Selective Role for β-Protein Kinase C in Signaling for O₂ Generation but Not Degranulation or Adherence in Differentiated HL60 Cells*

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A role for protein kinase C (PKC) isotypes is implicated in the activation of phagocytic cell functions. An antisense approach was used to selectively deplete β-PKC, both β- and βII-PKC, but not α-PKC, δ-PKC, or ζ-PKC in HL60 cells differentiated to a neutrophil-like phenotype (dHL60 cells). Depletion of β-PKC in dHL60 cells elicited selective inhibition of O₂⁻ generation triggered by fMet-Leu-Phe, immune complexes, or phorbol myristate acetate, an activator of PKC. In contrast, neither ligand-elicited β-glucuronidase (azurophil granule) release nor adherence to fibronectin was inhibited by β-PKC depletion. Ligand-induced phosphorylation of a subset of proteins was reduced in β-PKC-depleted dHL60 cells. Phosphorylation of p47phox and translocation of p47phox to the membrane are essential for activation of the NADPH oxidase and generation of O₂⁻. β-PKC deletion had no effect on the level of p47phox in dHL60 cells but did significantly decrease ligand-induced phosphorylation of this protein. Furthermore, translocation of p47phox to the membrane in response to phorbol myristate acetate or fMet-Leu-Phe was reduced in β-PKC-depleted cells. These results indicate that β-PKC is essential for signaling for O₂⁻ generation but not cell adherence or azurophil degranulation. Depletion of β-PKC inhibited ligand-induced phosphorylation of p47phox, translocation of p47phox to the membrane, and activation of O₂⁻ generation.

Protein kinase C (PKC) mediates signaling for multiple functions of phagocytic cells, neutrophils, monocytes, macrophages, and differentiated HL60 (dHL60) cells. Ligands such as the chemotactic peptide fMet-Leu-Phe and phagocytic stimuli (immune complexes) trigger responses that include O₂⁻ generation, degranulation, adherence, and actin filament assembly (1–8). These functions are essential for the microbicidal activity of phagocytic cells and are also proinflammatory.

PKC, a phospholipid-dependent family of serine/threonine kinases, acts in multiple signal transduction pathways. The cofactor requirements differ between different classes of PKC isotypes. Classical α-, β-, and γ-PKC are acidic phospholipid, diglyceride (DG), and Ca²⁺-dependent; novel forms δ-, ε-, η-, and ζ-PKC also require acidic phospholipid and DG, but are Ca²⁺ independent. The atypical PKC isotypes, ζ- and λ-PKC, require PS but are DG and Ca²⁺ independent (9–14). PKC isotypes differ in their tissue distribution and localization within the cell, suggesting that each isotype plays a specific role in signal transduction.

Neutrophils, monocytes/macrophages, and dHL60 cells contain multiple isotypes of PKC, including Ca²⁺-dependent isotypes α-PKC, β-PKC, and βII-PKC, Ca²⁺-independent DG-dependent isotype, δ-PKC, and atypical PS-dependent, Ca²⁺/DG-independent ζ-PKC (3, 14–16). PKC has been implicated in the signaling for several different responses of phagocytic cells because PMA triggers O₂⁻ generation and adherence but not the release of azurophil granules (1, 17). The PKC substrates involved in these processes are largely unknown. The cytoskeleton and integrins are involved in cell adherence, and several cytoskeletal proteins, including MARCKS, are PKC substrates (8).

Assembly of an active NADPH oxidase for generation of O₂⁻ requires translocation of cytosolic factors p47phox, p67phox, and rac2 to the plasma membrane, where they interact with the integral membrane protein cytochrome b₅₅₈ (18–25). p47phox is phosphorylated in ligand-activated phagocytic cells. p47phox contains multiple phosphorylation sites, including a number of classical PKC substrate sites (RXXX/TXRX) and is phosphorylated by β-PKC in vitro (3). In addition, p47phox phosphorylated in vitro by PKC, but not cAMP-activated kinase or mitogen-activated kinase, was active in a cell-free system for activation of the NADPH oxidase (4–7). However, in vitro activity does not necessarily predict a role for a particular PKC isotype in the intact cell, where access to both substrate and cofactors is critical in controlling signaling specificity. Specific functions for each PKC isotype remain to be established.

In the present study, an antisense strategy was used to probe a role for a specific PKC isotype in signaling for the ligand-activated responses of O₂⁻ generation and cell adherence in dHL60 cells. β-PKC was selectively depleted by an antisense strategy in dHL60 cells. Selective depletion of β-PKC in dHL60 cells decreased O₂⁻ generation but not degranulation or adherence. Depletion of β-PKC reduced ligand-induced phosphorylation of a subset of proteins including p47phox and reduced translocation of p47phox to the membrane, consistent with a selective role for β-PKC in signaling for assembly of an active NADPH oxidase.

MATERIALS AND METHODS

Reagents—Cytochalasin B, cytochrome c, phenolphthalein glucuronidase, protease inhibitors (leupeptin and aprotinin), BSA, PMA, fMet-Leu-Phe, and PMSF were purchased from Sigma. PMA was stored as a concentrated stock solution in Me₂SO and diluted with Krebs-
Ringer buffer before use, fMet-Leu-Phe was stored as a stock solution in ethanol and diluted in buffer prior to use. BSA and anti-BSA IgG (Cappel, Durham, NC) were used to form an immune complex by the method of Ward and Zvelebil (26).

Antisense, sense, and scrambled control oligonucleotides were synthesized by the PENN Nucleic Acid Facility as described previously (2), and by centrifuging for 20 min at 115,000 × g at 4 °C. PKC activity of cytosol fractions was assayed in the presence of Ca2+, PS, and DG by measuring the incorporation of 32P into histone type IIb or into PKC substrate peptide (Ser41-Pro19-casein) (4). The net PKC-dependent activity was determined by subtracting the PKC-independent, PS/DG activity from the Ca2+/PS/DG-dependent activity.

Selective Role for β-PKC in O2 Generation

The generation of superoxide anion (O2·-) by dHL60 cells was measured as superoxide dismutase-inhibitable cytochrome c reduction by either a continuous recording method (28) or endpoint analysis. Cells were activated by 1 μM fMet-Leu-Phe in the presence of 10 μM BSA/well, 50 μg/ml leupeptin, 24 μg/ml pepstatin, or 1 μM M antipain, 24 μg/ml cytochalasin B, or by 1 μM PMA, 10 μg/ml cytochalasin B, 50 μg/ml leupeptin, 24 μg/ml pepstatin, or 1 μM M antipain, 24 μg/ml cytochalasin B, or by 5 μg/ml cytochalasin B/anti-BSA immune complex in the absence of cytochalasin B.

Degranulation—The release of the azurophil granule-associated enzyme-β-glucuronidase triggered by fMet-Leu-Phe from dHL60 cells was measured in the presence of 5 μg/ml cytochalasin B to allow extracellular release of granule contents. Degranulation triggered by insoluble immune complex, BSA/anti-BSA, was measured in the absence of cytochalasin B. dHL60 cells (5 × 106 cells/well) were incubated for 5 min at 37 °C with buffer, fMet-Leu-Phe (1 μM) or BSA/anti-BSA (300 μg/ml).

Protein Phosphorylation in Activated dHL60 Cells—dHL60 cells (5 × 106 cells/well) were incubated for 60 min at 37 °C with “P-P” (250 μCi of [32P]orthophosphoric acid/ml). The 32P-labeled cells were stimu- lated with either buffer alone or fMet-Leu-Phe (1 μM) for 1 min in the presence of 5 μg/ml cytochalasin B. The reaction was stopped by placing the samples on ice. Cell lysates were prepared and run on 4–12% gradient SDS-PAGE. The gel was dried and subjected to autoradiography.

Adherence—Adherence of dHL60 cells to fibronectin-coated plates was determined by a colorimetric assay according to the method of Aumailley et al. (31). 96-well microtiter plates were coated with fibronectin as described by Nathan et al. (32). dHL60 cells (4 × 105 cells/well) were incubated for 30 min at 37 °C with either buffer, fMet-Leu-Phe (1 μM) or PMA (1 μg/ml). Nonadherent cells were removed by washing with Krebs-Ringer phosphate-buffered saline buffer, and the adherent cells were fixed with 70% ethanol. The bound cells were stained with crystal violet (0.1% in D.H.O). The plates were washed extensively to remove excess stain, and the cells were solubilized with 1% Nonidet P-40 in D.H.O. Optical density was read at 550 nm.

Preparation of Particulate Fractions from fMet-Leu-Phe-stimulated dHL60 Cells and Translocation of p47phox and PKC Isotypes to Cell Membranes—dHL60 cells (2.5 × 106 cells/ml) were incubated at 37 °C in the presence of buffer, 1 μM fMet-Leu-Phe (1 min) or 1 μM PMA (5 min). At the end of the incubation period, the cell suspension was transferred to an ice bath. The suspension was then centrifuged for 10 min at 300 × g, and the cell pellet was resuspended in buffer containing 131 mM NaCl, 1 mM EDTA, 100 mM potassium phosphate buffer, pH 7.0, 2 mM PMSF, 20 μg/ml leupeptin, 20 μg/ml aprotinin, and 20 μg/ml pepstatin (buffer A). The cells were disrupted using three 5-s bursts of a microprobe sonicator at low power at 4 °C and centrifugation for 20 min at 115,000 × g) to remove unbroken cells and nuclei. The supernatant was layered over a 15% sucrose cushion made up in buffer A and centrifuged at 4 °C for 20 min at 115,000 × g. The supernatant (cytosol) was mixed 4:1 with 4 × SDS sample buffer. The pellet was solubilized in hot Laemmli buffer; pellet (10 × 106 cell equivalents/lane) and cytosol (3 × 106 cell equivalents/lane) were run on 4–12% SDS-
Depletion of β-PKC Isotypes by Antisense Treatment—To deplete β-PKC, dHL60 cells were first treated with 1.3% Me₂SO for 4 days to initiate differentiation, followed by two treatments with β-PKC antisense oligonucleotide (βAS) and the cationic lipid DMRIE-C (2.5 μg/ml) at 24-h intervals (see “Materials and Methods”). Preliminary experiments demonstrated that cationic lipids were essential to enhance the potency of the oligonucleotides (33, 34). Phosphorothioate oligonucleotides were synthesized since these have a greater intracellular half-life than the native oligonucleotides. Since the half-life of PKC was long, reported to vary from 6.7 h to over 24 h, prolonged treatment with βAS was used (35). The cells were treated twice with the βAS/DMRIE-C mixture to prolong the exposure to βAS and to achieve decreased protein expression of β-PKC (35); a single treatment with βAS/DMRIE-C was not effective. The effect of antisense treatment on dHL60 cells following differentiation was examined over a concentration range of 0–1000 nM βAS (Fig. 1). Treatment with βAS resulted in decreased expression of β-PKC (Fig. 1). Depletion of β-PKC followed a bell-shaped concentration response curve; maximal depletion of β-PKC immunoreactivity occurred at 250 and 500 nM βAS (Fig. 1). For this experiment, β-PKC was reduced to 23 and 31% of control levels by 250 and 500 nM βAS, respectively. At higher concentrations of βAS oligonucleotide, there was increased β-PKC expression. Attempts to enhance the depletion of β-PKC by more prolonged incubation or by increases in cationic lipid levels were not successful due to a loss of cell viability.

Selective Depletion of β-PKC and Not α-PKC, δ-PKC, or ζ-PKC—dHL60 contained numerous PKC isotypes, α-PKC, β₁-PKC, β₁-δ-PKC, δ-PKC, and ζ-PKC, in agreement with other workers (Fig. 2A) (15). The predominant isotypes were α-, β₁-, and δ-PKC, whereas β₁-PKC and ζ-PKC were present at lower levels (Fig. 2A). No evidence for ε-PKC (Fig. 2A), γ-PKC, η-PKC, or θ-PKC (results not shown) was found in dHL60 cells. dHL60 cells were treated for 48 h with 300 nM β-PKC antisense (βAS) and β-PKC missense oligonucleotide (βMS) in the presence of DMRIE-C (2.5 μg/ml). Selectivity of depletion of β-PKC was assessed by probing the blot with antibodies to α-PKC, β₁-PKC, β₁-δ-PKC, δ-PKC, and ζ-PKC. Both β-PKC isotypes, β₁-δ-PKC and β₁-ε-PKC, were effectively depleted by βAS treatment but not by βMS treatment (Fig. 2B) or in sense oligonucleotide-treated cells (results not shown). β₁-PKC was reduced to 45.6 ± 5.3% (n = 10) of control (βMS) levels, and β₁-δ-PKC was reduced to 60.8 ± 4.4% (n = 10) of control levels. In contrast, a concentration of βAS, which was effective in depleting β-PKC, had no effect on the expression of other PKC isotypes including α-PKC, δ-PKC, and ζ-PKC (Fig. 2B). Calcium/PS/DG-dependent histone III phosphorylating activity was 21.6 pmol/10⁷ cell equivalents/min in cytosol from control βMS-treated cells; calcium/PS/DG-dependent phosphorylating activity in β-PKC-depleted cells was reduced to 73.6 ± 1.7% (n = 3, p < 0.001) of control activity. This finding demonstrates that βAS depleted, but did not abolish, calcium-dependent PKC activity. Thus, treatment with the β-PKC antisense oligonucleotide selectively depleted dHL60 cells of β-PKC but not calcium-dependent α-PKC or calcium-independent δ-PKC and ζ-PKC.

Effect of β-PKC Depletion on Superoxide Anion Generation by dHL60 Cells—Activation of dHL60 cells by a variety of stimuli elicits the assembly of an active NADPH oxidase enzyme complex, which generates superoxide anion (O₂⁻). Previous studies suggested that β-PKC may play an important role in the activation of the NADPH oxidase (3, 20). Therefore, the effect of β-PKC depletion on O₂⁻ generation triggered by different ligands was evaluated. The PKC activator PMA (1 μg/ml) triggered continuous generation of O₂⁻ by dHL60 cells following a lag period of approximately 1–3 min (Fig. 3A). The mean lag period for control βMS-treated cells was 205 ± 55 s (n = 5); the lag period was significantly increased to 222 ± 43% (n = 5) of control (p < 0.05) in βAS-treated cells. Thus, depletion of β-PKC delayed the onset of O₂⁻ generation in PMA-activated cells. Control βMS-treated cells generated 33.5 ± 6.2 nmol (n = 5) O₂⁻/10⁶ cells/15 min (Fig. 3C). Treatment of cells with DMRIE-C/oligonucleotide was not toxic to the cells since the rate of O₂⁻ generation in the absence of DMRIE-C/oligonucleotide was 39.14 ± 5.7 (n = 4) nmol O₂⁻/10⁶ cells/15 min as compared with the rate of 33.50 ± 6.2 (n = 5) nmol O₂⁻/10⁶ cells/15 min in DMRIE-C/βMS-treated cells, a difference that is
Selective Role for β-PKC in \( \mathcal{O}_2 \) Generation

not significant. When dHL60 cells were treated with βAS (Fig. 2B), PMA-elicited \( \mathcal{O}_2 \) production was significantly decreased to a level of 25.1 ± 6.2 (n = 5) nmol \( \mathcal{O}_2/10^6 \) cells/15 min (p < 0.01), a level that was 71.6 ± 7.8% of control (p < 0.02) (Fig. 3C). In addition, the \( V_{\text{max}} \) of PMA-induced \( \mathcal{O}_2 \) generation, defined as the maximal rate of \( \mathcal{O}_2 \) generation, was reduced in cells depleted of β-PKC. Calculation of the \( V_{\text{max}} \) demonstrated that in βMS-treated cells activated by 1 μg/ml PMA, the \( V_{\text{max}} \) was
2.51 ± 0.80 (n = 5) nmol/min/10^6 cells; the \( V_{\text{max}} \) of \( \beta\)-PKC-depleted cells was significantly reduced to 2.04 ± 0.70 nmol/min/10^6 cells (n = 5), which was 75.8 ± 7.8% of control \( \beta\)-MS-treated cells (p < 0.04).

The chemotactic peptide, fMet-Leu-Phe (1 \( \mu \)m), triggers \( O_2^\cdot \) generation by dHL60 cells in the presence of cytochalasin B (Fig. 3B). The onset of \( O_2^\cdot \) generation was rapid but ceased after 3–4 min. (Fig. 3D). Control (\( \beta\)-MS-treated) cells activated by 1 \( \mu \)m fMet-Leu-Phe generated 16.88 ± 2.6 nmol/10^6 cells/15 min (n = 8) (Fig. 3C). \( O_2^\cdot \) generation was significantly decreased in \( \beta\)-PKC-depleted (\( \beta\)-AS-treated) cells to 4.83 ± 2.1 nmol/10^6 cells/15 min (n = 8) (p < 0.001), a level that was 28.5% of control values (p < 0.01) (Fig. 3C). The kinetics of inhibition of \( O_2^\cdot \) generation following \( \beta\)-PKC depletion were examined in fMet-Leu-Phe-activated cells. The lag time of reaction was approximately 10–15 s. after addition of f-Met-Leu-Phe, and was not significantly altered by depletion of \( \beta\)-PKC. However, the \( V_{\text{max}} \) of \( \text{M}\text{et-Leu-Phe-depressed} \) \( O_2^\cdot \) generation was significantly decreased in \( \beta\)-PKC-depleted cells as compared with controls. The \( V_{\text{max}} \) of \( O_2^\cdot \) generation decreased significantly from a rate of 3.36 ± 0.57 nmol/min/10^6 cells (n = 8) in control \( \beta\)-MS-treated cells to a \( V_{\text{max}} \) of 1.45 ± 0.28 nmol/min/10^6 cells (p < 0.002; n = 8) in \( \beta\)-PKC-depleted cells. Ligands such as immune complexes, in addition to fMet-Leu-Phe, also trigger \( O_2^\cdot \) generation by dHL60 cells. \( \beta\)-AS treatment decreased \( O_2^\cdot \) generation in response to immune complex from 2.02 ± 0.14 nmol/10^6 cells/15 min (n = 3) in \( \beta\)-MS-treated cells to 1.04 ± 0.22 nmol/10^6 cells/15 min (n = 3) in \( \beta\)-AS-treated cells (Fig. 3C) and decreased the \( V_{\text{max}} \) from 0.68 ± 0.04 nmol/min/10^6 cells (n = 3) to 0.35 ± 0.07 nmol/min/10^6 cells (n = 3) (p < 0.01).

Thus, depletion of \( \beta\)-PKC in dHL60 cells results in inhibition of the rate of \( O_2^\cdot \) generation in response to a number of different ligands. The extent of inhibition of \( O_2^\cdot \) generation by \( \beta\)-PKC depletion was stimulus dependent; the greatest inhibition of \( O_2^\cdot \) generation by \( \beta\)-PKC depletion was observed in cells activated by fMet-Leu-Phe.

Depletion of \( \beta\)-PKC Isotypes Does Not Inhibit fMet-Leu-Phe-Elicited Degranulation by dHL60 Cells—Activation of dHL60 cells with ligands such as fMet-Leu-Phe triggers release of \( \beta\)-glucuronidase, an azurophil granule marker, simultaneously with the generation of \( O_2^\cdot \). The effect of depletion of \( \beta\)-PKC on the extracellular release of \( \beta\)-glucuronidase was monitored in dHL60 cells following stimulation with 1 \( \mu \)m fMet-Leu-Phe. Treatment of dHL60 cells with \( \beta\)-AS plus DMRIE-C, at a concentration that significantly depleted levels of \( \beta\)-PKC and inhibited \( O_2^\cdot \) production in response to fMet-Leu-Phe, had no significant effect on degranulation (Fig. 4); 10.5 ± 2.7% (n = 4) of total \( \beta\)-glucuronidase was released in response to fMet-Leu-Phe in control (\( \beta\)-MS-treated) cells, whereas 12.2 ± 2.5% (n = 4) of total \( \beta\)-glucuronidase was released from \( \beta\)-PKC-depleted cells. Similarly, degranulation triggered by BSA/anti-BSA in the absence of cytochalasin B was not affected by depletion of \( \beta\)-PKC; 7.5 ± 0.2% (n = 4) of total \( \beta\)-glucuronidase was released in response to BSA/anti-BSA in control (\( \beta\)-MS-treated) cells, whereas 8.3 ± 0.6% (n = 4) of total \( \beta\)-glucuronidase was released from \( \beta\)-PKC-depleted cells. Furthermore, total cell content of \( \beta\)-glucuronidase, a marker for HL60 cell differentiation, was not altered in \( \beta\)-PKC-depleted cells (Fig. 4). Therefore, depletion of \( \beta\)-PKC with a \( \beta\)-PKC antisense oligonucleotide selectively inhibits ligand-elicited \( O_2^\cdot \) generation but not \( \beta\)-glucuronidase release.

Effect of \( \beta\)-PKC Depletion on Adherence of dHL60 Cells to Fibronectin—A role for PKC in integrin-mediated adherence to fibronectin has been suggested since phorbol esters activate adherence mechanisms in phagocytic cells (7, 36). To ascertain whether \( \beta\)-PKC plays a role in integrin-mediated adherence in dHL60 cells, the effect of \( \beta\)-PKC depletion on basal and stimulated adherence to fibronectin was examined in dHL60 cells. Activation of control (\( \beta\)-MS-treated) cells by PMA (1 \( \mu \)g/ml) or fMet-Leu-Phe (1 \( \mu \)m) triggered increased cell adherence to fibronectin-coated wells (Fig. 5). PMA produced the greatest increase in adherence, to 242.1 ± 64.0% (n = 4) of resting levels in control (\( \beta\)-MS-treated) cells, whereas in \( \beta\)-PKC-depleted cells, a similar level of PMA-induced adherence, 246.8 ± 40.4% (n = 4) of resting levels, was observed. fMet-Leu-Phe (1 \( \mu \)m) triggered increased adherence to 175.1 ± 41.5% (n = 3) of resting levels in control (\( \beta\)-MS-treated) cells, whereas in \( \beta\)-PKC-depleted cells, a similar level of fMet-Leu-Phe-induced adherence, 214.38 ± 62.5% (n = 4) of resting levels, was observed. As shown in Fig. 5, \( \beta\)-AS treatment under conditions that significantly altered fMet-Leu-Phe and PMA-induced \( O_2^\cdot \) production had no significant effect on cell adherence to fibronectin in unstimulated or stimulated cells. Thus, depletion of \( \beta\)-PKC in dHL60 cells elicits selective inhibition of \( O_2^\cdot \) generation but not \( \beta\)-glucuronidase release or adherence to fibronectin.

Effect of \( \beta\)-PKC Depletion on Protein Phosphorylation Triggered by 1 \( \mu \)m fMet-Leu-Phe—\( \beta\)-AS- and \( \beta\)-MS-treated dHL60 cells were labeled with [32P]inorganic phosphate for 1 h at 37 °C and treated with buffer or fMet-Leu-Phe. Cell lysates were prepared, the phosphorylated proteins were separated on SDS-PAGE, and the gels were subjected to autoradiography. Activation of the cells for 1 min with 1 \( \mu \)m fMet-Leu-Phe triggered phosphorylation of multiple proteins, including 18-, 24-, 38-, 43-, 47-, 54-, 68-, and 80-kDa proteins (Fig. 6A). Phosphorylation of prominent 14-, 97-, and 105-kDa bands was not significantly altered in fMet-Leu-Phe-activated cells (Fig. 6A). Densitometry of the autoradiograph demonstrated that treatment of cells with antisense to \( \beta\)-PKC reduced the fMet-Leu-Phe-induced phosphorylation of the 18-, 43-, 47-, 54-, and 68-kDa proteins (Fig. 6B); the 47-kDa band is a candidate for p47Fyn. In contrast, phosphorylation of a band at 105 kDa was enhanced in \( \beta\)-PKC-depleted cells. The effect of \( \beta\)-PKC depletion on fMet-Leu-Phe-induced protein phosphorylation was selective, since treatment with \( \beta\)-AS had no significant effect on the fMet-Leu-Phe-induced phosphorylation of the 24- or 80-kDa bands. These results suggest that depletion of \( \beta\)-PKC inhibits fMet-Leu-Phe-induced phosphorylation of a discrete number of proteins including a 47-kDa band, which is a candidate for...
p47\textsuperscript{phox}, an important component of the NADPH oxidase.

**Depletion of β-PKC and Reduced Ligand-initiated Phosphorylation of p47\textsuperscript{phox}**—Phosphorylation of p47\textsuperscript{phox} and its translocation to the membrane-associated cytochrome b\textsubscript{558} is essential for assembly of the NADPH oxidase. β-PKC-depleted dHL60 cells were used to determine whether β-PKC was essential for phosphorylation of p47\textsuperscript{phox} in cells activated by fMet-Leu-Phe. dHL60 cells were pretreated with βAS or βMS and stimulated for 1 min with 1 µM fMet-Leu-Phe; the p47\textsuperscript{phox} was then immunoprecipitated. Western blotting and densitometry analysis demonstrated that control (βMS) and β-PKC-depleted (βAS) dHL60 cells contained equivalent amounts of p47\textsuperscript{phox} immunoreactivity (Fig. 7A). However, phosphorylation of the p47\textsuperscript{phox} band was reduced in the βAS-treated dHL60 cells as compared with the control (βMS-treated) cells; \textsuperscript{32}P counts eluted from the βAS band were 56% of control levels measured in the βMS band (Fig. 7B). β-PKC-depleted cells contained equivalent protein levels of p47\textsuperscript{phox} as compared with control cells. However, fMet-Leu-Phe-induced phosphorylation of p47\textsuperscript{phox} was decreased in the β-PKC-depleted cells, concordant with a role for β-PKC in phosphorylation of p47\textsuperscript{phox} in ligand-initiated signaling and in the assembly of an active NADPH oxidase.

**Effect of βPKC Depletion on p47\textsuperscript{phox} Translocation**—Phosphorylation and translocation of p47\textsuperscript{phox} to the membrane and association of phosphorylated p47\textsuperscript{phox} with cytochrome b\textsubscript{558} are essential steps in the assembly of an active NADPH oxidase complex. Activation of dHL60 cells by fMet-Leu-Phe or PMA elicited translocation of p47\textsuperscript{phox} from the cytosol to the membrane fraction (Fig. 8). Depletion of β-PKC by βAS treatment was associated with decreased translocation of p47\textsuperscript{phox} from the cytosol to the membrane in both fMet-Leu-Phe- and PMA-activated cells as compared with βMS-treated controls (Fig. 8). In contrast, the membrane-associated gp91\textsuperscript{phox} subunit of cytochrome b\textsubscript{558} was not affected by depletion of β-PKC (Fig. 8). In resting cells, βI-PKC and βII-PKC were predominantly in the cytosol (Fig. 8). Activation of dHL60 cells by 1 µM fMet-Leu-Phe for 1 min elicited translocation of both βI-PKC and βII-PKC from the cytosol to the membrane (Fig. 8). Activation of the cells with 1 µg/ml PMA for 5 min triggered an almost total disappearance of βI-PKC and βII-PKC from the cytosol and translocation of both isotypes to the membrane (Fig. 8). In the β-PKC-depleted cells, less βI and βII-PKC was associated with the membrane in fMet-Leu-Phe- and PMA-treated cells.
The inhibitory effect of β-PKC depletion on O₂⁻ generation was functionally selective. The simultaneously elicited release of the azurophil granule markers, β-glucuronidase and elastase (results not shown), from fMet-Leu-Phe-activated cells was not decreased in β-PKC-depleted cells. This finding is concordant with previous findings that activators of PKC, such as PMA, do not trigger azurophil degranulation and that the kinase inhibitor staurosporine does not inhibit azurophil degranulation (17). These findings also demonstrate that β-PKC depletion did not have a nonspecific effect on signaling for cell activation.

DHE60 cells adhere to fibronectin by a β1 integrin (36); adherence of DHE60 cells to a fibronectin-coated surface was triggered by PMA and fMet-Leu-Phe. Depletion of β-PKC in DHE60 cells did not inhibit either PMA or fMet-Leu-Phe-induced adherence to fibronectin. Thus, signaling for adherence must use other isotypes of PKC such as α- or δ-PKC that, like β-PKC, are also activated by PMA.

**DISCUSSION**

A role for PKC is implicated in the signaling for proinflammatory responses of phagocytic cells such as generation of O₂⁻, actin assembly, and cell adherence. PKC is a family of structurally related isotypes that have different cofactor and substrate specificities (9, 10). It has therefore been suggested that different isotypes of PKC transduce different signals, although there is little evidence to date to indicate specific functions for each isotype of PKC.

Multiple isotypes of PKC were demonstrated in DHE60 cells including α-, βI-, βII-, δ-, and γ-PKC, in agreement with other studies (15); the expression of these PKC isotypes is similar to the PKC isotypes observed in neutrophils (3, 14). In addition, the cofactors required for activation of PKC, calcium, DG, and PIP₃, are elevated in ligand-activated phagocytic cells, indicating a potential role for these PKC isotypes in activating responses such as O₂⁻ generation and adherence (37–40).

A role for β-PKC in ligand-initiated responses was probed by selective depletion of the β-PKC isotypes. Selective depletion by antisense was used since difficulty in distinguishing primary and secondary effects may be seen in overexpression studies either of the wild-type isotype or of a dominant negative mutant (41). Selective depletion of β-PKC but not depletion of α-, δ-, or γ-PKC was achieved using an antisense oligonucleotide designed against the translocation start site of β-PKC. This oligonucleotide depleted both β1-PKC and βII-PKC, which are derived by alternate splicing and only differ at the 3'-terminus. Depletion of β-PKC by 45–60% was associated with decreased O₂⁻ generation triggered by PMA, fMet-Leu-Phe, or immune complexes. The extent of inhibition of O₂⁻ generation in β-PKC-depleted cells was ligand dependent; the greatest inhibition was observed in fMet-Leu-Phe-activated cells and the smallest inhibition in PMA-activated cells. The greater degree of inhibition of O₂⁻ generation observed in response to fMet-Leu-Phe and immune complexes, as compared with PMA, may reflect differences in signaling initiated by these ligands. fMet-Leu-Phe may use β-PKC at more than one step in the signaling pathway. There is a noteworthy difference in the kinetics of O₂⁻ generation triggered by PMA, which is continuous, as compared with the fMet-Leu-Phe-induced response, which ceases after approximately 3 min (1, 17). In addition, continuous receptor occupancy is required to maintain fMet-Leu-Phe-induced O₂⁻ generation (42). Thus, a role for β-PKC might be involved in maintaining fMet-Leu-Phe-induced O₂⁻ generation. Finally, PMA is promiscuous and might recruit other PKC isotypes, whereas fMet-Leu-Phe may be constrained to recruit only β-PKC for a key step in activation of the NADPH oxidase.

**Selective Role for β-PKC in O₂⁻ Generation**

Generation triggered by PMA, fMet-Leu-Phe, or other stimuli is blocked by depletion of β-PKC. These findings are concordant with a role for β-PKC-dependent phosphorylation of p47phox in translocation of p47phox to the membrane and assembly of an active NADPH oxidase.

**Fig. 7. Effect of depletion of β-PKC on phosphorylation of p47phox in dHL60 cells activated by fMet-Leu-Phe.** DHL60 cells treated with βAS or βMS oligonucleotides were prelabeled with [32P]-P, (300 μCi) for 60 min at 37 °C. fMLP (1 μM) was added (zero time), and the reaction was stopped after 1 min. p47phox was immunoprecipitated from each sample, and the immune complexes were run on a 4–12% gradient SDS-PAGE and blotted to PVDF membrane. Representative experiment of two. A, Western blot using anti p47phox. B, autoradiogram of membrane. Molecular weight markers are indicated on the left margin and p47phox on the right margin.

**Fig. 8. Effect of depletion of β-PKC on translocation of cytosolic p47phox and β-PKC isotypes to cell membranes in resting, fMet-Leu-Phe-, and PMA-activated dHL60 cells.** DHL60 cells were treated with βAS or βMS oligonucleotides (see Materials and Methods). DHL60 cells were treated with buffer, 1 μM fMet-Leu-Phe (1 min) or 1 μg/ml PMA (5 min). Membrane (10 × 10⁶ cell equivalents/lane) and cytosol (3 × 10⁶ cell equivalents/lane) fractions were prepared, run on a 4–12% gradient SDS-PAGE, blotted to PVDF membrane, and probed with antibodies to p47phox, β1-PKC, βII-PKC, and gp91phox. Representative experiment of five.

(8). Densitometry demonstrated that in fMet-Leu-Phe-activated cells, 606 ± 121 DU (density units) (n = 5) of βII-PKC was associated with the membrane in β-PKC-depleted cells as compared with 1000 ± 162 DU (n = 5) in βMS-treated cells (60.1 ± 9.5% control, p < 0.03). In PMA-activated cells, 1125 ± 162 DU (n = 4) of βII-PKC was associated with the membrane in β-PKC-depleted cells as compared with 1493 ± 181 DU (n = 4) in βMS-treated cells (75.0 ± 2.9% control, p < 0.02). Similarly, membrane-associated β1-PKC was significantly reduced to 125 ± 17 DU (n = 5) in β-PKC-depleted cells activated with fMet-Leu-Phe as compared with 481 ± 144 DU (n = 5) in βMS-treated control cells (39.0 ± 3.7% control, p < 0.09). In PMA-activated cells, membrane-associated β1-PKC was significantly reduced to 300 ± 13 DU (n = 5) in β-PKC-depleted cells activated as compared with 991 ± 98 DU (n = 5) in βMS-treated control cells (38.4 ± 6.7% control, p < 0.01). These results are concordant with a role for β-PKC-dependent phosphorylation of p47phox in translocation of p47phox to the membrane and assembly of an active NADPH oxidase.
Identifying key substrate(s) is important in defining a role for β-PKC in signal transduction. Addition of fMet-Leu-Phe to dHL60 cells triggered phosphorylation of numerous proteins. In β-PKC-depleted cells, phosphorylation of a subset of proteins was reduced in response to mILP, indicating a selective effect of β-PKC depletion on ligand-induced phosphorylation. Phosphorylation of a cytosolic component of the NADPH oxidase, p47phox, and translocation and binding of p47phox to cytochrome b558 are essential steps in ligand-initiated activation of the NADPH oxidase. Indeed, depletion of β-PKC reduced the fMet-Leu-Phe-induced phosphorylation of a 47-kDa band and phosphorylation of immunoprecipitated p47phox. However, the level of p47phox, which is a differentiation marker in HL60 cells, was not altered by depletion of β-PKC. Thus, phosphorylation of p47phox is triggered by β-PKC in activated dHL60 cells.

Phosphorylation of p47phox is required for a conformational change in p47phox, which releases binding of p47phox to itself and to p40phox (43), and allows translocation and binding of p47phox to membrane-associate cytochrome b558 (43–46). In the present study, fMet-Leu-Phe and PMA triggered phosphorylation and translocation of p47phox from the cytosol to the membrane in dHL60 cells. In β-PKC-depleted dHL60 cells, the ligand-induced translocation of p47phox to the membrane was reduced, concurrent with a role for β-PKC in the phosphorylation and translocation of p47phox to the membrane-associated cytochrome b558 and activation of the NADPH oxidase. Several serines in the C terminus of p47phox, including Ser-304, Ser-309, and Ser-379, are consensus sequences for phosphorylation by PKC (47), and we have demonstrated that in vitro β-PKC phosphorylates p47phox (3). The importance of a role for phosphorylation of p47phox by PKC for assembly of an active NADPH oxidase was demonstrated in a neutrophil cell-free system (4). Phosphorylation of Ser-379 was essential for translocation of p47phox to the membrane and activation of the NADPH oxidase, whereas a double mutation of Ser-303 and Ser-304 inhibited oxidase activity but not translocation of p47phox (5, 6). The present finding that β-PKC depletion suggested both phosphorylation and translocation of p47phox suggests that Ser-379 was a target for phosphorylation by β-PKC.

Previous studies demonstrated translocation of β-PKC from cytosol to the membrane or cytoskeleton in PMA-activated neutrophils (3, 20, 48). Such translocation of PKC would allow β-PKC to access the activating lipid cofactors PS and DG, which are located in the membrane, as well as membrane-associate substrates. In the present study, both β-PI-PKC and βII-PKC were translocated to the membrane in dHL60 cells activated by fMet-Leu-Phe and by PMA. The amount of βII-PKC and βII-PKC recruited to the membrane in response to ligand was reduced in β-PKC-depleted cells. Thus, it is presently not possible to distinguish whether βII-PKC or βII-PKC is essential for activation of O2 generation. As these studies demonstrated a role for β-PKC in ligand-induced phosphorylation and translocation of p47phox, the findings do not preclude role(s) for other kinases in activation of O2 generation, either in phosphorylation of p47phox, which has multiple phosphorylation sites, or in other aspects of signaling. Roles for δ-PKC and a phosphatidate-activated kinase have been suggested in activation of the NADPH oxidase (16, 49).

Selective depletion of β-PKC by an antisense strategy demonstrated a selective role for β-PKC in signaling for fMet-Leu-Phe, immune complex, and PMA-induced O2 generation but not for ligand-initiated cell adherence to fibronectin or for azurophil degranulation. Depletion of β-PKC decreased ligand-induced phosphorylation of p47phox, translocation of p47phox and β-PKC to the membrane, and inhibited O2 generation.