Protein Kinases Potentially Capable of Catalyzing the Phosphorylation of p47-phox in Normal Neutrophils and Neutrophils of Patients With Chronic Granulomatous Disease

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A procedure for uncovering novel protein kinases was used to search for enzymes in neutrophils that may catalyze the phosphorylation of the 47-kD subunit of the NADPH oxidase system (p47-phox). This component of the oxidase can undergo phosphorylation on multiple sites. The method is based on the ability of renatured kinases to recognize exogenous substrates fixed in gels. We report that neutrophils contain several uncharacterized protein kinases that catalyze the phosphorylation of a peptide substrate that corresponds to amino acid residues 297 through 331 of p47-phox. Some of these enzymes are strongly activated on stimulation of the cells with phorbol 12-myristate 13-acetate (PMA). The results indicate that the phosphorylation of p47-phox in neutrophils may be more complicated than previously appreciated and may involve multiple protein kinases. In addition, we have examined both the renaturable protein kinases and the properties of protein kinase C (PKC) in neutrophils from patients with chronic granulomatous disease (CGD) who are deficient in cytochrome b558. Previous studies have shown that these cells exhibit incomplete phosphorylation of p47-phox on stimulation. In this study, we were unable to detect any alterations in the renaturable protein kinases or PKC in CGD neutrophils that could explain these defects in the phosphorylation of p47-phox.

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Boston, MA. Purity was assessed by high performance liquid chromatography (HPLC) (Beckman Instruments, Fullerton, CA) and the composition was checked by amino acid analysis (Waters Pico-tag System; Millipore Co, Milford, MA). [γ-32P]ATP (3,000 Ci/ mmol) was purchased from DuPont-New England Nuclear. Boston, MA. Guanidine hydrochloride (99+%), Tween-40 and ATP (potassium salt, from equine muscle) were obtained from Sigma Chemical Co (St Louis, MO). Murine monoclonal antibodies (MoAbs) that recognize the α- and β-isozymes of PKC were purchased from Seikagaku America Inc, St Petersburg, FL. Antimouse IgG alkaline phosphatase conjugate, 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and nitroblue tetrazolium (NBT) were purchased from Promega, Madison, WI. Sources of all other materials are listed elsewhere.20-23

Methods

Preparation of human neutrophils. Human neutrophils (purity >95%) were prepared as described by Badwey et al.22 The cells were suspended (2×10^6 cells/mL) in phosphate buffered saline (PBS) (138 mMol/L NaCl, 2.7 mMol/L KCl, 8.1 mMol/L Na2HPO4, and 1.47 mMol/L KH2PO4, pH 7.35) and treated with 2.5 mMol/L diisopropyl fluorophosphate (DFP) for 10 minutes at 4°C. The DFP-treated cells were collected by centrifugation at 300g for 6 minutes (4°C) and resuspended in PBS.

Preparation of solubilized neutrophils for assaying the renaturable protein kinases and SDS-PAGE. Neutrophils (8×10^6/mL) were suspended in prewarmed disposable 3-mL polypropylene test tubes at 37°C. The standard reaction mixture consisted of the PBS medium noted above supplemented with 0.90 mMol/L CaCl2, 0.50 mMol/L MgCl2, and 7.5 mMol/L D-glucose. Cells were incubated in this reaction medium for 3 minutes at 37°C before stimulation by the addition of 320 mMol/L PMA. At the appropriate time, the entire reaction mixture (1.0 mL) was transferred to a microfuge tube containing 0.25 mL of 5% concentrated solubilization buffer and rapidly mixed. The final composition of this buffer after mixing was 2.3% (wt/vol) Triton X-100, 10.0% (vol/vol) glycerol, 5.0% (vol/vol) 2-mercaptoethanol, and 0.002% (wt/vol) bromophenol blue. After immersion in a boiling water bath for 4 min, the samples were subjected to SDS-PAGE in 9% (wt/vol) polyacrylamide slab gels (0.075×12×14 cm) containing a “fixed” peptide that corresponds to residues 297 through 302 of the p47-peptide. The peptide substrate (0.50 mg/mL) was dissolved in 9.0% (vol/vol) polyacrylamide gel solution and rapidly mixed. The final composition of this buffer after mixing was 2.3% (wt/vol) SDS, 62.5 mMol/L Tris (pH 6.8), 5.0 mMol/L EDTA, 10.0% (vol/vol) glycerol, 5.0% (vol/vol) 2-mercaptoethanol, and 0.002% (wt/vol) bromophenol blue. After immersion in a boiling water bath for 4 minutes, the samples were subjected to SDS-PAGE in 9% (wt/vol) polyacrylamide slab gels (0.075×12×14 cm) containing a “fixed” peptide that corresponds to residues 297 through 331 of p47 phox (see below). The buffer system of Laemmli was used.22 Marker proteins were run on each gel in the first and last lanes.

Detection of renaturable protein kinases in polyacrylamide gels. In this procedure, protein kinases were detected directly in gels containing the p47-peptide. The peptide substrate (0.50 mg/mL) was dissolved in 9.0% (vol/vol) polyacrylamide gel solution just before polymerization and served as the phosphate acceptor for the kinases. A small amount of the peptide did not dissolve under these conditions and was removed by centrifugation. Previous studies have reported that exogenous proteins can remain fixed in the gel throughout electrophoresis and do not affect the mobilities of the proteins in the samples.23 A uniform distribution of the p47-peptide in the gel after PAGE was confirmed by staining with Coomassie blue. Methods for preparing extracts of neutrophils and for performing SDS-PAGE are described above. The methods for protein renaturation and detection of kinase activity described by Kamataniya and Fujisawa 20 and as modified by Gotoh et al.15 were used. Briefly, SDS was removed from the gel after electrophoresis by washing with 20% (vol/vol) 2-propanol in 50 mMol/L Tris (pH 8.0). Proteins in the gel were then treated with 6.0 mol/L guanidine - HCl and renatured with 50 mMol/L Tris (pH 8.0) containing 50 mMol/L 2-mercaptoethanol and 0.04% (vol/vol) Tween-40. Protein kinases were detected by incubating each gel (14×12×0.075 cm) at room temperature for 60 minutes in a 25-mL solution containing 10 mMol/L HEPES (pH 8.0), 2.0 mMol/L diethiolethiol, 0.10 mMol/L EGTA, 5.0 mMol/L MgCl2, and 25 mMol/L/L [γ-32P]ATP (1 μCi/mL). Finally, the gels were washed extensively with 5.0% (vol/vol) trichloroacetic acid containing 1.0% (wt/vol) sodium phosphate and dried.

Autoradiography. The dried gels were exposed to XRP-5 Kodak film (Eastman Kodak Co, Rochester, NY) using a Cronex intensifying screen (DuPont Inc, Wilmington, DE) at −70°C for 20 to 70 hours.

Preparation of cellular fractions. Neutrophils were suspended at a concentration of 3×10^7/mL or 1.8×10^7/mL in freshly prepared, ice-cold extraction buffer (20 mMol/L Tris [pH 7.5], 2.0 mMol/L EGTA, 2.0 mMol/L EDTA, 1.0 mMol/L phenylmethylsulfonyl fluoride [PMSF], leupeptin [50 μg/mL] and 50 mMol/L L-mercaptoethanol)20 and disrupted by sonication on ice four times for periods of 15 seconds each. These conditions of disrupting the cells in a “Ca2+-depleted buffer” have been shown to maximize the recovery of the nonmembrane-inserted form of PKC in the soluble fraction.26 The sonicate was centrifuged at 100,000g (4°C) for 1 hour. The resulting supernatant was removed and stored on ice while the pellet was resuspended to the original volume in extraction buffer. Both fractions were mixed with an equal volume of glycerol and stored on dry ice or at −70°C for not more than 4 days.

Stimulation of neutrophils for measuring translocation of PKC. Neutrophils (2×10^6/mL) were incubated for 3 minutes at 37°C in PBS supplemented with 0.50 mMol/L MgCl2, 0.90 mMol/L CaCl2, and 7.5 mMol/L D-glucose. Cells were treated with 320 mMol/L PMA for 2 minutes followed by immersion of the reaction mixture in ice water for 2 minutes. Neutrophils were collected by centrifugation (300g for 6 minutes at 4°C) and treated with DFP (see above). Subcellular fractions were prepared as described in the previous section.

PKC assay. PKC was assayed by measuring the incorporation of 32P into histone type III-S at 30°C26 with the modifications described by Badwey et al.20 Activity specific for PKC is defined as the difference between the activities observed with cofactors (Ca2+, phosphatidylserine, and diglyceride) and without. Activity measured at 7 minutes was directly proportional to the quantity of the cell fractions present in the assay between 0.75×10^6 to 2.25×10^6 cell-equivalents. The reaction was linear with respect to time between 2 and 10 minutes at 1.5×10^6 cell-equivs/assay.

Immunoblotting. Aliquots of the cell fractions (1.8×10^6 cell-equivalents) were mixed with an equal volume of 2X concentrated “loading buffer.” The final composition of this buffer after mixing with the cell-fractions was 50 mMol/L Tris (pH 6.8), 2.0% (vol/vol) SDS, 4.0% (vol/vol) glycerol, 1.0% (vol/vol) β-mercaptoethanol, and 0.002% (wt/vol) bromophenol blue. After immersion in a boiling water bath for 4 minutes, the samples were subjected to SDS-PAGE in 10% (wt/vol) polyacrylamide slab gels (0.075×16×18 cm). The buffer system of Laemmli was used.22 The proteins were immediately transferred electrophoretically to Immobilon-P membranes (Millipore) using 192 mMol/L glycine and 25 mMol/L/L Tris as the transfer buffer. Transfer was at 50 V for 90 minutes. The membranes were blocked with 5.0% gelatin in 10 mMol/L HEPES (pH 7.4) plus 500 mMol/L NaCl. The blocking buffer was removed and the membranes were incubated with the primary antibody against PKC (0.1 or 1.0 μg/mL) overnight at room temperature in 10 mMol/L HEPES (pH 7.4), 500 mMol/L NaCl, 0.2% Tween 20 and 1.0% (wt/vol) gelatin. The membranes were subsequently washed five times with washing buffer (10 mMol/L HEPES [pH 7.4], 500 mMol/L NaCl, 0.2% Tween 20) and then incubated with the secondary antibody (antimouse IgG alkaline phosphatase conjuga-
gate) for 1 hour at room temperature with the same buffer system used with the primary antibody. Membranes were then washed four times in washing buffer and once in washing buffer without Tween-20. Alkaline phosphatase was visualized with a kit from Promega that used BCIP and nitroblue tetrazolium (eg, ref 27).

Analytical data. Unless otherwise noted, all of the autoradiographic observations were confirmed in at least three separate experiments performed on different preparations of cells.

RESULTS

Renaturable protein kinases capable of catalyzing the phosphorylation of the p47-phox peptide. Lysates of neutrophils were separated by SDS-PAGE and renatured in situ as described in Materials and Methods. Protein kinases were detected by autoradiography after exposure of the gels to \([\gamma^3P]ATP\). Fig 1 presents results from a typical experiment in which the gels contained either no added substrate (A) or the p47-peptide (B). Gels containing proteins from unstimulated or stimulated neutrophils but lacking the peptide exhibited three diffuse bands of radioactivity with molecular masses of approximately 96, 84, and 62 Kd (A). These bands are likely to represent protein kinases that undergo autophosphorylation and/or catalyze the phosphorylation of endogenous proteins (cf ref 28) (see below).

Gels containing the p47-peptide and proteins from unstimulated neutrophils also displayed the 96-, 84- and 62-Kd bands with the latter being more intensely labeled. In addition, two heavily labeled bands with molecular masses of 56 and 44 kDa, along with several minor bands (eg, 120 Kd), were also observed (Fig 1B, lane a). Stimulation of the cells with PMA for 1 minute resulted in a marked increase in the content of \(^{32}P\) in the 96-Kd band and the appearance of two or three new minor bands in the 74- to 66-Kd range (Fig 1B, lane b). Neutrophils stimulated with this agonist for 10 minutes also exhibited an intense band at 105 Kd and a diminution of the 120-Kd band (Fig 1B, lane c). The bands in the 74- to 66-Kd range that appeared in the PMA-stimulated neutrophils were also more heavily labeled after 1 minute compared with 10 minutes (Fig 1B, compare lanes b and c). The positions of the 105- and 96-Kd bands are designated by solid arrows. Additional examples of the increased labeling of the 96- and 105-Kd bands after stimulation of the cells with PMA can be seen (see Figs 2 and 4). The finding that certain bands were only observed (or markedly increased in intensity) in the presence of the p47-phox peptide suggests that these bands correspond to the locations of protein kinases in the gel that can use the immobilized peptide as a substrate. We cannot completely rule out the possibility that the presence of the peptide may enhance the renaturation and/or autophosphorylation of certain kinases. However, the bands that exhibited enhanced labeling with the peptide were not affected when albumin or gelatin (0.50 mg/mL) were incorporated into the gel (data not shown).

Labeling of the various bands by \([\gamma^3P]ATP\) could be

![Fig 1. Renaturable protein kinases in neutrophils. Lysates of neutrophils were separated by SDS-PAGE on gels that contained either no exogenous substrate (A) or the p47-phox peptide (B). Phosphorylation of the peptide in the gel catalyzed by renatured kinases using \([\gamma^3P]ATP\) was assayed as described in Materials and Methods. The autoradiograms shown are from cells treated with: (a) 0.25% (vol/vol) Me2SO for 1 minute (unstimulated cells); (b) 320 nmol/L PMA for 1 minute; and (c) 320 nmol/L PMA for 10 minutes. The pattern of renaturable protein kinases from unstimulated cells (eg, lane a) did not change between 1 and 10 minutes. Molecular mass markers are provided on the left. Molecular masses of the labeled bands are designated on the right according to their apparent molecular weights in kilodaltons. The arrows in panel B indicate the positions of the 105- and 96-Kd kinases.](attachment:image)
caused by one or more of the following: (1) the phosphorylation of the p47-peptide or endogenous proteins catalyzed by protein kinases; (2) autophosphorylation of protein kinases; and (3) simple binding of [γ-32P]ATP to proteins (cf ref 28). The third possibility was investigated and eliminated by a pulse-chase experiment in which the renatured proteins were first treated with 25 μmol/L [γ-32P]ATP (1 μCi/mL) for 60 minutes and then washed with 0.50 mmol/L nonradioactive ATP for 60 minutes. This treatment did not alter the labeling of the samples from unstimulated or stimulated neutrophils (Fig 2A and B). These data indicate that the incorporation of 32P into the various bands was caused by the action of protein kinases: either by their ability to catalyze phosphorylation of the p47 peptide or endogenous proteins, or by their ability to undergo autophosphorylation. Exposure of the labeled proteins to 1.0 mol/L KOH for 60 minutes resulted in a massive loss of radioactivity from all regions of the gel (Fig 2C). This indicates that the vast majority of the phosphorylation reactions catalyzed by the renaturable kinases occurred on serine and threonine residues.39

The location of PKC in the gels was determined by Western blotting (Fig 3). A prominent immunoreactive band was observed using an MoAb to α-PKC (lane b) that corresponded very closely to the 84-Kd band (lane a) labeled with 32P on exposure of the gel to [γ-32P]ATP. Additional studies on PKC are described below.

Figure 4 compares the renaturable protein kinases in normal neutrophils (A) and neutrophils from a patient with CGD caused by a mutation in the 91-Kd subunit of cytochrome b$_{558}$ (panel B). Most patients with mutations in the gene loci for either the 22- or 91-Kd subunits of cytochrome b$_{558}$ are missing both subunits of the cytochrome.35 All of the patients with mutations in cytochrome b$_{558}$ used in this study were of this type. No consistent differences were observed between the normal samples and those from three CGD patients with mutations in the 91-Kd subunit. In particular, neutrophils deficient in cytochrome b$_{558}$ also exhibited activation of the 96- and 105-Kd kinases on stimulation with PMA (Fig 4B, lane b).
Fig 4. Renaturable protein kinases capable of catalyzing the phosphorylation of the p47-phox peptide in CGD neutrophils deficient in cytochrome b$_{558}$. Autoradiograms compare the renaturable protein kinases in normal neutrophils (A) and in CGD neutrophils devoid of cytochrome b$_{558}$ caused by a mutation in the 91-Kd subunit of the cytochrome (B). Neutrophils were treated for 10 minutes with either (a) 0.25% (vol/vol) Me$_2$SO (unstimulated cells) or (b) 320 nmol/L PMA before assay. The 105- and 96-Kd kinases are designated by the solid arrows.

PKC in CGD. Previous studies have suggested that the incomplete phosphorylation of p47-phox in CGD neutrophils deficient in cytochrome b$_{558}$ may be caused, in part, by alterations in PKC. Therefore, we examined the level of activity of this enzyme in neutrophils from cytochrome-deficient patients as well as its partial isozyme pattern and ability to undergo translocation. The histone III-S kinase activity specific for PKC in the soluble fraction of unstimulated normal human neutrophils was 750 ± 58 pmol P/min/10$^7$ cell-equivalents (SD, n = 16). A kinase activity that phosphorylates histone III-S is also present in the particulate fraction of these cells (107 ± 16 pmol P/min/10$^7$ cell-equivalents [SD, n = 15]) and is thought to represent, at least in part, the “membrane-inserted” form of PKC (cf refs 31-33). Figure 5 summarizes the soluble activities specific for PKC in normal neutrophils (A) and neutrophils from patients with CGD caused by mutations in the 91-Kd subunit of cytochrome b$_{558}$ (B) or p47-phox (D). Data on single individuals with mutations in the 22-Kd subunit of cytochrome b$_{558}$ (C) or p67-phox (E) are also provided. While activity for PKC was present in all samples, a wide range of values was observed in neutrophils from patients with mutations in the 91-Kd component of cytochrome b$_{558}$. It is unlikely that a partial deficiency in PKC was responsible for the incomplete phosphorylation of p47-phox in these patients because the defects in phosphorylation were observed in all patients missing cytochrome b$_{558}$, even those who exhibited normal levels of activity for PKC (≥700 pmol P/min/10$^7$ cells). Neutrophils that contained low levels of activity for soluble PKC contained normal amounts of activity for the particulate kinase (eg, patients 3 and 4 in Table 1).

Soluble fractions from normal neutrophils and neutrophils deficient in cytochrome b$_{558}$ were immunoblotted with a low concentration of antisera (0.10 μg/mL) specific for β-PKC or α-PKC (Fig 6). All samples displayed a major immunoreactive species (approximately 84 Kd) of similar

Table 1. Alterations in the Activity of PKC in Neutrophils After Stimulation With PMA: Studies on Normal Neutrophils and Neutrophils Deficient in Cytochrome b$_{558}$

<table>
<thead>
<tr>
<th>PKC Activity*</th>
<th>Unstimulated Cells</th>
<th>Stimulated Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Soluble Fraction</td>
<td>Particulate Fraction</td>
</tr>
<tr>
<td>Normal individuals</td>
<td>(pmol P/min/10$^7$ cell-equivalents ± SD)</td>
<td></td>
</tr>
<tr>
<td>Patient 11</td>
<td>748 ± 80</td>
<td>106 ± 7</td>
</tr>
<tr>
<td>Patient 2</td>
<td>856</td>
<td>100</td>
</tr>
<tr>
<td>Patient 3</td>
<td>725</td>
<td>119</td>
</tr>
<tr>
<td>Patient 4</td>
<td>245</td>
<td>115</td>
</tr>
<tr>
<td>Patient 5</td>
<td>389</td>
<td>113</td>
</tr>
<tr>
<td>Patient 6</td>
<td>631</td>
<td>148</td>
</tr>
</tbody>
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* The activity in the soluble fraction is that specific for PKC as defined in Materials and Methods. Activity in the particulate fraction represents the total activity that phosphorylates histone III-S. The percentage of the particulate activity in unstimulated cells that corresponds to PKC is not known.
† The primary defect in patients 1 through 4 was a mutation in the gene encoding the 91-Kd subunit of cytochrome b$_{558}$. Patient 5 had a mutation in the gene for the 22-Kd subunit of cytochrome b$_{558}$. All patients were missing both subunits of cytochrome b$_{558}$ in their neutrophils.
Protein kinases that phosphorylate p47-phox

Fig 6. Selected PKC isotypes in normal neutrophils and in neutrophils deficient in cytochrome b\textsubscript{558}. The soluble fractions from unstimulated neutrophils were immunoblotted with a low amount of MoAb (0.1 \(\mu\)g/mL) specific for the \(\beta\) (A) and \(\alpha\) (B) isozymes of PKC. The samples shown were from normal neutrophils (lane a), neutrophils with a mutation in the 91-Kd subunit of cytochrome b\textsubscript{558} (lanes b and c), and neutrophils with a mutation in the 22-Kd subunit of cytochrome b\textsubscript{558} (lane d).

Intensity with antisera to P-PKC (Fig 6A). Very little, if any, staining was observed with antisera specific for \(\alpha\)-PKC under these conditions (Fig 6B). However, increasing the concentration of the antisera for \(\alpha\)-PKC 10-fold (ie, to 1.0 \(\mu\)g/mL) showed a prominent immunoreactive species with a molecular mass of approximately 84 Kd along with several other bands of less intensity (Fig 7). The staining intensity of this 84-Kd band was similar in normal cells and cells deficient in cytochrome b\textsubscript{558} (Fig 7; compare lanes a and b). Previous studies have shown that both the \(\alpha\)- and \(\beta\)-isozymes of PKC are present in human neutrophils with the \(\beta\)-isozyme predominating.\textsuperscript{8,34} Additional evidence that this 84 kDa band was, in fact, \(\alpha\)-PKC was provided by the observation that it was markedly reduced in cells treated with PMA (Fig 7, lane c; see below). Results presented in Figs 6 and 7 are representative of data obtained from five patients deficient in cytochrome b\textsubscript{558}.

Neutrophils stimulated with PMA are known to exhibit a dramatic diminution in the soluble activity of PKC, a small but significant part of which is recovered in the particulate fraction (ie, translocation) (eg, refs 33, 35, 36). Table I compares this phenomenon in normal neutrophils and neutrophils deficient in cytochrome b\textsubscript{558}. Cells from all five patients exhibited a marked loss in the activity of soluble PKC on treatment with PMA, and an increase in the activity of the particulate kinase was observed in 4 of the 5 cases. The loss in total kinase activity is likely caused by a proteolytic degradation of this enzyme during cell stimulation.\textsuperscript{34,35}

Treatment of neutrophils with PMA also resulted in the loss of the 84-Kd band in the soluble fraction that reacted with antisera to \(\beta\)-PKC (Fig 8) and \(\alpha\)-PKC (eg; Fig 7, lane c). Neutrophils stimulated with PMA did not exhibit a significant increase in the particulate form of PKC on immunoblotting with either antisera (data not shown).

Discussion

We have used a peptide substrate of 35 amino acids to search for novel protein kinases in neutrophils that may catalyze the phosphorylation of p47-phox. Previous studies have shown that PKC and cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA) can catalyze the phosphorylation of this protein.\textsuperscript{7,9} We now report that neutrophils contain several uncharacterized protein kinases that use the p47-peptide as a substrate (Fig 1). Stimulation of the cells with PMA results in a marked increase in the activity of two major kinases with molecular masses of approximately 96 and 105 Kd (Fig 1B, lanes b and c). It is likely that these enzymes undergo covalent modifications (eg, phosphorylation) during cell-stimulation that increase their catalytic activity because the enhanced activity persists after SDS-PAGE and treatment with guanidine (eg, ref 28). These kinases exhibited molecular masses that are clearly different from that of PKC (84 Kd, Fig 3) and PKA (38-Kd catalytic subunit) (eg, ref 28) and therefore may represent novel enzymes that can use p47-phox as a substrate. Several minor renaturable kinases with molecular masses between 74 and 66 Kd were also stimulated in neutrophils treated with PMA.
with PMA (Fig 1B, lane b). Still other renaturable protein kinases were observed that catalyze phosphorylation of the p47-phox peptide but do not change their levels of activity in response to PMA. The renaturation assay we used cannot detect changes in the activity of protein kinases that are the result of conformational changes (eg, PKC) or alterations in quaternary structure (eg, PKA) because these modifications would be lost during SDS-PAGE. Thus, some of the renaturable kinases that appear active in unstimulated cells may actually be inactive in vivo and regulated by these types of physical alterations.

The exact sites phosphorylated on p47-phox are not known. Moreover, the assay used here is limited in that it only detects those kinases that can undergo renaturation and are active with the peptide. Nevertheless, our data indicate that the phosphorylation of p47-phox in neutrophils may be more complicated than previously appreciated and could involve a number of uncharacterized protein kinases. While we cannot prove that any of these enzymes actually catalyze the phosphorylation of p47-phox in vivo, it is noteworthy that approximately 80% of the total PKC activity undergoes inactivation within 2 minutes of treatment of the cells with PMA (Table 1). Neutrophils exhibit an intense phosphorylation of p47-phox for at least 10 minutes after stimulation with PMA and p47-phox is known to undergo a continual cycle of phosphorylation and dephosphorylation in these cells (eg, refs 19 and 37). This finding indicates that either a small quantity of PKC can maintain the phosphorylation of p47-phox, or that additional protein kinases may also catalyze these events. It is unlikely that PKA is responsible for the phosphorylation of p47-phox in neutrophils stimulated with PMA because the cellular levels of cAMP do not increase in cells treated with this stimulus.

As noted above, the most heavily phosphorylated isoforms of p47-phox are not observed in cytochrome b556-deficient neutrophils stimulated with PMA. In this study, we were unable to detect any alterations in the renaturable protein kinases (Fig 4) or PKC (Figs 5-8) that could explain these defects. Previous studies have also reported that cellular fractions from neutrophils deficient in p47-phox contain normal levels of PKC activity and are capable of phosphorylating recombinant p47-phox protein. Therefore, the data support a scheme of hierarchic phosphorylation of p47-phox in which the final phosphorylation reactions occur after this protein binds to the plasmalemma at a site created by, or dependent on, cytochrome b556 (cf refs 1, 14, and 15). Consistent with this scheme, the most heavily phosphorylated isoforms of p47-phox are still observed in an unusual and rare form of CGD in which the neutrophils contain normal amounts of a mutant cytochrome b556 rendered nonfunctional by a proline → histidine mutation at residue 415 in its 91-Kd subunit. Whether binding of p47-phox to the membrane induces a conformational change in this protein that exposes additional phosphorylation sites and/or simply brings this protein into close apposition with the relevant kinase(s) is not known.

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Protein kinases potentially capable of catalyzing the phosphorylation of p47-phox in normal neutrophils and neutrophils of patients with chronic granulomatous disease

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