane was mixed with either 25 μg neutrophil cytosol for 60 min at 25 °C in the absence (−) or presence (+) of 10 μM 10:0 PA (A) or 100 ng of conventional PKC (160 pmol phosphate transferred/min) for 30 min at 30 °C in the presence of either 10 mM EGTA (−) or 20 μg/ml PS, 2 μg/ml 1,2-dioleoyl-rac-glycerol, and 0.6 mM Ca2+ (+) (B). Proteins were separated by 8–15% SDS-PAGE, transferred to nitrocellulose, and subjected to autoradiography. Following autoradiography, p22phox protein was detected using Western blotting. The scans of autoradiographs shown are representative of 13 experiments (A) and three experiments (B).
DISCUSSION

It has been established that protein kinase-mediated reactions are critical for the activation of the neutrophil respiratory burst enzyme, NADPH oxidase, and that several enzyme components are phosphorylated during cell stimulation. Phosphorylation of p47\textsuperscript{phox} can be mediated by PKC, cAMP-dependent protein kinases, mitogen-activated protein kinases, p21-activated kinases, and a potentially novel PA-activated protein kinase (36, 37, 62, 67–69). Recently, it was found that the protein kinase responsible for p47\textsuperscript{phox} phosphorylation is the same protein kinase that phosphorylates p47\textsuperscript{phox}. Indeed, the potentially novel PA-activated protein kinase we have partially purified could substitute for cytosol as the source of the protein kinase phosphorylating p47\textsuperscript{phox} (Fig. 4B). Thus, this as yet unidentified protein kinase can utilize at least two NADPH oxidase components as substrates. Based on previous studies correlating PA levels in neutrophils with NADPH oxidase activity, (19, 28–34), we speculate that the PA-activated protein kinase is responsible for phosphorylation of one or both of these components in intact cells. Studies to identify the PA-activated protein kinase are underway and must be completed before we can explore its function in intact neutrophils.

Interestingly, differences in lipid activator specificity for the phosphorylation of p22\textsuperscript{phox} and p47\textsuperscript{phox} were observed in vitro. Phosphorylation of p22\textsuperscript{phox} was more selective for PA, compared with other phospholipids, than was the phosphorylation of p47\textsuperscript{phox} (Fig. 2A and Ref. 37). The basis for this difference is unclear, but it may be related to physical differences in the two substrates. Studies were performed using p22\textsuperscript{phox} as an integral membrane protein, whereas p47\textsuperscript{phox} is provided as a soluble protein. Perhaps, PA selectively induces membrane association of the protein kinase and, thus, accessibility to p22\textsuperscript{phox}. Alternatively, PA may be specifically needed to induce a required conformational change in p22\textsuperscript{phox} before phosphorylation can occur. PA is known to directly interact with flavocytochrome b\textsubscript{558} in intact cells. Phosphorylation by Lipid-dependent Protein Kinases

We found that only the conventional PKC isoforms could phosphorylate p22\textsuperscript{phox} (Fig. 5A, top panel). This is in contrast to results using p47\textsuperscript{phox} as substrate, in which all classes of PKC isoforms were effective (Fig. 5A, bottom panel). It is unlikely that gross differences in substrate accessibility account for these differences, because all classes of PKC isoforms are known to become membrane-associated in the presence of their activators (reviewed in Refs. 76–78). Indeed, we found that the autophosphorylation of each PKC isoform was stimulated by the presence of membrane fractions (source of p20\textsuperscript{phox}) (data not shown), indicative of an interaction between the PKC and the membrane. The differences between p20\textsuperscript{phox} and p47\textsuperscript{phox} as substrates for PKC may have functional implications. Our results predict that p47\textsuperscript{phox}, but not p20\textsuperscript{phox}, could become phosphorylated by calcium-independent PKC isoforms (PKC \(d\), PKC \(z\)) in the absence of an increase in intracellular Ca\(^{2+}\). Indeed, Dusi and co-workers (79) found that p47\textsuperscript{phox} became phosphorylated in calcium-depleted neutrophils treated with formylmethionyl-leucyl-phenylalanine and concanavalin A. Thus, these results and our data suggest that a calcium-independent mechanism for p47\textsuperscript{phox} phosphorylation and NADPH activation could involve novel or atypical PKC isoforms.

Under both PKC and PA-activated protein kinase conditions, p22\textsuperscript{phox} is phosphorylated on threonine residues and undergoes a retardation in migration by SDS-PAGE. Thus, all of the p22\textsuperscript{phox} becomes phosphorylated. According to sequence simi-
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...sequence are putative phosphorylation motifs (Thr147, PKC; Thr132, casein kinase II) (Wisconsin Package version 9.0, Genetics Computing Group) and are candidate phosphorylation sites. In conclusion, p22phox can be phosphorylated by an unknown PA-activated protein kinase as well as by conventional PKC isoforms. Furthermore, p47phox can be phosphorylated by all of the PKC isoforms known to be expressed in human neutrophils. Thus, there is selectivity of various PKC isoforms for the phosphorylation of NADPH oxidase components. This information will aid in understanding the role of PKC isoforms in the activation of NADPH oxidase in intact neutrophils. Furthermore, we have identified a second substrate, p22phox, for a PA-dependent protein kinase. Because multiple NADPH oxidase components are now known to be substrates for several protein kinases (Refs. 11, 36–38, 62, and 67–72), it is clear that the phosphorylation-dependent activation of this enzyme is complex.

Acknowledgments—We thank Dr. Algirdas Jesaitis for the generous gift of a 1 mg/ml solution of 125I-rhIL-1β during the p22phox immunoprecipitation experiments; Dr. Michael Kleinberg for the gift of purified, relipidated flavocytochrome b558; Dr. Tom Leto for the GST-p22 (127–195) plasmid, the GST-p47 plasmid, and p47phox; Dr. Mike Thomas for synthesizing 1-(5-isoquinolinesulfonyl)piperazine. We also thank Dr. Susan Seger for insightful discussion and Dianne G. Greene for technical assistance with lipid phosphorys assays.

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