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Protein Kinase C \( \zeta \) Phosphorylates a Subset of Selective Sites of the NADPH Oxidase Component \( p47^{phox} \) and Participates in Formyl Peptide-Mediated Neutrophil Respiratory Burst

Pham My-Chan Dang,* Alexandre Fontayne,* Jacques Hakim,* Jamel El Benna,* and Axel Périanin†

Generation of superoxide anion by the multiprotein complex NADPH phagocyte oxidase is accompanied by extensive phosphorylation of its 47-kDa protein component, \( p47^{phox} \), a major cytosolic component of this oxidase. Protein kinase \( C \zeta \) (PKC \( \zeta \)), an atypical PKC isoform expressed abundantly in human polymorphonuclear leukocytes (PMN), translocates to the PMN plasma membrane upon stimulation by the chemoattractant fMLP. We investigated the role of PKC \( \zeta \) in \( p47^{phox} \) phosphorylation and in superoxide anion production by human PMN. In vitro incubation of recombinant \( p47^{phox} \) with recombinant PKC \( \zeta \) induced a time- and concentration-dependent phosphorylation of \( p47^{phox} \) with an apparent \( K_m \) value of 2 \( \mu \)M. Phosphopeptide mapping analysis of \( p47^{phox} \) showed that PKC \( \zeta \) phosphorylated fewer selective sites in comparison to "conventional" PKCs. Serine 303/304 and serine 315 were identified as targets of PKC \( \zeta \) by site-directed mutagenesis. Stimulation of PMN by fMLP induced a rapid and sustained plasma membrane translocation of PKC \( \zeta \) that correlated to that of \( p47^{phox} \). A cell-permeant-specific peptide antagonist of PKC \( \zeta \) inhibited both fMLP-induced phosphorylation of \( p47^{phox} \) and its membrane translocation. The antagonist also inhibited the fMLP-induced production of oxidant (IC\(_{50}\) of 10 \( \mu \)M), but not that induced by PMA. The inhibition of PKC \( \zeta \) expression in HL-60 neutrophil-like cells using antisense oligonucleotides (5 and 10 \( \mu \)M) inhibited fMLP-promoted oxidant production (27 and 50%, respectively), but not that induced by PMA. In conclusion, \( p47^{phox} \) is a substrate for PKC \( \zeta \) and participates in the signaling cascade between fMLP receptors and NADPH oxidase activation.

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P olymorphonuclear leukocytes (PMN)\(^\text{2}\) play a vital role in the first line of cellular host defense against microorganisms. This function relies in part on the ability of PMN to generate large amounts of superoxide anion (\( O_2^- \)) and related reactive oxygen species, a phenomenon known as the respiratory burst (1). The superoxide-generating enzyme, NADPH oxidase, is a multiprotein complex that comprises a membrane-bound flavocytochrome \( b \) composed of two subunits (a 91-kDa glycoprotein and a 22-kDa protein) and four main cytosolic factors (\( p47^{phox} \), \( p67^{phox} \), \( p40^{phox} \), and a small G protein, Rac2) (2–6). NADPH oxidase activation can be triggered by various stimuli such as chemotactic peptides, phorbol esters, and opsonized particles. Upon activation of NADPH oxidase, \( p47^{phox} \) becomes phosphorylated on several sites (7–9) and translocates to the plasma membrane (8, 10–12), where it interacts with cytochrome \( b \) (13). Various protein kinases have been involved in the regulation of NADPH oxidase activity (14–17), among which the protein kinase C (PKC) family appears to play a major role (18–20).

PKC comprises a family of isoenzymes that play a key role in signaling events and cell functions (21, 22). PKC has been classified into the following three subgroups on the basis of their molecular structure and mode of activation: 1) "conventional" PKCs (\( \alpha \), \( \beta_1 \), \( \beta_2 \), and \( \gamma \)), which require phosphatidylserine (PS) and are activated by calcium and diacylglycerol (DAG); 2) "novel" PKCs (\( \delta \), \( \epsilon \), \( \mu \), \( \eta \), and \( \theta \)), which require PS and DAG but not calcium for activation; and 3) "atypical" PKCs (\( \zeta \), \( \lambda \)), which are calcium-independent and are not activated by DAG (21, 22). Among the atypical PKC, PKC \( \zeta \) can be stimulated by phospholipids, including phosphatidic acid (PA), phosphatidylinositol trisphosphates, and ceramides. Activation of PKCs in intact cells is generally associated with the translocation of the enzyme from the cytosol to particulate compartments (18, 23–25).

Immunological studies have shown that human PMN express five PKC isoforms: \( \alpha \), \( \beta_1 \), \( \beta_II \), \( \delta \), and \( \zeta \) (23, 24, 26–29). The respective contribution of each PKC isoform in regulating PMN respiratory burst has not yet been elucidated, although it has been shown that PKC \( \beta \) may modulate the respiratory burst of both electroporation-activated PMN (15) and HL-60 cells differentiated into PMN-like cells (30). PKC \( \zeta \), which like PKC \( \beta \) is expressed at high levels in human PMN (23), was shown to regulate PMN adhesion and chemotaxis (31). The contribution of PKC \( \zeta \) to the respiratory burst has not been documented so far, although it has been shown that PKC \( \zeta \) translocates to the plasma membrane and that its activity is increased in fMLP-stimulated PMN (31–33).

In the present work we have studied the role of PKC \( \zeta \) in the signaling pathway between stimulated fMLP receptors and NADPH oxidase activation. The results indicate that PKC \( \zeta \) phosphorylates selective residues of \( p47^{phox} \) and plays a predominant role in the activation of NADPH oxidase.
role in the stimulation of superoxide production in fMLP-stimulated human PMN.

Materials and Methods

Materials

1- β-[32P]ATP (sp. act. 6000 Ci/mmol) and [32P]phosphoric acid (9000 Ci/mmol) were obtained from NEN Life Science Products (Boston, MA). Protease inhibitors (PMSF, diisopropylfluorophosphate, leupeptin, pepstatin, and aprotinin), cytochrome c, fMLP and PMA, PA, PS, and DAG were obtained from Sigma-Aldrich (St. Louis, MO). Liposome reagent and Ab to PKC ζ were obtained from Life Technologies (Cergy Pontoise, France). Myristoylated peptide inhibitor of PKC ζ (Myr-SIYRKGARRWRK) and PKC η (Myr-TRKRQARMRRRQHIQRQI) were obtained from BioSource International QCB (Camarillo, CA). The nonmyristoylated PKC ζ inhibitor peptide (RRGARRWRK) was synthesized by NeoSystem (Strasbourg, France). The purity of both peptides was >97%. Trypsin was obtained from Boehringer Mannheim (Meylan, France). Recombinant p47phox and Ab against p47phox were kindly provided by Dr. B. M. Babior (Scirpps Research Institute, La Jolla, CA). Wild-type p47phox (M) was derived from the published coding sequence of PKC ζ (34). Recombinant mouse PKC ζ was kindly provided by Dr. P. J. Parker (London, U.K.) (36–37). Recombinant human PKC ζ was purchased from Biomol (Tel Aviv, Israel). HL-60 cells were obtained from the European Collection of Animal Cell Cultures (Salisbury, U.K.). Thiololated oligodeoxynucleotides, antisense and sense, respectively. The proteins were expressed in Escherichia coli and purified by one-step immunoaffinity chromatography (35). Recombinant mouse PKC ζ was expressed in Sf9 insect cells (36). The proteins expressed in Sf9 insect cells were purified by HPLC. Phosphorylation of recombinant p47phox in vitro by PKC ζ

Phosphorylation of recombinant p47phox by recombinant PKC ζ was performed in 40 μl of a mixture containing 50 mM glycerol-2-phosphate, 0.4 mM EGTA, 10 mM magnesium acetate, 35 mM Tris-HCl buffer (pH 7.5), various concentrations of wild-type or mutant p47phox, and 0.15 U/ml recombinant PKC ζ (1 U corresponding to 1 nmol phosphate incorporated in peptide e/min) without activators. This PKC ζ preparation has been used previously to phosphorylate peptide α and myristoyl alanine-rich C kinase substrate peptide (37). Phosphorylation was initiated with 50 μM [γ-32P]ATP (specific radioactivity, 150–300 Ci/mmol) and conducted at 30°C. After 30 min of incubation, 20 μl of the reaction mixture was spotted onto Whatman p81 paper (Tewksbury, MA) followed by three washes with 5% H₃PO₄, and the radioactivity was counted. The remaining fraction (20 μl) was mixed with 3× Laemmli sample buffer (Bio-Rad, Irvine, CA) and subjected to SDS-PAGE (10% acrylamide gel) (39), then blotted (40) for autoradiography analysis with Amersham Hyperfilm MP (Arlington, Heights, IL).

Cyanogen bromide (CNBr) cleavage of p47phox and two-dimensional phosphopeptide mapping analysis

The phosphorylated p47phox was subjected to SDS-PAGE and transferred to nitrocellulose. The band of interest was excised and incubated in dark with 12.5 mg/ml CNBr in 70% (v/v) formic acid for 16 h at room temperature. The reaction mixture was quenched with 1 vol of water and lyophilized in a Speed-Vac (Savant, Holbrook, NY). The digested peptides were then separated by Tris-1[2-hydroxy-1,1-bis(hydromethyl)ethyl]glycine (Tricine)-SDS-PAGE as previously described (16, 41, 42). For peptide mapping, the phosphorylated p47phox blotted onto a nitrocellulose membrane was cut out and resuspended in 200 μl of 50 mM (NH₄)HCO₃ buffer (pH 7.8) and digested overnight with 25 μg trypsin at 37°C. After drying, the resulting phosphopeptides were resolved on thin-layer cellulose plates by electrophoresis at pH 1.9 for 30 min at 1000 V and 4°C in the first dimension, followed by ascending chromatography developed with isobutyric acid buffer (isobutyric acid:butanol:pyridine:acetic acid:water, 25/1/5/1/10 (v/v/v/v)/). The resulting phosphopeptides were detected by autoradiography at −70°C.

Preparation of PMN

PMN were isolated from heparinized venous blood of healthy volunteers by two-step sedimentation on dextran and Ficoll-Hypaque (23). Red cells were removed by hypotonic lysis, and the leukocyte suspension (95–97% PMN) was washed and resuspended either in HBSS containing 1.2 mM calcium (pH 7.4) or in phosphate-free buffer (10 mM HEPES (pH 7.4), 137 mM NaCl, 5.4 mM KCl, 5.6 mM glucose, 0.8 mM MgCl₂, and 0.025% BSA).

Chemiluminescence measurement

A suspension of 5 × 10⁵ cells was incubated in 0.3 ml HBSS at 37°C in the absence (control) or presence of various concentrations of PKC antagonist for 30 min in the presence of 0.025% BSA. Cells were then incubated with 10 μM luminol and stimulated with 20 nM fMLP or PMA (100 ng/ml). Results are expressed as the percentage of the peak of chemiluminescence or in cpm.

Phosphorylation of p47phox in PMN and immunoprecipitation

Cells were incubated in phosphate-free buffer containing 0.5 mCi of [32P]-labeled phosphoric acid/10⁵ cells/ml for 60 min at 30°C as previously reported (44). After washing, PMN (40 × 10⁶/2 ml) were treated in the absence or presence of PKC peptide antagonists for 30 min in the presence of 0.025% BSA, then stimulated with either PMA (250 ng/ml) for 6 min or fMLP (20 nM) for 3 min. The reaction was stopped with ice-cold buffer and centrifugation at 4°C (4000 g, 6 min). The cells were suspended at 1 × 10⁶ cells/ml in homogenization buffer containing 20 mM Tris-HCl (pH 7.5), 0.5% Triton X-100, 250 mM sucrose, 2 mM EDTA, 5 mM EGTA, 1 mg/ml DNase, 1.5 mM NaF, 1 mM p-nitro-phenyl-phosphate, 1 mM β-glycerophosphate, and an antiprotease mixture consisting of 10 μl/mg leupeptin, pepstatin, aprotinin, 1 mM diisopropylfluorophosphate, and 1 mM PMFS and disrupted by sonication (3 × 10 s) at 4°C. Homogenates were centrifuged for 1 h at 100,000 × g (TL100 Ultracentrifuge; Beckman Coulter, Fullerton, CA), and the supernatant was incubated overnight with anti-p47phox Abs (dilution 1/200). Proteins were immunoprecipitated with p-glycoprotein G-Sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ), washed, and eluted electrophoresed as described (44).

Fractionation of PMN

PMN were treated in HBSS containing 0.025% BSA in the absence or presence of PKC antagonist for 30 min and stimulated with fMLP or PMA under the conditions described above. Membranes were prepared as described (44). Briefly, the PMN suspensions (10⁶ cells/ml) were sonicated for 5 s on ice in 1 ml relaxation buffer containing 10 mM PIPES, (pH 7.3), 3 mM MgCl₂, 100 mM KCl, and 5 mM NaCl, supplemented with 0.5 mM PMSF, 1 mM EDTA, 10 μg/ml leupeptin, and 10 μg/ml pepstatin. Cytosolic and membrane fractions were prepared by centrifugation (200,000 × g, 30 min) of the homogenates on 15–34% sucrose gradient at 4°C. A membrane preparation equivalent of 5 × 10⁶ cells was used for SDS-PAGE.

In vitro phosphorylation of PMN endogenous p47phox induced by PA

Phosphorylation of endogenous substrates was performed with 20 μl of cytosol fraction from unlabeled PMN (2 × 10⁶ cell equivalent) in a final volume of 100 μl containing 50 mM glycerol-2-phosphate, 0.4 mM EGTA, 10 mM magnesium acetate, 10 mM Tris-HCl buffer (pH 7.5) to which PA (100 μM) or DAG/PS/calcium (0, 2/0, 1/1, 2 mM) was added. In some experiments, cytosolic fractions were pretreated in the presence or absence of nonmyristoylated PKC ζ peptide antagonist. The reaction was initiated with [γ-32P]ATP at a final concentration of 50 μM (specific radioactivity, 150–300 Ci/mmol). Incubation was conducted at 30°C for 30 min, and the reactions were stopped with 3% Laemmli buffer. Proteins were heat-denatured, subjected to SDS-PAGE (10% acrylamide gel), and blotted for autoradiography and Western blot analysis.

Culture of HL-60 cells and oligonucleotide treatment

Cells were grown in suspension in RPMI 1640 medium (Life Technologies, Cergy Pontoise, France) supplemented with 10% (v/v) heat-inactivated FCS, 10 mM HEPES, 2 mM l-glutamine, streptomycin (50 μg/ml), and penicillin (50 U/ml) in a 5% CO₂ atmosphere. To induce myeloid differentiation, cells were seeded at a density of 5 × 10⁵–1.0 × 10⁶/ml and treated with 1.3% DMSO for 4–6 days. For antisense treatment, we adapted a procedure described by Xu et al. (38), using an oligonucleotide complementary to the 5’ end of the PKC ζ messenger, starting at the translation start codon. Three days after cell treatment with DMSO, HL-60 cells were washed, resuspended in DMEM, and treated with 5 and 10 μM PKC ζ oligonucleotides (antisense or sense) in the presence of 15 μg/ml lipofectin for 6 h at 37°C in 5% CO₂ atmosphere. The medium was then replaced by fresh culture medium containing oligonucleotides with 1.3% DMSO without lipofectin. After 48 h, cells were washed and suspended in HBSS, and superoxide anion production was measured by chemiluminescence.
Mapping of p47phox phosphorylation targets for PKC ζ, a potential substrate for PKC ζ isoforms and p47phox is a good substrate for PKC ζ as described in Materials and Methods, except that various concentrations of p47phox were used. Reactions were stopped with Laemmli sample buffer or on Whatman P81 paper, and samples were electrophoresed for autoradiography or Cerenkov counting. A shows an autoradiogram revealing the phosphorylation of p47phox. A Lineweaver-Burk plot of the data is shown in B. Data are representative of three experiments.

Results

In vitro phosphorylation of p47phox by PKC ζ

The phosphorylation of p47phox on multiple serines is required for NADPH oxidase activation. Previous studies have reported that p47phox is a good substrate for PKCs. However, the contribution of each isoform to the multiphosphorylation process of p47phox is not known. We and others have reported that PKC ζ is expressed in human neutrophils and translocates to the plasma membranes upon PMN activation (23, 24, 31, 32). To determine whether p47phox is a potential substrate for PKC ζ, we analyzed the phosphorylation of recombinant p47phox in vitro by a constitutively active recombinant PKC ζ. The results show (Fig. 1, right) that PKC ζ phosphorylated p47phox as a function of the incubation time. In this experiment, which was conducted at 30°C in the absence of enzyme activators, phosphorylation of p47phox began to be visible after 3 min and was maximal at 30 min. In the absence of p47phox, a weakly phosphorylated band of 80 kDa was detected (Fig. 1, left), which probably corresponded to auto- or phosphorylated PKC ζ because the band was recognized by an anti-PKC ζ Ab in Western blotting experiments (results not shown). Phosphorylation of p47phox by PKC ζ was also dependent on the concentration of the protein (Fig. 2A). The apparent Kₘ value of PKC ζ for p47phox, calculated from a Lineweaver-Burk representation of the data, was ~2.0 μM (Fig. 2B), indicating that p47phox is a good substrate for PKC ζ.

Mapping of p47phox phosphorylation targets for PKC ζ in vitro

In PMN stimulated with PMA or IMLP, p47phox is phosphorylated on multiple serine residues (42). Phosphorylated residues were located in the carboxyl-terminal portion of the protein resulting from CNBr cleavage of p47phox (42), and previous studies identified serine residues that were specifically phosphorylated by “conventional” PKC, mitogen-activated protein kinase (MAP kinase), and protein kinase A (PKA) (16). To map the p47phox amino acid residues that were phosphorylated by PKC ζ, we analyzed the phosphorylated protein by CNBr cleavage and tryptic peptide mapping. Cleavage of p47phox by CNBr followed by Tricine-SDS-PAGE showed that the phosphorylated sites were located in a single peptide of ~14 kDa (Fig. 3, autoradiography) corresponding to the C-terminal portion of the protein because it was recognized by an Ab directed against the 10 carboxyl aa 380–390 of p47phox (Fig. 3, Western blot).

Analysis of the two-dimensional phosphopeptide mapping of p47phox phosphorylated by PKC ζ (Fig. 4A) showed the presence of only four phosphopeptides, compared with the much larger number of phosphorylated peptides found in the phosphopeptide map of the p47phox phosphorylated by “conventional” PKCs (Fig. 4B). This result suggests that PKC ζ specifically phosphorylates a subgroup of serine residues on p47phox.

In previous studies on “conventional” PKCs, five phosphorylated serine residues were shown to be critical for p47phox activation (16). To investigate whether PKC ζ phosphorylation targets

FIGURE 1. PKC ζ phosphorylates p47phox in vitro. PKC ζ was incubated in the presence of 50 μM ATP (1 μCi [γ-32P]ATP) alone (left) or in the presence of 5 μg of recombinant p47phox for various times (right). Reactions were stopped with Laemmli sample buffer, and samples were electrophoresed for autoradiography. Data are representative of four experiments.

FIGURE 2. Phosphorylation of p47phox by PKC ζ as a function of the substrate concentration. p47phox was phosphorylated by recombinant PKC ζ as described in Materials and Methods, except that various concentrations of p47phox were used. Reactions were stopped with Laemmli sample buffer or on Whatman P81 paper, and samples were electrophoresed for autoradiography or Cerenkov counting. A shows an autoradiogram revealing the phosphorylation of p47phox. A Lineweaver-Burk plot of the data is shown in B. Data are representative of three experiments.

FIGURE 3. CNBr cleavage of phosphorylated p47phox. Recombinant p47phox phosphorylated by PKC ζ was cleaved by CNBr. Intact and cleaved p47phox were analyzed by 10% SDS-PAGE or 16.5% Tricine-SDS-PAGE, and autoradiography (Autor), Western blot (W. Blot), and detection were performed with a specific Ab directed against the carboxyl-terminal (C-term) sequence. Data are representative of three experiments.
In stimulated PMN, PKC ζ and p47phox translocate to plasma membrane fractions, and PKC ζ phosphorylates p47phox

Stimulation of superoxide production by fMLP in PMN is associated with the phosphorylation and translocation of p47phox to the plasma membrane (reviewed in Ref. 6). We reported previously that fMLP induced a translocation of PKC ζ to the plasma membrane of human PMN (32). Here, we studied whether this PKC ζ translocation paralleled that of p47phox, which could be consistent with a coordinated spatial redistribution of the two proteins. In PMN stimulated by fMLP, PKC ζ translocated to the plasma membrane in a rapid and sustained manner (Fig. 5). The membrane translocation of p47phox induced by fMLP was similar to that of PKC ζ. Densitometric analysis of the immunoblots showed a good correlation in the membrane translocation of the two proteins (r = 0.88, p < 0.01). In addition, a myristoylated membrane-permeable peptide antagonist (10 μM) corresponding to the pseudo-substrate region of PKC ζ inhibited membrane translocation of p47phox by ~45% (Fig. 6, upper panel). This peptide, which was shown to inhibit protein kinase activity in vitro (46, 47), was used to demonstrate that PKC ζ is involved in the regulation of integrin-dependent adhesion and chemotaxis of intact human neutrophils (31). A control myristoylated peptide directed against PKC η, used at the same concentration (10 μM), was ineffective on fMLP-induced translocation of phosphorylated p47phox. This peptide was chosen as a control because the PKC η isoform seems not to be expressed in human PMN, as determined by immunoblotting experiments (23, 25). The PKC ζ peptide antagonist did not alter the translocation of p47phox induced by PMA, indicating that this peptide did not interfere with PMA-sensitive PKCs (i.e., “conventional” and “novel” PKCs). The PKC ζ antagonist (10 μM) also inhibited the fMLP-induced phosphorylation of p47phox in 32P-loaded PMN, but not the p47phox phosphorylation induced by PMA (Fig. 6, lower panel), whereas the control peptide was without effect. These results are consistent with the hypothesis that fMLP-activated PKC ζ phosphorylates and activates p47phox in intact PMN.

Studies with inhibitory peptides and antisense RNA indicate that PKC ζ participates in the fMLP-dependent activation of NADPH oxidase

The data obtained above suggest that PKC ζ regulates NADPH oxidase activation. Incubation of PMN in the presence of the myristoylated peptide antagonist of PKC ζ for 15 min induced a concentration-dependent inhibition of the fMLP-induced respiratory burst, as assessed by a chemiluminescence assay (Fig. 7). This effect was not due to a cytotoxic effect of the peptide because peptide-treated PMN excluded trypan blue (data not shown). Unlike the PKC ζ antagonist, the control PKC η peptide antagonist did not inhibit fMLP- and PMA-induced PMN superoxide production, but rather slightly potentiated their effect. The concentration of the PKC ζ antagonist used here (5–15 μM) did not appear to inhibit other PKC isoforms. This was first suggested by the observation that the myristoylated peptide did not alter PMA-induced superoxide production, in agreement with previous data (31). In
addition, measurement of in vitro phosphorylation of endogenous p47phox upon stimulation of conventional PKC by diglycerides and calcium in a cytosolic fraction of PMN was not altered by low peptide concentrations (5–15 μM) (Fig. 8). By contrast, this low peptide concentration did inhibit p47phox phosphorylation induced by a PKCζ activator (PA). Higher peptide concentrations (20–50 μM) inhibited the activation of conventional PKC (Fig. 8). Taken together, our results indicate that the down-regulation of PMN respiratory burst by the myristoylated PKCζ-derived peptide results from inhibition of PKCζ function.

To further examine the functional contribution of PKCζ, we down-regulated its expression with antisense phosphorothioate oligonucleotides. For this purpose, HL-60 cells differentiated into neutrophils were used rather than fresh blood PMN because this latter cell type is inappropriate for the antisense strategy, given their low transcriptional activity and short survival. After differentiation of HL-60 cells into neutrophil-like cells with DMSO, cells were incubated with 5 and 10 μM PKCζ sense (control) and antisense oligonucleotides. Immunoblot experiments (Fig. 9, lower panel) showed that this treatment efficiently inhibited the expression of PKCζ relative to control cells (cells incubated with the sense oligonucleotide). The antisense oligonucleotide did not alter the expression of other PKC isoforms (α, β, and δ) or of p47phox (Fig. 9, lower panel), indicating the specificity of the oligonucleotide treatment toward PKCζ. Both concentrations of PKCζ antisense oligonucleotide significantly inhibited the fMLP-promoted production of superoxide anion relative to controls (Fig. 9, upper panel). In contrast, the PMN respiratory burst induced by PMA was not altered. This fact is consistent with the observation that the expression level of “conventional” and “novel” PKCs was not altered by the oligonucleotide and shows that the NADPH oxidase complex remained fully functional in these experiments. These results, obtained with the two different approaches, strongly indicate that PKCζ is a major effector of fMLP receptors in the signaling pathway leading to NADPH oxidase activation.

Discussion

Classical chemoattractants such as fMLP, the complement-derived C5a, or platelet-activating factor induce various rapid PMN responses such as adhesion, chemotaxis, exocytosis, and production of superoxide anion. Both classical PKC isoforms (α, β, and δ) and novel PKCζ are involved in PMN activation and their role is context dependent. The identification of a specific role for PKCζ in the fMLP-induced respiratory burst is consistent with the recent findings that PKCζ is involved in the regulation of adhesion and chemotaxis of PMN by fMLP. This study demonstrates that PKCζ specifically regulates NADPH oxidase activation and chemiluminescence response in PMN stimulated by fMLP. In contrast, PKCε has been implicated in PMA-induced responses. This suggests that PKCζ and PKCε are differentially involved in the regulation of PMN activation by classical chemoattractants.

FIGURE 7. Effect of PKC antagonists on PMN chemiluminescence. PMN were incubated in the absence (control) or presence of either PKCζ antagonist (5, 10, and 15 μM) (A) or PKCη antagonist (10 and 15 μM) (B) for 15 min and stimulated with 20 nM fMLP or 250 ng/ml PMA. Data represent the peak of chemiluminescence and are expressed as percentage of control values (25 × 10⁶ cpm/ml, 5 × 10⁶ cells for PMA, and 8 × 10⁶ cpm/0.5 × 10⁶ cells for fMLP).
of superoxide, which all contribute to the bactericidal function of PMN (1). Signaling pathways triggered by chemoattractant receptors are mediated through activation of a pertussis-toxin-sensitive G protein and involve the activation of tyrosine kinases and the generation of second messengers by various effectors. Among the multiple effectors involved in PMN functions, PKC play an important role in the activation of superoxide-generating NADPH oxidase (48). p47phox is essential for enzyme activation and assembly in PMN (6). In the present study, we show that p47phox is a good substrate for PKCζ. PKCζ selectively phosphorylated only a few serine residues on p47phox, mainly serines 303/304 and 315. In addition, using two different strategies based on the inhibition of PKCζ activity or enzyme expression, we provide the first evidence that PKCζ is indeed involved in the regulation of IMLP-induced PMN respiratory burst.

In intact cells, p47phox is phosphorylated on several serine residues located between positions 303 and 379. Previous studies have shown that p47phox is phosphorylated in vitro by different types of protein kinases, including “conventional” PKC (14, 16) such as PKCβ (17), MAP kinases (p42-ERK2 and p38) (16, 49), PKA (16, 50), p21-activated kinases (51), undefined protein kinases (17, 52), and PKCζ (53). Phosphopeptide mapping of p47phox revealed that “conventional” PKC, MAP kinase, and PKA phosphorylate different groups of serine residues, suggesting that these different kinases may have different regulatory effects on NADPH oxidase (16). The comparison of phosphopeptide maps described here further indicates that some specificity exists among members of the PKC family for the phosphorylation of p47phox. Indeed, PKCζ selectively phosphorylated serine residues at position 303/304, and, to a lesser extent, at position 315, as well as two other residues that remain to be determined. PKCζ did not phosphorylate the other residues that were phosphorylated by “conventional” PKCs, indicating that PKCζ and conventional PKCs may have discrete specificities for p47phox functions. In addition, the sites previously found to be phosphorylated by MAP kinase (S345/S348A) and by PKA (S320A) and one or both peptides containing S328A and S359/370A (16) were not phosphorylated by PKCζ.

Site-directed mutagenesis studies using EBV-transformed lymphocytes expressing exogenous wild-type or p47phox mutants in which serine residues were replaced by alanine residues indicated that each individual serine residue so far identified is phosphorylated independently (34). Our data regarding phosphorylation of the three p47phox mutants (S315A, S320A, and S328A) by PKCζ are in agreement with this assumption. However, in the case of the p47phox S303/304A mutant, the phosphorylation of some residues by PKCζ was strongly blunted. The decreased phosphorylation might result from an altered conformation of p47phox caused by the mutation. However, this possibility can be ruled out because the p47phox mutant S303/304A is still phosphorylated in PMA-activated EBV-lymphoblasts (34) and by “conventional” PKCs in vitro (data not shown). Alternatively, the strong decrease in the phosphorylation of the p47phox mutant suggests that phosphorylation of S303/304A may facilitate the subsequent phosphorylation
of other residues. Finally, the double mutation S(303 + 304)A inhibited superoxide production in B lymphoblasts (54), underlining the importance of the phosphorylation of these two serine residues in oxidase activation in intact cells.

p47phox is extensively phosphorylated during PMN stimulation (42) and plays the main role in transporting the cytosolic oxidase components to the plasma membrane (13). IMLP and PMA, two major activators of NADPH oxidase, induce the phosphorylation of several serine residues including S303/304A and S315A (42). In human PMN stimulated by IMLP, PKC ζ was shown to be activated. To examine the contribution of PKC ζ to NADPH oxidase activity, we used a synthetic peptide antagonist of PKC ζ to down-regulate the enzyme activity. The sequence of this peptide corresponds to the pseudosubstrate region of the N-terminal regulatory domain that maintains the enzyme in an inactive form in the absence of activator (55). This peptide, which was previously found to block PKC ζ-dependent signal transduction in Xenopus oocytes and mouse fibroblasts (46–56), inhibited ~50% of the IMLP-induced translocation of p47phox and PMN respiratory burst. The specificity of this inhibition was suggested by the absence of alteration of PMA-induced respiratory burst. Because PMA is a direct activator of both “conventional” and “novel” PKCs (21, 57), these data further suggest that the peptide antagonist does not interfere with oxidase activation mediated by these two classes of PKC. From these experiments, it appears that PKC ζ may contribute substantially to the stimulation of PMN respiratory burst by chemotactants. Consistent with this assumption, the peptide antagonist inhibited the phosphorylation and translocation of p47phox induced by IMLP but not by PMA. Further evidence for the involvement of PKC ζ in NADPH oxidase regulation comes from the antisense-mediated inhibition of PKC ζ expression in neutrophil-like cells from differentiated HL-60 cells. Again, this treatment, which caused a significant inhibition of the respiratory burst induced by IMLP, was ineffective on PMA-mediated superoxide production. The oligonucleotide treatment did not alter the expression of other PKC isozymes (α, β, and δ), further indicating its specificity for PKC ζ. The sense sequence of PKC ζ we used is also shared by a recently cloned human serine/threonine kinase termed kpm (58). However, the sense sequence within kpm is located more than 1500 bp away from the initiation codon, which makes the antisense oligonucleotides less effective than those targeting around the translational start codon, as in the case of PKC ζ. In addition, kpm is expressed during mitosis and has been shown to regulate cell cycle; there is no evidence that kpm can be activated by PKC ζ activators. Finally, an important contribution of PKC ζ in respiratory burst is further illustrated by the strong inhibition of respiratory burst and p47phox phosphorylation by the PKC ζ peptide antagonist (pseudo-substrate region), the sequence of which is not present in kpm.

PKC ζ can be activated in vitro by various lipidic second messengers that are generated by different signaling effectors. Among these messengers, phosophatidylinositol-3,4,5 trisphosphate is formed by phosphatidylinositol 3-kinase (PI3-kinase); PA is produced by phospholipase D (PLD) or DAG kinase; and ceramide is generated by sphingomyelinase. These signaling effectors are all stimulated in PMN by IMLP and may have a potential role in regulating the respiratory burst (48), although their respective contribution to the stimulation of PKC ζ remains unknown. It was shown recently that in PMN from PI3-kinase-γ knockout mice the PMN respiratory burst induced by IMLP was markedly inhibited (59, 60). Interestingly, in this model the translocation of p47phox as well as Rac to the plasma membrane was not decreased (59), indicating that signaling pathways other than PI3-kinase-γ may be required for redistribution of p47phox and Rac. The PLD pathway appears to be a possible candidate for this regulation. This hypothesis is supported by the observation that PLD is a major source of PA and diglycerides in stimulated PMN (45, 61) and may thus contribute to the activation of PKC ζ and other PKC isoforms. In addition, the PLD-derived PA production was associated with a priming of PMN respiratory burst (45, 62, 63), and this priming was accompanied by an enhanced phosphorylation of p47phox (19, 64). This study supports the hypothesis that several pathways might participate in p47phox phosphorylation. In PMA-activated cells, “conventional” PKCs, and possibly “novel” PKCs, are activated and phosphorylate p47phox. However, in more physiological conditions such as fMLP stimulation of neutrophils, other pathways, and so other PKC isoforms (such as PKC ζ), are activated and participate in p47phox phosphorylation. The cooperativity of different protein kinases or multiple isoforms of PKC in phosphorylating p47phox is explained by the fact that these kinases phosphorylate specific targets on p47phox.

In conclusion, this study shows that p47phox is a substrate for PKC ζ in vitro. Phosphorylation of p47phox occurs on the serine residue 303/304, on serine 315, and on two unidentified residues. The inhibition of PKC ζ activity or enzyme expression in human PMN by the use of a specific antagonist or antisense oligonucleotide markedly inhibited IMLP-induced superoxide production, membrane translocation of PKC ζ, and phosphorylation of p47phox. These data provide evidence of the involvement of PKC ζ in the signaling pathways leading to NADPH oxidase activation by fMLP receptors, but not by PMA, and suggest a role for PKC ζ in regulating the bactericidal function of PMN.

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