Phosphorylation of p47^{phox} Sites by PKC α, βII, δ, and ζ: Effect on Binding to p22^{phox} and on NADPH Oxidase Activation

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ABSTRACT: Production of superoxide anions by the multicomponent enzyme of human neutrophil NADPH oxidase is accompanied by extensive phosphorylation of p47^{phox}, one of its cytosolic components. p47^{phox} is an excellent substrate for protein kinase C (PKC), but the respective contribution of each PKC isoform to this process is not clearly defined. In this study, we found that PKC isoforms known to be present in human neutrophils (PKC α, β, δ, and ζ) phosphorylate p47^{phox} in a time- and concentration-dependent manner, with apparent K_m values of 10.33, 3.37, 2.37, and 2.13 μM for PKC α, βII, δ, and ζ, respectively. Phosphopeptide mapping of p47^{phox} showed that, as opposed to PKC ζ, PKC α, βII, and δ are able to phosphorylate all the major PKC sites. The use of p47^{phox} mutants identified serines 303, 304, 315, 320, 328, 359, 370, and 379 as targets of PKC α, βII, and δ. Comparison of the intensity of phosphopeptides suggests that Ser 328 is the most phosphorylated serine. The ability of each PKC isoform to induce p47^{phox} to associate with p22^{phox} was tested by using an overlay technique; the results showed that all the PKC isoforms that were studied induce p47^{phox} binding to the cytosolic fragment of p22^{phox}. In addition, PKC α, βII, δ, and ζ were able to induce production of superoxide anions in a cell-free system using recombinant cytosolic proteins. Surprisingly, PKC ζ, which phosphorylates a subset of selective p47^{phox} sites, induced stronger activation of the NADPH oxidase. Taken together, these results suggest that PKC α, βII, δ, and ζ expressed in human neutrophils can individually phosphorylate p47^{phox} and induce both its translocation and NADPH oxidase activation. In addition, phosphorylation of some serines could have an inhibitory effect on oxidase activation.

Phagocytes such as polymorphonuclear leukocytes (PMN)[1] play an important role in the first line of host defense against invading microorganisms. The release of superoxide anions ($O_2^{\bullet -}$) is one of the main mechanisms used by PMN to kill bacteria, viruses, and fungi. This phenomenon is known as the respiratory burst and is due to activation of an enzyme called NADPH oxidase (*1*). This enzyme is a multicomponent system which is inactive in resting cells and the components of which are distributed between the cytosol and membranes. The major membrane component, cytochrome b_{558}, is composed of two subunits (gp91^{phox} and p22^{phox}); the cytosolic components are four major proteins (p47^{phox}, p67^{phox}, p40^{phox}, and a small G protein Rac2) (*2*−*5*). When cells are activated by stimuli such as *N*-formyl peptide (fMLP) and phorbol myristate acetate (PMA), some of the cytosolic components become phosphorylated and migrate to the membranes, where they assemble as an active complex (*5*−*7*). One of the phosphorylated cytosolic proteins is p47^{phox}, which is phosphorylated on several serine residues by several kinases (*6*−*8*). Phosphorylation of this protein is crucial for translocation of the cytosolic components and assembly of the active NADPH oxidase (*9*−*11*). Treatment of neutrophils with fMLP, opsonized zymosan, or PMA (a direct PKC activator) induces $O_2^{\bullet -}$ production, the degree of which correlates with PKC activation and p47^{phox} phosphorylation (*6*, *12*, *13*). These data support a major role of PKC in NADPH oxidase activation.

Neutrophils contain five of the eleven known isoforms of PKC (*14*−*17*). These comprise three conventional isoforms designated α, βI, and βII, which are dependent on phosphatidylserine (PS), diglyceride (DG), and calcium; one novel isoform designated PKC δ, which also requires phosphatidylserine and diglyceride but is calcium-independent; and one atypical isoform designated PKC ζ, which is diglyceride- and calcium-independent but can be activated by phosphatidylserine, phosphatidic acid, or phosphatidylinositol (*3*−*5*). These PKC isoforms are activated in human neutrophils stimulated with PMA, fMLP, or opsonized zymosan (*12*−*14*). Recent reports show that PKC β plays a major role in oxidase activation (*18*, *19*). We have recently reported that PKC ζ phosphorylates selective sites of p47^{phox} and could participate in the fMLP-induced respiratory burst (*20*). In addition, p47^{phox} is phosphorylated in vitro by a mixture of PKC isoforms, but the specific role of each neutrophil PKC

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[1] Abbreviations: PMN, polymorphonuclear leukocytes; $O_2^{\bullet -}$, superoxide anion; fMLP, *N*-formylmethionylleucylphenylalanine; PMA, phorbol 12-myristate 13-acetate; PS, phosphatidylserine; DG, diglyceride; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); PMSF, phenylmethylsulfonyl fluoride; DFP, diisopropyl fluorophosphate; GST, glutathione *S*-transferase; Wt, wild-type; PAGE, polyacrylamide gel electrophoresis; SOD, superoxide dismutase; SD, standard deviation.

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isoform in p47^{phox} phosphorylation and NADPH oxidase activation is not clearly defined.

In this study, we examined, in vitro, the phosphorylation of p47^{phox} by recombinant PKC isoforms known to be expressed in human neutrophils, namely, PKC α, β, δ, and ζ, and the consequences of this phosphorylation on p47^{phox} binding to p22^{phox} and NADPH oxidase activation. The results suggest that all the studied isoforms can participate in signaling pathways that phosphorylate p47^{phox}, and may trigger $O_2^{\bullet -}$ production.

MATERIALS AND METHODS

Materials. Leupeptin, pepstatin A, aprotinin, phosphatidylserine, diacylglycerol, lysozyme L6876, tryspsin, ATP, NADPH, superoxide dismutase, calyculin A, guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS), FAD, lucigenin, sucrose, phenylmethylsulfonyl fluoride (PMSF), and thrombin were from Sigma. Dextran, Ficoll-Hypaque, PreScission protease, glutathione, and glutathione-Sepharose 4B beads were from Amersham Pharmacia. GF-109203X (GFX), diisopropyl fluorophosphate (DFP), and SDS−PAGE reagents were from Bio-Rad. Human recombinant protein kinase C βII and δ were from Calbiochem. Human recombinant protein kinase C α and ζ were from Biomol. Sequencing grade trypsin was from Boehringer Mannheim. [γ-^{32}P]ATP was from NEN. Pfu polymerase was from Stratagene. PGEX-1λT-p47^{phox}, pGEX-6P3-p67^{phox}, and anti-p47^{phox} antibody were a gift from B. Babior (Scripps Research Institute, La Jolla, CA). pGEX-2T-Rac1 was a gift from A. Hall (MRC Laboratory for Molecular Cell Biology and Department of Biochemistry, University College London, London, England), and p22^{phox} cDNA cloned into the pBluescript vector was a gift from M. Dinauer (Herman B. Wells Center for Pediatric Research, Indianapolis, IN).

Neutrophil Preparation and Fractionation. Neutrophils were obtained from healthy volunteers by dextran sedimentation and Ficoll-Hypaque fractionation of freshly drawn citrate-coagulated blood. Neutrophils were treated with DFP and resuspended at a density of 10^8 cells/mL in relaxation buffer [10 mM Pipes buffer (pH 7.3), 100 mM KCl, 3 mM NaCl, and 3.5 mM $MgCl_2$]. Plasma membranes and cytosol were obtained by nitrogen cavitation and centrifugation on a Percoll gradient as described by Borregaard et al. (*21*). Cytosol and membranes were aliquoted and stored at −70 °C until they were used.

Construction of the C-Terminal Cytoplasmic Region of p22^{phox}. The DNA fragment encoding the C-terminal cytoplasmic region of p22^{phox}, p22^{phox}-(132−195), was obtained by PCR with Pfu polymerase and wild-type (Wt) p22^{phox} cDNA cloned into the Bluescript vector as a template. The fragment was amplified with a 5′ primer (GGAATTCACGCCATCGAGCCCAAG) that contained an *Eco*RI site (underlined) and a sequence that annealed to nucleotides 393−410 and a 3′ antisense primer (CCGCTCGAGTCACACGACCTCGTC) containing an *Xho*I site (underlined), an antisense sequence that annealed to nucleotides 573−587, and the antisense stop codon. The PCR product was ligated into the *Eco*RI and *Xho*I sites of the pGEX-6P1 vector and transformed into the DH5α strain for protein expression. The sequence of the construct was confirmed by DNA sequencing.

Preparation of Recombinant Proteins. Recombinant fusion proteins {glutathione *S*-transferase (GST) fused to wild-type p47^{phox} or p47^{phox} mutants [S(303+304)A, S315A, S320A, S(315+320)A, S328A, S(359+370)A, S379A, S(328−379)A], GST−p67^{phox}, GST−Rac1, and GST−p22^{phox} (132−195)} were expressed in *Escherichia coli* and purified in a single step by affinity chromatography on glutathione-Sepharose 4B beads as previously described (*22*). Where indicated, fusion proteins were cleaved with thrombin or PreScission protease. Proteins were concentrated by Centricon centrifugation and then assessed with the Bio-Rad assay kit and stored at −70 °C until they were used.

In Vitro Phosphorylation of p47^{phox}. p47^{phox} is phosphorylated as a fusion protein or as a cleaved protein at various concentrations (0−2.5 μM). In a calcium-dependent PKC assay, α and βII were incubated in a reaction mixture containing kinase buffer (20 mM Hepes, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 0.5 mM DTT). In a calcium-independent PKC assay, δ and ζ were incubated in a reaction mixture containing calcium-free kinase buffer (20 mM Hepes, 10 mM $MgCl_2$, 0.5 mM EGTA, and 0.5 mM DTT), with or without 1 μg/mL diacylglyceride (DG), and 18.6 μg/mL phosphatidylserine (PS), 50 μM (1 μCi) [γ-^{32}P]ATP, and PKC (80 and 540 ng). For kinetic studies, the PKC specific activity was standardized at 0.1794 nmol of P_i/min (determined using peptide ε as a substrate), and the reaction was run in a volume of 100 μL at 30 °C for times ranging from 0 to 40 min. For K_m studies, the incubation time was 15 min. The phosphorylation reactions were stopped with an equal volume of 2× Laemmli sample buffer. The reaction mixes were then boiled for 10 min and subjected to SDS−PAGE using a 10% running gel for p47^{phox} and its mutants and a 13% running gel for the histone control. For kinetic and affinity studies, gels were stained with Coomassie blue and autoradiographed; p47^{phox} radioactivity was quantified in a Beckman LS 6000sc scintillation counter.

Two-Dimensional Phosphopeptide Mapping of p47^{phox}. Phosphorylated p47^{phox} and its mutants were subjected to 10% SDS−PAGE and transferred to nitrocellulose. The band of interest was isolated and trypsinized for 18 h at 37 °C. The reaction was stopped, and the sample was dried and washed three times in water before being redissolved in pH 1.9 electrophoresis buffer [1/5.6 (v/v) formic acid/water mixture]. The sample was applied to one corner of a thin-layer cellulose plate and run at 1100 V and 4 °C for 30 min. The plate was then submitted to isobutyric acid buffer chromatography [25/1/2.5/1.8/10 (v/v) isobutyric acid/butanol/pyridine/acetic acid/water mixture]. Radioactivity was detected by autoradiography, and phosphopeptides were quantified with an Instantimager.

In Vitro Binding of Phosphorylated p47^{phox} to the p22^{phox}(132−195) Tail in an Overlay Assay. Samples of the recombinant GST−p22^{phox} tail (1 μg) first migrated on 10% SDS−PAGE, were transferred to nitrocellulose, and were blocked for 1 h at room temperature in Tris buffer [150 mM NaCl and 25 mM Tris-HCl (pH 7.4)] containing 5% nonfat dried milk. Separately, p47^{phox} (10 μg/mL) was phosphorylated as described above in a reaction volume of 100 μL for 30 min at 30 °C. The PKC specific activity was standardized to have the same amount of radioactivity incorporated in p47^{phox} phosphorylated by PKC α, βII, or δ. The reaction was stopped by adding 4 μM staurosporine, and the reaction

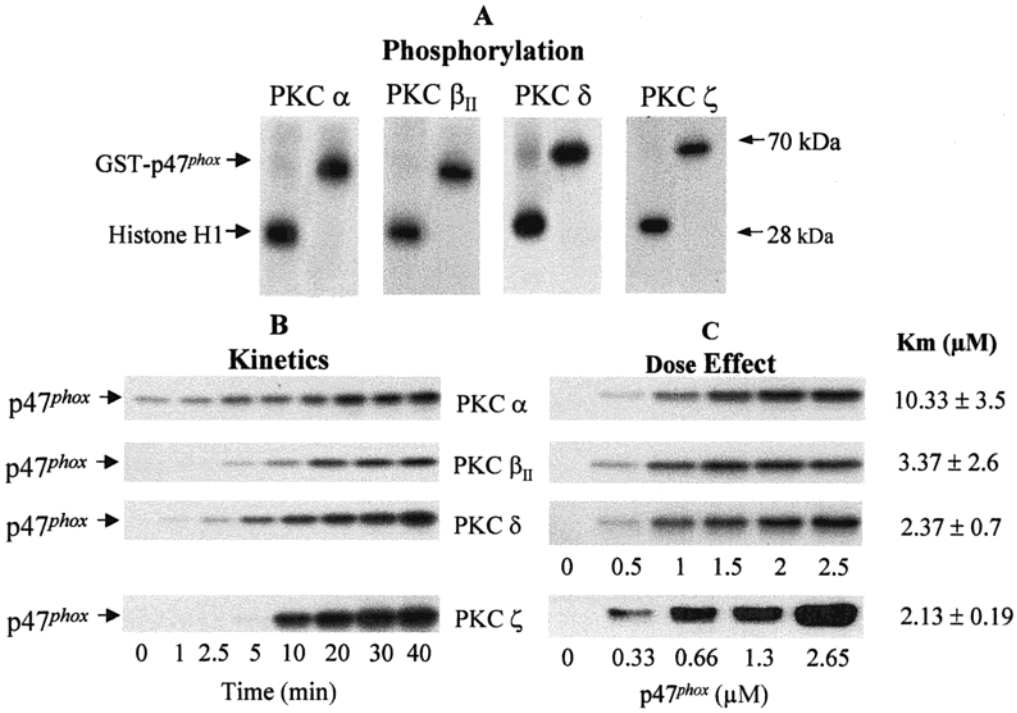

FIGURE 1: In vitro phosphorylation of p47^{phox} by PKC α, βII, δ, and ζ. The GST−p47^{phox} fusion protein and histone H1 were incubated with the different PKC isoforms for 30 min at 30 °C. Reactions were stopped by adding Laemmli sample buffer and boiling, before electrophoretic migration and autoradiography were carried out as described in Materials and Methods (A). Kinetic studies of p47^{phox} phosphorylation were performed with the PKC specific activity standardized at 0.1794 nmol of P_i/min (B). The P47^{phox} concentration dependence of phosphorylation by the different isoforms was studied for 15 min (C). Apparent K_m values were calculated for each PKC isoform with a Lineweaver−Burk representation (C, right). Kinetic and concentration dependence data are representative of three and four experiments, respectively. K_m values are expressed as means ± SD of four different experiments.

mix was diluted 20-fold in binding buffer [500 mM NaCl, 3% BSA, 0.1% Tween 20, and 20 mM Tris-HCl (pH 7.4)]. p22^{phox} membranes were incubated with phosphorylated p47^{phox} for 1 h at room temperature. To stop the binding, membranes were washed three times with binding buffer. Binding of phosphorylated p47^{phox} was assessed by Western blotting and autoradiography.

NADPH Oxidase Activation in a Cell-Free System. Human neutrophil membranes (40 μg of protein/mL) were mixed with human recombinant p67^{phox} (20.5 μg/mL), Rac1 (16 μg/mL), and p47^{phox} (80 μg/mL) phosphorylated by various PKC isoforms, the specific activity of which had been standardized to 0.6 nmol of P_i/min, in a reaction mixture containing relaxation buffer (10 mM Pipes, 100 mM KCl, 3 mM NaCl, and 3.5 mM $MgCl_2$) supplemented with 10 μM FAD, 25 μM GTPγS, 250 nM calyculin A, 40 μM ATP, 0.5 mM $CaCl_2$, 50 μM lucigenin, and 7.5 μg/mL PS and 0.5 μg/mL DG in the presence or absence of SOD (150 units) or 10 μM GF-109203X. The reaction mix was incubated for 20 min at room temperature to phosphorylate p47^{phox}. $O_2^{\bullet-}$ production was initiated by adding 200 μM NADPH and quantified in terms of Lucigenin-amplified chemiluminescence (Autolumat LB 953) for 60 min. As the kinetics of activation were the same for all the PKC isoforms that were tested, we used the height of the peaks to analyze the results.

Statistical Analysis. Data are presented as means ± SD of at least three different experiments for each condition. Differences between groups were examined for statistical significance using ANOVA and by the Fisher protected least significant difference test.

RESULTS

In Vitro Phosphorylation of p47^{phox} by PKC Isoforms. To determine if p47^{phox} is phosphorylated by PKC isoforms known to be present in human neutrophils, the recombinant GST−p47^{phox} fusion protein was incubated with each human recombinant PKC isoform as described above. For this study, the PKC specific activity was standardized at 0.1794 nmol of P_i/min (as determined using peptide ϵ substrate). Figure 1A shows the autoradiographs of the corresponding SDS−PAGE gels. Each PKC isoform that was tested (PKC α, βII, δ, and ζ) phosphorylated the GST−p47^{phox} fusion protein [as well as p47^{phox} alone, but not GST alone (data not shown)]. The histone H1 positive control was always phosphorylated, confirming the active state of the kinases. Figure 1B shows the kinetic autoradiographs of p47^{phox} phosphorylation by each PKC isoform. This phosphorylation was time-dependent, the extent increasing linearly to a maximum between 20 and 30 min for PKC α, βII, and δ . In contrast to the other isoforms, phosphorylation by PKC ζ was first detected at 10 min, as observed previously (*20*), and was maximal at 30 min. Phosphorylation of p47^{phox} by PKC α, βII, δ, and ζ was also dependent on the protein concentration (Figure 1C). The apparent K_m values of each PKC isoform for p47^{phox}, calculated from Lineweaver−Burk representations, were 10.33 ± 3.5, 3.37 ± 2.6, 2.37 ± 0.7, and 2.13 ± 0.19 μM (n = 4) for PKC α, βII, δ, and ζ, respectively, indicating that p47^{phox} is a good substrate for these PKC isoforms with the following order of affinities ($1/K_m$): PKC ζ > PKC δ > PKC βII > PKC α.

Two-Dimensional Phosphopeptide Mapping of p47^{phox} Phosphorylated by PKC Isoforms. Earlier studies carried out

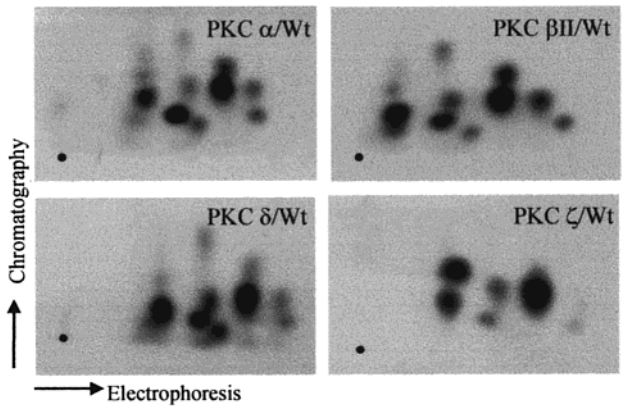

FIGURE 2: Phosphopeptide maps of p47*phox* phosphorylated by PKC α, *β*II, *δ*, and *ζ*. p47*phox* was phosphorylated by each PKC isoform for 30 min at 30 °C, then cleaved with trypsin, and subjected to thin-layer electrophoresis and chromatography as described in Materials and Methods. The PKC isoform that was used is indicated in the upper right-hand corner of each map, and the sample application point is indicated in the lower left-hand corner by a black spot. On the electrophoresis axis, the cathode is on the right. Wt, wild type. Data are representative of five different experiments.

on neutrophils and EBV-transformed lymphocytes have shown that, under stimulation with PMA or fMLP, p47*phox* is phosphorylated on multiple serine residues ranging from Ser 303 to Ser 379 (*23*, *24*). We have recently shown that PKC *ζ* phosphorylates a subset of selective sites on p47*phox* (*20*). Here we examined whether the other isoforms present in neutrophils also target a specific subset of serines. We used two-dimensional phosphopeptide mapping to analyze p47*phox* peptides phosphorylated by PKC α, *β*II, and *δ* relative to PKC *ζ*. The overall peptide maps of recombinant p47*phox* phosphorylated by PKC α, *β*II, and *δ* (Figure 2) yielded the same map as p47*phox* phosphorylated in neutrophils (*23*, *24*), with up to 10 phosphorylated peptides. However, the phosphorylation intensity of some peptides appeared to be isoform-dependent. The map of p47*phox* phosphorylated by PKC *ζ* was completely different from those of PKC α, *β*II, and *δ*, as it contained only five peptides.

Comparative Analysis of Individual Phosphopeptides. As mentioned above, PKC α, *β*II, and *δ* phosphorylated p47*phox* on the same number of peptides, but the degree of phosphorylation of each peptide seemed to be different with each PKC isoform. To analyze the levels of phosphorylation, the radioactivity associated with each spot was expressed relative to the total phosphorylation level of p47*phox*. A diagram of the phosphopeptide map for PKC α, *β*II, and *δ* phosphorylation is shown in Figure 3A. The results of three separate experiments showed (Figure 3B) that peptides g and c (g > c) were the most intensively phosphorylated, as both represented more than 50% of the phosphorylation of p47*phox*. The phosphorylation intensity of the peptides was ranked in the following order: g > c > e > f > d > b > h > a. The intensity difference between PKC *δ* and *β* was statistically significant ($p < 0.05$) for peptide b only. These data suggest that PKC α, *β*II, *δ*, and *ζ* may have discrete specificities for NADPH oxidase regulation, targeting selective serines on p47*phox*.

Identification of the Phosphopeptides on the Peptide Map of p47phox. To identify the phosphorylated serines, we analyzed the phosphopeptide maps of p47*phox* mutants in which serines 303−379 were mutated to alanines. As the results obtained for PKC α, *β*II, and *δ* were the same, we present (Figure 4) only the results for PKC α. Compared to the wild type, mutation of serines 303 and 304 to alanines resulted in the disappearance of the peptide shown by dots (peptide f on the diagram). This mutation also altered the phosphorylation of other peptides (arrows). Mutation of serines 315 and 320 individually resulted in the elimination of specific phosphopeptides (e+h and d, respectively), as confirmed with the double mutant S(315+320)A. Mutation of Ser 328 to Ala eliminated one major peptide (g) and altered several peptides (arrows). The S379A mutant resulted in the elimination of specific peptides (a and b). With mutant S(328−379)A, several peptides disappeared, but the peptides corresponding to serines (303+304), 315, and 320 remained phosphorylated. The effects of the mutants on p47*phox* phosphopeptides are summarized in Table 1. Taken together,

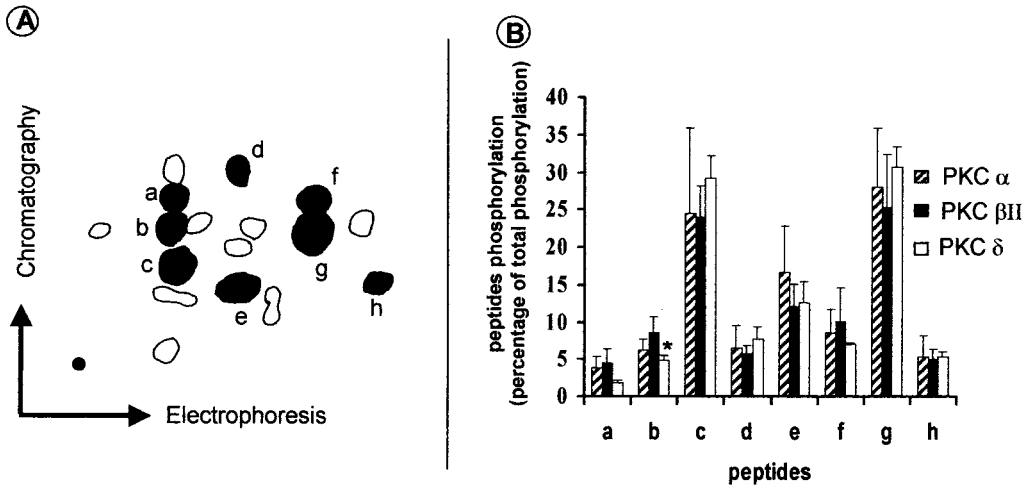

FIGURE 3: Diagram of the phosphopeptide map of p47*phox* phosphorylated by PKC α, *β*II, and *δ* and quantification of phosphorylated peptides. The diagram (A) of the p47*phox* phosphopeptide map represents the major phosphopeptides (filled area) designated "a−h" for phosphorylation by PKC α, *β*II, and *δ*. Empty areas represent unidentified minor phosphopeptides. The application point of each sample is indicated in the lower left-hand corner by a black spot. The quantification of major phosphopeptides (B) was done by using an Instantimager. Results are expressed as mean peptide phosphorylation (% of total phosphorylation) ± SD of three different experiments. Asterisks indicate $p < 0.05$.

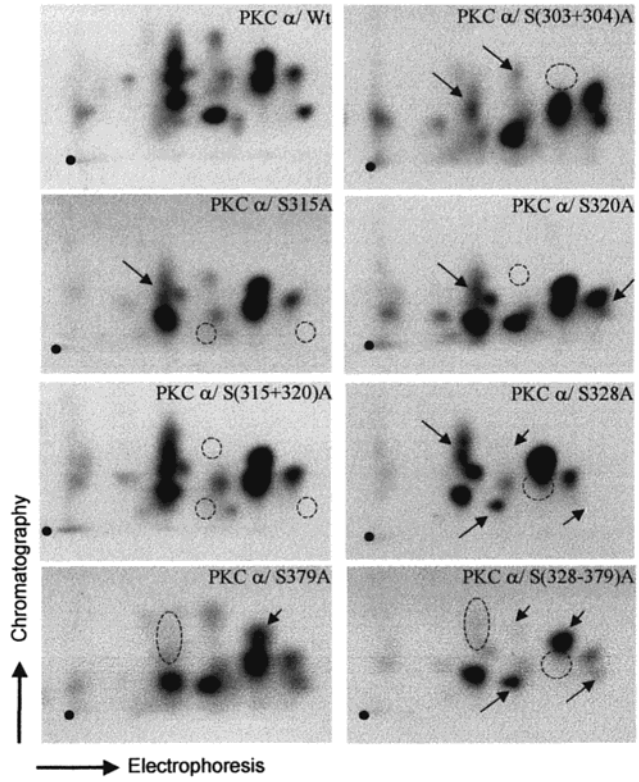

FIGURE 4: Phosphopeptide maps of p47^{phox} and mutants phosphorylated by PKC α. Recombinant p47^{phox} mutants were phosphorylated with PKC α, and phosphopeptide maps were determined as described in Materials and Methods. The mutations are indicated in the upper right-hand corner of each map. Missing phosphopeptides in p47^{phox} mutants are circled with dotted lines. The arrows show the decrease in phosphopeptide intensity in p47^{phox} mutants. The sample application point is indicated by the black spot in the lower left-hand corner of each panel. On the electrophoresis axis, the cathode is on the right. Data are representative of two different experiments.

Table 1: Summary of the Effect of p47^{phox} Mutants on the Phosphorylated Peptides[a]

mutant	missing peptides[b]	decreased peptides[b]
S(303+304)A	f	a−d
S315A	e and h	a and b
S320A	d	a, b, and h
S(315+320)A	e, h, and d	none
S328A	g	a, b, d, e, and h
S379A	a and b	f
S(328−379)A	a, b, and g	d−f and h

[a] For peptides a−h, see the chart in Figure 3A. [b] Radioactivity was quantified using Instantimager analysis.

these results suggest that serines (303+304), 315, 320, 328, and 379, corresponding to peptides f, (e+h), d, g, and (a+b) in the diagram, respectively, are the phosphorylation targets of PKC α, βII, and δ isoforms.

The S(359+370)A Mutation Blocks the Phosphorylation of Other Serines on p47^{phox}. Johnson et al. (*24*) have previously shown, in whole cells, that p47^{phox} mutant S(359+370)A inhibits NADPH oxidase activation and phosphorylation of other serines present on p47^{phox}. We examined if Ser 359 and 370 controlled the phosphorylation of other serines when phosphorylated by each PKC isoform alone. As shown in Figure 5, the phosphorylation of p47^{phox} by PKC α, βII, δ, and ζ was inhibited by the S(359+370)A mutation. This implied that phosphorylation of these two serines might

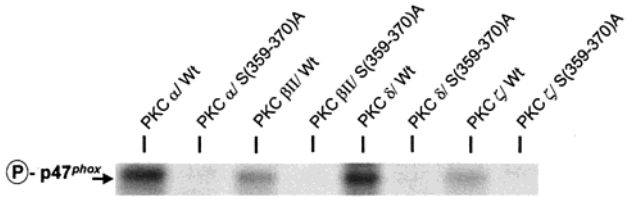

FIGURE 5: Effect of the S(359+370)A mutant on the phosphorylation of p47^{phox} by PKC α, βII, δ, and ζ. Wild-type p47^{phox} and the S(359+370)A mutant were phosphorylated by PKC isoforms for 30 min at 30 °C. Samples were denatured and subjected to 10% gel SDS−PAGE as described in Materials and Methods. Radioactivity was revealed by autoradiography. Data are representative of two different experiments.

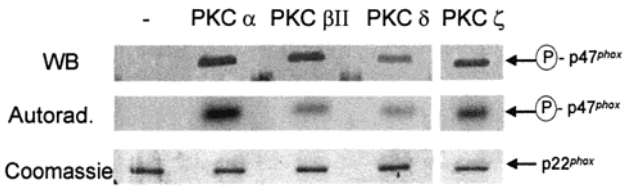

FIGURE 6: Binding of phosphorylated p47^{phox} to p22^{phox}. Wild-type p47^{phox} was phosphorylated by PKC isoforms for 30 min at 30 °C. Reactions were stopped by the addition of staurosporine and dilution as described in Materials and Methods. Phosphorylated p47^{phox} was incubated with nitrocellulose containing p22^{phox}. Membranes were autoradiographed and blotted with anti-p47^{phox}. p22^{phox} was detected by SDS−PAGE and Coomassie blue staining. Data are representative of three different experiments.

be an important initial step in the phosphorylation of other serine residues by each individual PKC isoform.

Effect of p47^{phox} Phosphorylation by PKC Isoforms on Its Binding to the Cytosolic Fragment of p22^{phox} and NADPH Oxidase Activation in a Cell-Free System. In activated cells, translocation of the cytosolic complex is crucial for NADPH oxidase activation. This translocation is controlled by p47^{phox} phosphorylation and is mediated by interaction of the SH3 domains of p47^{phox} with the proline-rich region of p22^{phox}. We examined the capacity of each PKC isoform to induce p47^{phox} binding to the cytosolic fragment of p22^{phox}, corresponding to amino acids 132−195. As shown in Figure 6, nonphosphorylated p47^{phox} was unable to bind to the cytosolic portion of p22^{phox}. However, p47^{phox} phosphorylated by PKC α, βII, δ, and ζ binds the p22^{phox} tail. To confirm that p47^{phox} was equally phosphorylated by each isoform, parallel SDS−PAGE runs were performed and the radioactivity of the p47^{phox} band was quantified (data not shown). The PKC inhibitor GF-109203X (10 μM) inhibited p47^{phox} binding induced by PKC α, βII, δ, and ζ (Figure 7), suggesting that binding of p47^{phox} requires PKC-dependent phosphorylation activity and is not due to nonspecific interactions.

NADPH oxidase can be activated in a cell-free system in a PKC-dependent manner (*25*, *26*). We used this system to evaluate NADPH oxidase activation by the different PKC isoforms. As shown in Figure 8, PKC α, βII, δ, and ζ induced NADPH oxidase activation and $O_2^{\bullet-}$ production, while GF-109203X (10 μM) and SOD (150 units) inhibited $O_2^{\bullet-}$ production induced by the isoforms, indicating that this production is a phosphorylation- and NADPH oxidase activation-dependent process. Atypical PKC ζ induced a higher level of superoxide production than PKC α, β, and δ ($p < 0.05$), suggesting that phosphorylation of some sites could have an inhibitory effect on oxidase activity.

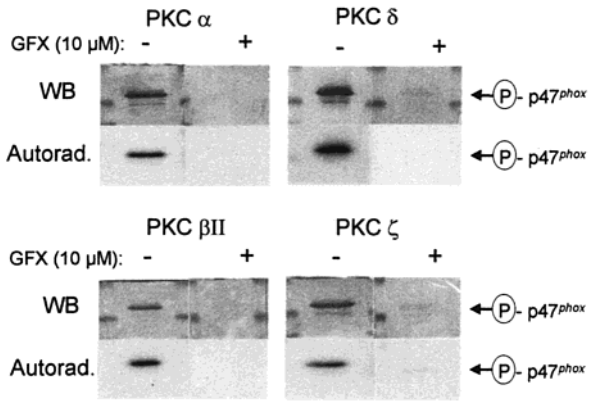

FIGURE 7: Effect of GF-109203X on binding of p47*phox* to p22*phox*. p47*phox* was incubated with PKC isoforms for 30 min at 30 °C in the absence or presence of GF-109203X (GFX) (10 μM). Reactions were stopped by the addition of staurosporine and dilution as described in Materials and Methods. p47*phox* was incubated with nitrocellulose containing p22*phox*. Samples were autoradiographed and blotted with anti-p47*phox* for quantification. Data are representative of three different experiments.

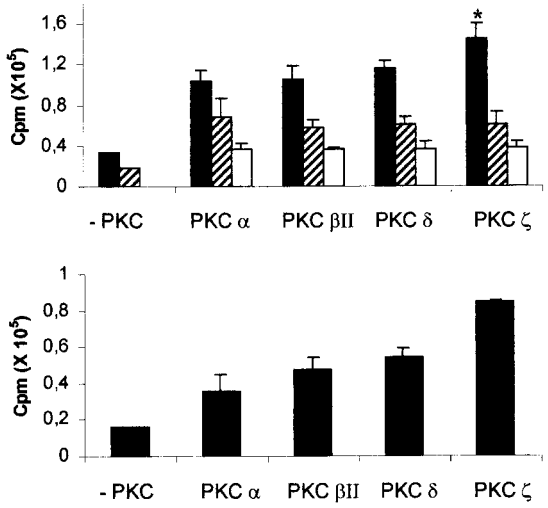

FIGURE 8: Activation of NADPH oxidase by PKC isoforms in a cell-free system. Neutrophil membranes were preincubated at room temperature for 20 min with recombinant proteins (p67*phox*, Rac1, and p47*phox*) and PKC isoforms (■) in the presence of SOD (cross-hatched bars) or GF-109203X (□). Superoxide production was assessed with a chemiluminometer for 60 min as described in Materials and Methods. Basal superoxide production was quantified without PKC and with activators. The "bottom panel" represents the SOD-inhibitable superoxide production. Data are representative of three different experiments. The asterisk indicate $p < 0.05$.

Taken together, these results show that (1) all PKC isoforms are able to induce p47*phox* binding to p22*phox* and NADPH oxidase activation, (2) the binding of p47*phox* to p22*phox* and oxidase activation require a minimal number of phosphorylated serines, as PKC ζ only phosphorylates a subset of sites yet induces binding to p22*phox* and enzyme activity, and (3) the phosphorylation of some sites by PKC α, β, and δ could have an inhibitory effect on oxidase activity.

Effect of p47phox Mutants on the Binding to p22phox and NADPH Oxidase Activation. We next analyzed the effect of p47*phox* mutants on the binding to p22*phox* and NADPH oxidase activation. As shown in Figure 9, the mutants S(359+370)A and S(303+304)A inhibited the binding of p47*phox* to p22*phox* for all the isoforms that were tested.

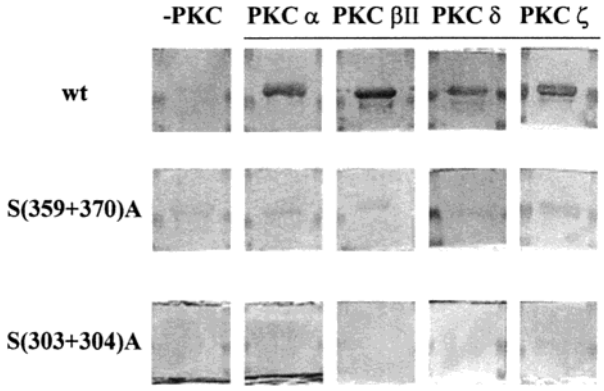

FIGURE 9: Effect of S(359+370)A and S(303+304)A mutants on binding of p47*phox* to p22*phox*. Wild-type p47*phox* and mutants were incubated with PKC isoforms for 30 min at 30 °C. Reactions were stopped by the addition of staurosporine and dilution as described in Materials and Methods. Wild-type p47*phox* and mutants were incubated with nitrocellulose containing p22*phox*. Samples were autoradiographed and blotted with anti-p47*phox* for quantification. Data are representative of three different experiments.

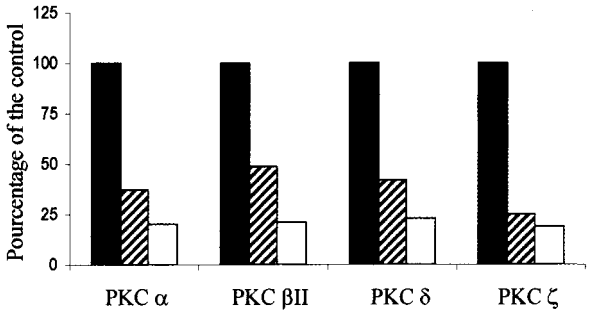

FIGURE 10: Effect of S(359+370)A and S(303+304)A mutants on activation of NADPH oxidase by PKC isoforms in a cell-free system. Neutrophil membranes were preincubated at room temperature for 20 min with recombinant proteins (p67*phox* and Rac1), wild-type p47*phox* (■), S(359+370)A (cross-hatched bars), and S(303+304)A (□) in the presence of each PKC isoforms. Superoxide production was assessed with a chemiluminometer for 60 min as described in Materials and Methods and expressed as the percentage of total production with wild-type p47*phox*.

Consequently, these two mutants inhibited NADPH oxidase activation in the PKC-activating cell-free system (Figure 10). The other mutants only partially affected binding and activation (data not shown), except for S379A which has previously been demonstrated to cause inhibition of NADPH oxidase activation (*25*).

DISCUSSION

p47*phox* phosphorylation plays an important role in the activation and regulation of the NADPH oxidase. The involvement of the PKC family in this process has been suggested by studies based on the use of chemical agents, particularly, PMA, a direct PKC activator that can strongly activate NADPH oxidase and phosphorylate p47*phox*. In addition, these effects are inhibited by PKC inhibitors such as staurosporine and GF-109203X (*25*, *27*). Recently, an in vivo study by Dekker et al. (*18*) has shown that neutrophils from PKC β knockout mice resulted in a 50% decrease in the level of superoxide production compared to that in neutrophils from normal mice. The use of PKC β antisense in HL60 cells by Korchak et al. (*19*) has also suggested that PKC β is involved in the phosphorylation of p47*phox* and $O_2^{\bullet -}$

generation. In this study, we show that, individually, PKC isoforms α, βII, δ, and ζ phosphorylate p47^{phox} and induce both translocation of the phosphorylated p47^{phox} and activation of NADPH oxidase in a cell-free system. The phosphorylation map of p47^{phox} phosphorylated by PKC ζ was very different from the maps obtained with PKC α, βII, and δ. Although these latter isoforms phosphorylated the same sites, the degree of phosphorylation of certain peptides differed among them. These results suggested that PKC isoforms might be involved in p47^{phox} phosphorylation in whole cells, and might have different roles based on their targeting of specific serine residues. Furthermore, despite this differential phosphorylation of p47^{phox} sites, PKC isoforms α, βII, δ, and ζ all induced p47^{phox} binding to p22^{phox} and NADPH oxidase activation in a cell-free system. Although PKC ζ phosphorylates fewer sites, it induced a higher level of NADPH oxidase activation, suggesting that phosphorylation of some serines by PKC α, β, and δ could attenuate oxidase activity.

The K_m results suggest that p47^{phox} is a better substrate for PKC ζ, δ, and β than PKC α. This in vitro result may differ in the context of an intact cell membrane, the order of translocation depending on the stimuli used. Indeed, a correlation between translocation of PKC ζ, α, and β and translocation of p47^{phox} was described (*12*, *17*, *20*), but not with translocation of PKC δ. Furthermore, Reeves et al. (*28*) have recently shown that PKC β and δ bind to p47^{phox} in intact neutrophils with different time courses.

In a previous study, a mixture of PKC isoforms from rat brain yielded a p47^{phox} phosphopeptide map similar to that obtained with p47^{phox} from PMA-activated neutrophils and B lymphocytes (*8*). PKA phosphorylated two or three peptides on p47^{phox}, while MAPK phosphorylated only one. In our study, PKC α, βII, and δ phosphorylated eight major p47^{phox} peptides, and we also confirm our previous results for PKC ζ (*20*), which phosphorylates only few peptides. PKC α and βII phosphorylated p47^{phox} sites to the same degree, but PKC δ phosphorylated some peptides less intensively. As serine accessibility is the same for all PKC isoforms, such differences could be explained by differences in the affinity of PKC α, βII, δ, and ζ for each serine residue of p47^{phox} and the surrounding amino acids, as suggested by Nishikawa et al. (*29*), who observed this phenomenon by using synthetic substrates for PKC isoforms. Analysis of mutants phosphorylated by PKC α, βII, and δ showed that serine 328 was the most extensively phosphorylated residue and that the other phosphorylated serines were Ser 303, 304, 315, 320, and 379. We confirmed that PKC ζ phosphorylates Ser 303, 304, and 315 (data not shown) (*20*). All the mentioned serines are phosphorylated in intact neutrophils and lymphoblasts (*8*). We also found that the S(359+370)A mutation inhibited the phosphorylation of all other p47^{phox} serines by each PKC isoform and the binding to p22^{phox}. These results suggest that serines 359 and 370 are required for the phosphorylation of other serine residues by PKC isoforms. Thus, phosphorylation of serines 359 and 370 might induce conformational changes that permit the phosphorylation of the other serine residues by PKCs, as suggested by Johnson et al. (*24*), who found that mutants with anionic charges (glutamate or aspartame) in positions 359 and 370 permitted phosphorylation of other serine residues.

Despite differential phosphorylation of p47^{phox} sites between PKC ζ and α, βII, and δ, p47^{phox} was always able to bind to p22^{phox} and to activate NADPH oxidase in a cell-free system. PKC α, βII, and ζ induced binding of p47^{phox} to p22^{phox} more efficiently than PKC δ. It is conceivable that PKC α, βII, and ζ might phosphorylate the optimal combination of serines required to induce the conformational changes that are necessary for the binding of p47^{phox} SH3 domains to the proline-rich sequence of p22^{phox}. PKC ζ might phosphorylate the minimal number of sites required for binding to p22^{phox}. Many studies have shown that p47^{phox} phosphorylation results in conformational changes that lead to its translocation to cytochrome b_{558} (*30*−*34*). Our results support these previous reports and show that all the PKC isoforms that were studied (PKC α, βII, δ, and ζ) can induce p47^{phox} binding to p22^{phox} and therefore to cytochrome b_{558}. PKC ζ phosphorylated only a few serines but, nonetheless, induced p47^{phox} binding to p22^{phox}, suggesting that a restricted number of serines must be phosphorylated to induce p47^{phox} translocation. This agrees with a recent study by Ago et al. (*10*) in which replacement of serines 303, 304, and 328 with aspartates induced p47^{phox} translocation and NADPH oxidase activation. The other phosphorylated serines of p47^{phox} could have other functions, such as recruiting other kinases or phosphatases, or stabilizing the active complex. All the PKC isoforms that were tested induced NADPH oxidase activation, PKC ζ showing the highest activity. This unexpected result could be explained by the possibility that phosphorylation of some serines by PKC α, β, and δ could have an inhibitory effect on oxidase activity. This is supported by the report from Yamaguchi et al. (*35*), who showed that hyperphosphorylated p47^{phox} lost its ability to activate NADPH oxidase in intact neutrophils.

The results of this study suggest that all PKC isoforms present in human neutrophils are able to activate NADPH oxidase. Differential phosphorylation of p47^{phox} by these isoforms could be important for fine regulation of NADPH oxidase activity.

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