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Reconstitution of Chemotactic Peptide-Induced Nicotinamide Adenine Dinucleotide Phosphate (Reduced) Oxidase Activation in Transgenic COS-phox Cells

Rong He,* Masakatsu Nanamori,* Hairong Sang,* Hong Yin,* Mary C. Dinauer,† and Richard D. Ye2*†

A whole-cell-based reconstitution system was developed to study the signaling mechanisms underlying chemoatractant-induced activation of NADPH oxidase. This system takes advantage of the lack of formyl peptide receptor-mediated response in COS-phox cells expressing gp91phox, p22phox, p67phox, and p47phox, which respond to phorbol ester and arachidonic acid with O₂⁻ production. By exogenous expression of signaling molecules enriched in neutrophils, we have identified several critical components for fMLP-induced NADPH oxidase activation. Expression of PKCδ, but not PKCα, -βIII, and -ζ, is necessary for the COS-phox cells to respond to fMLP. A role of PKCδ in neutrophil NADPH oxidase was confirmed based on the ability of fMLP to induce PKCδ translocation and the sensitivity of fMLP-induced O₂⁻ production to rottlerin, a PKCδ-selective inhibitor. Optimal reconstitution also requires phospholipase C-β2 and PI3K-γ. We found that formyl peptide receptor could use the endogenous Rac1 as well as exogenous Rac1 and Rac2 for NADPH oxidase activation. Exogenous expression of p40phox potentiated fMLP-induced O₂⁻ production and raised the level of O₂⁻ in unstimulated cells. Collectively, these results provide first direct evidence for reconstituting fMLP-induced O₂⁻ production in a nonhemopoietic cell line, and demonstrate the requirement of multiple signaling components for optimal activation of NADPH oxidase by a chemoatractant.


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Rac small GTPase, and possibly for phosphorylation and final assembly of the phox proteins. An understanding of the related signaling mechanisms is important for the control of undesirable activation of phagocytes, which contributes to the release of oxidants and tissue damage. Genetic studies involving targeted deletion of phospholipase C-β (PLCβ) 2/3 and PI3Kγ have shown that these enzymes are important for chemoattractant-induced NADPH oxidase activation (reviewed in Ref. 14). However it is not clear whether activation of these signaling molecules is sufficient for O2\textsuperscript{2-} production, and how they interact with kinases and GTPases that are directly responsible for the phosphorylation and translocation of phox proteins. An area of significant interest is the involvement of individual PKC isoforms in fMLP-induced NADPH oxidase activation. Unlike PKA that can activate multiple PKCs, signaling through FPR may only stimulate selected PKC isoforms. Another unanswered issue is the vast disparity among chemoattractant and chemokine receptors in their ability to activate NADPH oxidase. Although all these receptors are capable of mediating leukocyte chemotaxis, only a small fraction of them can stimulate O2\textsuperscript{2-} production. These unanswered questions necessitate the development of reconstitution systems in which receptor-mediated NADPH oxidase can be studied in detail.

To accomplish this goal, we explored several possibilities for reconstituting FPR-mediated NADPH oxidase activation in intact cells. A previous study demonstrates that expression of p69\textsuperscript{phox}, p67\textsuperscript{phox}, and p47\textsuperscript{phox} in the erythroleukemia cell line K562 renders the cells responsive to PMA in O2\textsuperscript{2-} production (15). Reconstitution of NADPH oxidase activity was also achieved by expression of p47\textsuperscript{phox} in EBV-transformed B cells that lack this cytosolic factor (16). In both cases, the reconstituted cells produced a relatively small amount of O2\textsuperscript{2-} compared with neutrophils. More recently, one of our laboratories generated a stable COS-7 line expressing p67\textsuperscript{phox}, p22\textsuperscript{phox}, p67\textsuperscript{phox}, and p47\textsuperscript{phox} (the COS-phox cell line) (17). Stimulation of these cells with PMA and arachidonic acid led to potent production of O2\textsuperscript{2-}, suggesting the possibility of using these genetically amenable cells to identify signaling molecules downstream of the activated FPR. Whereas K562 is a cell line of hemopoietic lineage and may already have the necessary signaling components for FPR signaling, COS-7 is an epithelial cell line that lacks the hemopoietic specific proteins required for FPR signaling (18). We exploited this property of the COS-phox cells to examine the roles of selected signaling molecules in FPR-mediated NADPH oxidase activation by taking a gain-of-function approach. In this study, we report reconstitution of fMLP-induced NADPH oxidase activity in the transgenic COS-phox cells. Our results indicate that the novel PKC isoform, PKCδ, plays an important role in fMLP-induced O2\textsuperscript{2-} production in both the transfected COS-phox cells and human neutrophils. Our results also suggest a role of p40\textsuperscript{phox} in positive regulation of fMLP-induced NADPH oxidase activation.

Materials and Methods

Reagents

The N-formyl peptide fMLP, PMA, and isoouminol were purchased from Sigma-Aldrich (St. Louis, MO). HRP and superoxide dismutase (SOD) were purchased from Roche (Indianapolis, IN). Pertussis toxin was obtained from List Laboratories (Campbell, CA). LY294002, GF109203X, and rottlerin were obtained from Calbiochem (San Diego, CA). Polyclonal antibody against G\textsuperscript{i2} was prepared against a synthetic peptide with an epitope tag and HA-epitope tag (Covance, Richmond, CA), rabbit polyclonal Abs against nonphosphorylated and phospho-PKC\textsuperscript{δ} (Th505) (Cell Signaling Technologies, Beverly, MA).

Expression vectors

A full-length cDNA for human FPR was subcloned into the pRK5 vector (BD Pharmingen). Plasmids containing cDNA inserts for wild-type Goi proteins were gifts from Drs. C. Knall and G. Johnson (National Jewish Center, Denver, CO). The PLCβ2 expression vector was a gift from Dr. D. Wu (University of Connecticut Health Center, Farmington, CT). Preparation and characterization of the HA-tagged PKCs, PKCβ, and PKC\textsuperscript{δ} expression constructs were described in a previous publication (19). The GFP expression vector EGF-P-N1 was from Clontech (Palo Alto, CA). A full-length cDNA for human Rac1 (Guthrie Research Institute, Sayre, PA) was subcloned into the pRK5 expression vector (BD Pharmingen). A Myc-tagged Rac1 expression construct was provided by Dr. U. Knaus (Scirpiss Research Institute, La Jolla, CA). Myc-tagged p110\textsuperscript{y} and p101 constructs were provided by Dr. A. Sinaraki (University of Rochester, Rochester, NY). The p40\textsuperscript{phox} expression vector was a gift from Dr. S. Chanock (National Cancer Institute, National Institutes of Health, Bethesda, MD).

Preparation of human neutrophils

Peripheral blood was drawn from healthy donors, using a protocol approved by the Institutional Review Board at the University of Illinois (Chicago, IL). Neutrophils were prepared using Percoll gradient centrifugation based on the method of Ulmer and Fadl (20), as detailed in a previous publication (21). The prepared cells contained ~97% neutrophils with viability ≥98%. Neutrophils were resuspended in serum-free RPMI 1640 medium at a density of 2 × 10\textsuperscript{6} cells/ml before use. Blood cells from different donors (n ≥ 3) were used in experiments.

Cell culture and transient transfection

The transgenic COS-phox cells were generated as described previously (17). The stable cell line was maintained at 37°C with 5% CO\textsubscript{2} in DMEM supplemented with 10% heat-inactivated FBS, 2 mM l-glutamine, 100 IU/ml penicillin, and 50 μg/ml streptomycin. Cells were grown by limiting dilution to the presence of 0.6 mg/ml hygromycin (Sigma-Aldrich), 0.8 mg/ml neomycin (Invitrogen Life Technologies, Carlsbad, CA), and 1 μg/ml puromycin (Calbiochem). LipofectAMINE 2000 reagent (Invitrogen Life Technologies) was used for transient transfection of 6–7 μg of DNA into COS-phox cells grown in a 100-mm culture dish (0.5 × 10\textsuperscript{6} cells per dish). Cells were analyzed 21–24 h after transfection. Transient transfection efficiency, determined by flow cytometry, based on fluorescence of a cotransfected GFP, was 45–55%.

Measurement of NADPH oxidase activity

Superoxide production by COS-phox cells and neutrophils was determined by an isoluminol-ECL assay (22), in 6-mm diameter wells of 96-well, flat-bottom, white tissue culture plates (E&K Scientific, Campbell, CA). COS-phox cells were harvested with enzyme-free cell-dissociation buffer (Invitrogen Life Technologies), and washed once with 0.5% BSA/HBSS. Cells were then resuspended in 0.5% BSA/RPMI 1640 buffer at the density of 3.5 × 10\textsuperscript{6} cells/ml, and preincubated in the dark with 100 μM isoluminol and 40 U/ml HRP at room temperature for 5 min. Aliquot (200 μl) of the cells was added into the well and assayed for chemiluminescence (CL) at 37°C in a Wallac 1420 Multilabel Counter (PerkinElmer Life Sciences, Boston, MA). The CL counts per second (cps) was continuously recorded, at 1-min intervals, for 5–15 min before and 20–30 min after stimulation with PMA or fMLP. Supernatant containing 250 U of SOD, in addition to the stimulators, were run in parallel. The relative level of superoxide production was calculated based on the integrated CL during the first 20 min (COS-phox cells) or first 10 min (neutrophils) after agonist stimulation.

Analysis of protein expression

Whole–cell extracts were generated as described previously (21). In brief, the transfected COS-phox cells were lysed with 200–300 μl of PAGE buffer containing protease inhibitors (Protease Inhibitor Mixture Set I; Calbiochem). Each sample was sonicated for 15 s on ice (60 Sonic Disemembrator; Fisher, Hampton, NH) and heated at 95°C for 5 min. Whole-cell extracts were analyzed by 10% denaturing SDS gels, and protein profiles were then transferred to nitrocellulose membranes (Hybond ECL; Amersham Biosciences, Piscataway, NJ) for Western blotting using ECL detection (Pierce, Rockford, IL). Flow cytometry measurements were used to determine the cell surface expression of FPR. Briefly, the transfected COS-phox cells were incubated with an anti-FPR mAb SFI (BD Pharmingen), washed in PBS containing 0.2% BSA, and then incubated with FITC-conjugated anti-mouse IgG (1:}
200). The green fluorescence of each single cell was detected using a FACScan flow cytometer (BD Biosciences, Mountain View, CA). All incubations were done on ice for 60 min.

Rac activation assay

Activation of Rac was determined as previously described (17), based on the affinity of Rac-GTP for the p21-binding domain (PBD) of PAK1 (23). The PBD-GST fusion protein was expressed in Escherichia coli strain HB101 and purified. Twenty-one hours posttransfection, COS-phox cells were detached with dissociation buffer, washed, and resuspended in RPMI 1640 containing 0.5% BSA to 5 × 10^6 cells/0.5 ml. The cells were then stimulated with fMLP or left untreated as indicated in the figures. Twenty micrograms of PAK1 PBD-GST recombinant protein was added, and cells were lysed by the addition of lysis/wash buffer (6 mM Na2HPO4, 4 mM NaH2PO4, 1% Nonidet P-40, 150 mM NaCl, 2.5 mM MgCl2) supplemented with 2 mM PMSF, Protease Inhibitor Cocktail III (Calbiochem), 0.1 mM Na3VO4, and 50 mM NaF. The lysate was cleared, 30 μl of glutathione-Sepharose 4B beads (Amersham Biosciences) was added, and the binding reaction was conducted for 1 h at 4°C. Beads were pelleted and washed three times with wash buffer, and then finally resuspended in 30 μl of Laemmli sample buffer. Aliquots of supernatant (Rac-GTP) and pull-down samples (Rac-GTP) were electrophoresed, and the proteins were analyzed as described above.

PKC phosphorylation and translocation

Isolated neutrophils (1–5 × 10^7 cells) were stimulated with either PMA or fMLP over a time course of 0–30 min, and stopped by addition of a 10-fold excess volume of cold HBSS. Cells were treated with 1 mM diisoproplfluorophosphate, suspended in hypotonic lysis buffer (50 mM Tris-HCl (pH 7.5), 5 mM MgSO4, 0.5 mM EGTA, 0.1% 2-ME) supplemented with 1 mM PMSF and protease inhibitors at a concentration of 1×10^6 cells/ml, and sonicated (5 s, three times, at level 2) on ice. The sonicated samples were centrifuged at 800 × g for 10 min at 4°C, and the supernatants were subjected to differential centrifugation at 150,000 × g for 90 min at 4°C to yield cytosolic fractions (supernatants) and membrane/particulate fractions (pellets). Membrane/particulate fractions were subjected to differential centrifugation at 150,000 × g for 90 min at 4°C to yield cytosolic fractions (supernatants) and membrane/particulate fractions (pellets). Membrane/particulate fractions were subjected to Western blotting to detect PKCθ translocation by using a specific anti-PKCθ Ab (Cell Signaling Technologies). For PKC phosphorylation assay, the stimulated neutrophils were directly lysed with 200–500 μl of SDS-PAGE buffer containing protease inhibitors, sonicated for 15 s on ice, and heated at 95°C for 5 min. Whole-cell extracts were analyzed by 10% denaturing SDS gels and subjected to differential centrifugation at 150,000 × g for 90 min at 4°C to yield cytosolic fractions (supernatants) and membrane/particulate fractions (pellets). Membrane/particulate fractions were subjected to Western blotting to detect PKCθ translocation by using a specific anti-PKCθ Ab (Cell Signaling Technologies). For PKC phosphorylation assay, the stimulated neutrophils were directly lysed with 200–500 μl of SDS-PAGE buffer containing protease inhibitors, sonicated for 15 s on ice, and heated at 95°C for 5 min. Whole-cell extracts were analyzed by 10% denaturing SDS gels and phospho-PKCθ was detected by Western blotting using a specific anti-phospho-PKCθ Ab (Cell Signaling Technologies).

Statistics

Paired Student’s t test was performed to determine statistical significance. A p value of <0.05 was considered to be significant.

Results

Expression of FPR in COS-phox is insufficient for reconstitution of fMLP-induced O2\textsuperscript{−} production

The COS-phox cell line was generated by stable expression of gp91\textsuperscript{phox}, p22\textsuperscript{phox}, p67\textsuperscript{phox}, and p47\textsuperscript{phox} in the monkey epithelial cell line COS-7 (17). Expression of these components enables the epithelial cells to respond to arachidonic acid and PMA with potent O2\textsuperscript{−} production (17) (Fig. 1). The PMA-induced O2\textsuperscript{−} generation was dose dependent, could be inhibited by SOD (Fig. 1, A and B), and displayed kinetics similar to that of neutrophils (Fig. 1A and data not shown). To determine whether COS-phox cells respond to fMLP, we expressed the human FPR by means of liposome-mediated transfection. Twenty-four hours after transfection, 45–55% of cells expressed FPR as detected by flow cytometry using an anti-FPR Ab (5F1) and a FITC-conjugated secondary Ab (solid line). However, fMLP was unable to induce O2\textsuperscript{−} production in COS-phox without or with the exogenous FPR (R). SOD (250 U) was included in one sample. The histogram is representative of three independent experiments with similar results. B, Bar chart showing CL integrated during the first 20 min after agonist stimulation. Data shown are mean ± SEM from three experiments. C, A representative histogram showing expression of FPR as detected by flow cytometry using an anti-FPR mAb (SF1) and a FITC-conjugated secondary Ab (solid line).

Because COS-phox cells do not contain these hemopoietic cell-specific enzymes, we speculated that exogenous expression of PLC\textbeta\textsubscript{2} and/or PI3Kγ might render the cells responsive to fMLP. To our surprise, COS-phox cells cotransfected to express PLC\textbeta\textsubscript{2} and FPR, PI3Kγ and FPR, or PLC\textbeta\textsubscript{2} and PI3Kγ with FPR still could not respond to fMLP with detectable O2\textsuperscript{−} production (data not shown), suggesting that these two enzymes were insufficient for reconstitution of FPR-mediated NADPH oxidase activation.

Additional signaling molecules including the Gai proteins, the small GTPases Rac1, and selected PKC isoforms (shown in Fig. 7)
production when expressed alone, the combined expres-

production was monitored

production as well

in the 6PL-transfected COS-phox

generation requires G

production. COS-phox cells

produced in re-

when the cells were stimulated with fMLP. After normalization

production requires functional coupling

generated in

production in the transfected COS-phox

production, suggesting that FPR can

expression constructs coding for FPR, G

as spontaneous production of O2

A, A representative histo-

FIGURE 2. Reconstitution of fMLP-induced O2

FIGURE 3. FPR-mediated O2

production in trans-

fected COS-phox cells. COS-phox cells were transiently transfected with

production activity

Below), were expressed in COS-phox either alone or in combina-

tions. Although none of these molecules could rescue FPR-medi-

However, substitution of the exogenous Gs

requirement for functional coupling

expression vectors.

Dephosphorylation of PKCθ by pertussis toxin (PTX)

expression vectors coding for the respective cat-

expression vectors coding for the respective cata-

measurement of O2

stimulated with 50 ng/ml PMA (Fig. 1B). Inclusion of SOD

in the assay buffer abolished fMLP-induced O2

response to 1

base, confirming an important role of Go2 in coupling

Goi proteins. A, Neutrophils were either treated with pertussis toxin (PTX; 100 ng/ml; 16 h) or left untreated, and stimulated with fMLP. O2

Neutrophils were either treated with pertussis toxin (PTX; 100 ng/ml; 16 h)

or left untreated, and stimulated with fMLP. O2

an important role of Go2 in coupling FPR to the downstream signaling pathways. We have also observed that a substitution with the exogenous Go3 for Go2 resulted in similar level of O2

production, suggesting that FPR can couple to either protein for NADPH oxidase activation.

The relative contributions of exogenous vs endogenous PLCβ

and PI3Kγ to FPR-mediated NADPH oxidase activation

We next investigated whether PLCβ2 and PI3Kγ, insufficient by

Themselves for reconstitution of the FPR-mediated response, are

necessary for the fMLP-induced O2

Because FPR-mediated O2

requirement of Goi coupling to FPR for the reconstitution of

NADPH oxidase activity

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in a ~75% decrease in $O_2^+$ production (Fig. 4). These findings suggest possible involvement of the two hemopoietic-specific enzymes in $O_2^+$ production by fMLP in the transfected cells. The results also indicate that the endogenous PLCβ and PI3K isoforms can partially fulfill the functions of PLCβ2 and PI3Kγ.

Roles of Rac1 and Rac2 in the FPR reconstitution assay

The small GTPase Rac is essential for NADPH oxidase activation in neutrophils and in cell-free reconstitution assays (24, 25). We investigated whether Rac also plays a critical role in fMLP-induced $O_2^+$ generation in the transfected COS-phox cells. In the experiments described below, COS-phox cells were transfected with expression constructs coding for FPR, Goi2, PI3Kγ, PLCβ2, and PKCδ, with or without the expression vectors for human Rac1 and Rac2. Although the initial successful reconstitution included an expression construct of Rac1, substantial amount of $O_2^+$ could be produced in its absence (Fig. 5A, first group). This result suggests that FPR is able to use the endogenous Rac1 for activation of NADPH oxidase. In recent years, a critical role of Rac2 in neutrophil NADPH oxidase activation has been reported based on studies of Rac2−/− mice (10, 26). We investigated whether activation of Rac2 is a unique property of hemopoietic cells by exogenous expression of Rac2 in the transfected COS-phox cells. As shown in Fig. 5, Rac2 is absent from COS-phox cells and its expression resulted in a statistically significant ($p < 0.05$) enhancement of the fMLP-induced $O_2^+$ production. Activation of Rac under these conditions was determined based on the ability of the N-terminal domain of p21-activated kinase 1 to bind and pull down the activated Rac (23). Results shown in Fig. 5C demonstrate that fMLP induced rapid but transient increases in this binding as detected by Western blotting with either an anti-Rac1 Ab for the endogenous Rac1 or an anti-myc Ab for the exogenous Rac1 and Rac2 recovered from the pull-down assay. The kinetics of Rac activation remained unchanged when Rac1 is overexpressed, with peak activation detected at around 1 min after fMLP stimulation. This is consistent with the rapid generation of $O_2^+$ in fMLP-stimulated neutrophils and reconstituted COS-phox cells. Activation of Rac2 also peaked at ~1 min (Fig. 5C), and $O_2^+$ production in Rac2-transfected cells was similarly rapid as was observed in Rac1-transfected cells (data not shown).

Expression of p40phox enhances fMLP-induced $O_2^+$ production in reconstituted COS-phox cells

p40phox is a cytosolic, SH3 domain-containing protein found in myeloid cells (27), but not in COS-7 cells (Fig. 6A). Although

![FIGURE 5.](http://www.jimmunol.org/) Effect of endogenous and exogenous Rac on fMLP-induced $O_2^+$ generation. A, COS-phox cells were transiently transfected with FPR and the five expression constructs without exogenous Rac, or with exogenous Rac1 or Rac2. The integrated CL (first 20 min) showing percent differences in $O_2^+$ production without the exogenous Rac (No ExRac), and with the exogenous Rac1 (ExRac1) or Rac2 (ExRac2). Data shown are mean ± SEM from three experiments. B, A representative gel picture derived from RT-PCR detection of the Rac1 and Rac2 transcripts in COS-phox cells. Control, No reverse transcription products added. cDNA std, Rac1 or Rac2 cDNA (10 ng) was used as standards and positive controls. C, Activation of Rac based on binding to PBD-GST, with total Rac proteins shown as reference for the amounts of proteins used in the assays. An anti-Rac1 Ab was used for detecting the endogenous and exogenous Rac1, and the anti-myc Ab 9E10 was used for detecting the expression of Rac2, which was myc-tagged. fMLP stimulation (1 μM) was conducted for 1, 2, and 5 min as indicated. Three experiments were performed, and a set of representative blots is shown.

p40phox is known to form an active complex with p47phox and p67phox for their membrane translocation (27), its role in NADPH oxidase activation has not been fully established. COS-phox cells respond to PMA and arachidonic acid with potent $O_2^+$ production (17), and our initial reconstitution of fMLP-induced NADPH oxidase activity was achieved without p40phox (Fig. 2). These observations indicate that p40phox is not essential for NADPH oxidase activation in either PMA- or fMLP-stimulated COS-phox cells.

Because the current literature suggests that p40phox can be either stimulatory or inhibitory in NADPH oxidase activation, we examined its potential involvement in FPR-reconstituted COS-phox
PKCδ is critical to fMLP-induced $O_2^\bullet$ production in COS-phox cells and in human neutrophils

Phosphorylation of p47$^{phox}$ by PKC is essential for translocation of the cytosolic factors and assembly of a functional NADPH oxidase complex (28). In neutrophils, the conventional PKC isoforms PKCα and PKCβII are believed to mediate PMA-induced $O_2^\bullet$ production (29). The PKC expression profile in COS-7 cells is different from that of neutrophils. COS-7 cells contain a high level of PKCα, a moderate level of PKCζ, but only small amounts of PKCδ and PKCβII. In contrast, neutrophils express high levels of PKCβII and PKCδ, a moderate level of PKCα, and very little PKCζ (17). To determine which PKC is important for the fMLP-induced $O_2^\bullet$ production, we expressed the four individual PKC isoforms together with other necessary components in COS-phox cells. As shown in Fig. 7, cells transfected to express FPR, Gαi2, PI3K, PLC, and Rac1 responded to fMLP stimulation with a small increase in $O_2^\bullet$ generation over basal level. Cotransfection with a PKCδ expression vector markedly enhanced fMLP-stimulated $O_2^\bullet$ production (by 4-fold), whereas cotransfection with vectors encoding the other three PKC isoforms resulted in statistically insignificant ($p > 0.05$) changes in $O_2^\bullet$ production.

Because COS-phox is a nonhemopoietic cell line and can have quite different properties than neutrophils, we next examined whether PKCδ is important for fMLP-induced NADPH oxidase activation in human neutrophils. Freshly prepared blood neutrophils were stimulated with either PMA or fMLP, and membrane translocation of PKCδ was determined at various time points after stimulation (Fig. 7C). Translocation of PKCδ was evident 10 min after PMA stimulation. In fMLP-stimulated neutrophils, PKCδ translocation appeared much earlier and was detectable after 1 min (Fig. 7C). This profile is consistent with the kinetics of fMLP-induced $O_2^\bullet$ production.

We next determined the effects of PKC inhibitors on the fMLP-induced $O_2^\bullet$ production. Treatment of human neutrophils with GF109203X, a broad PKC inhibitor with selectivity for PKCα and other conventional PKCs, effectively suppressed PMA-induced $O_2^\bullet$ production at 0.1–1 μM concentrations (Fig. 8, A and B). In comparison, GF109203X was less effective on fMLP-induced $O_2^\bullet$ production.
At 1 μM, GF109203X inhibited the PMA-induced O₂⁻ production by ~85%, but only ~15% for the fMLP-induced O₂⁻ production. These results contrast sharply with data derived from a parallel study in which rottlerin, a PKCδ-selective inhibitor (IC₅₀ = 3–6 μM), was used. Rottlerin inhibited fMLP-induced O₂⁻ production by 65–100% at concentrations of 3–12 μM. Under the same experimental conditions, rottlerin had minimal effect on the PMA-induced O₂⁻ production (Fig. 8, C and D).

Discussion

Investigation of chemoattractant-induced O₂⁻ generation has been hampered by the lack of a whole-cell model for reconstitution of receptor-mediated signaling pathways. As a result, our current understanding of how chemoattractants activate NADPH oxidase relies heavily on information obtained with pharmacological activators such as PMA and arachidonic acids. The mechanistic information derived from these studies does not accurately reflect signaling events initiated by chemoattractant receptors. In the current study, we attempted to characterize the proximal signaling events immediately downstream of FPR activation and associate these events with kinase and GTPase activities directly responsible for NADPH oxidase activation. Our approach is based on the assumption that key signaling components for fMLP-induced O₂⁻ production may be identified by expression of the relevant proteins in cells that lack them. The transgenic COS-phox cell line is excellent for this study, because it is not of hemopoietic origin and thus does not have the special signaling components found in neutrophils. The experimental data presented above confirmed our initial prediction and demonstrated the utility of the COS-phox cells in reconstituting NADPH oxidase activation through a cell surface receptor.

PKC-mediated phosphorylation of NADPH oxidase components is a critical step in the initiation of a series of events that led to membrane translocation of the cytosolic factors (28, 30, 31). Several PKC isoforms are found to interact with and phosphorylate p47(phox) in its C-terminal region containing two SH3 domains. In PMA-stimulated neutrophils, translocation of PKCα and PKCβII to the cytoskeletal fraction correlates with p47(phox) translocation, suggesting that these conventional PKC isoforms are involved in PMA-induced O₂⁻ generation (29). PKC-regulated phosphorylation of p47(phox) is also observed in fMLP-induced NADPH oxidase activation (32–35), but the responsible PKC isoforms have not been identified. A major observation of the current study is that PKCδ plays a key role in FPR-mediated NADPH oxidase activation in both transgenic COS-phox cells and human neutrophils. In the COS-phox cells, exogenous expression of PKCδ led to a potent induction of fMLP-elicited O₂⁻ production. In contrast, exogenous expression of PKCα, PKCβII, and PKCζ did not significantly enhance the fMLP-induced response. In human neutrophils, a role of PKCδ in fMLP-elicited O₂⁻ generation was suggested by two findings. First, fMLP stimulates membrane translocation of this kinase with temporal correlation to the induced O₂⁻ production. Second, the PKCδ-selective inhibitor rottlerin exerts a much stronger effect on fMLP-induced O₂⁻ production than the PMA-stimulated response. PKCδ is a novel PKC isoform and is activated in a Ca²⁺-insensitive and diacylglycerol-dependent manner. PKCδ and PKCβII are the two most abundant PKC isoforms in neutrophils, whereas COS-7 cells express only a low level of PKCδ (17). Like several other PKC isoforms, PKCδ responds to PMA stimulation with binding to p47(phox) (36) and translocation to the cytoskeletal fraction (29). However, the lack of temporal correlation with p47(phox) binding (36) and translocation (29) casts doubt on the importance of PKCδ translocation in PMA-induced neutrophil NADPH oxidase activation. More recently, Yaffe and colleagues (37) studied NADPH oxidase activation in cytosol-depleted neutrophil cores and identified PKCδ as a key component for reconstitution of PMA-stimulated NADPH oxidase activity. Their conclusion was based on the observations that rottlerin (10 μM)
inhibits PMA-induced $O_2^-$ production in neutrophil cores, and that selective depletion of PKCδ from cytosol impairs its ability to restore the PMA-stimulated response in neutrophil cores. These findings provide additional evidence for a role of PKCδ in NADPH oxidase activation. A function of PKCδ in leukotriene $B_4$-stimulated eosinophil $O_2^-$ production was also reported (38). However, eosinophils differ from neutrophils, and leukotriene $B_4$ primes but does not directly activate neutrophil NADPH oxidase. Therefore, further investigation is necessary to determine whether PKCδ is required for neutrophil NADPH oxidase activation induced by a potent chemoattractant such as fMLP.

Although the above experimental results are important in establishing a correlation between PKCδ activation and $O_2^-$ production, PKCδ may not be the only PKC isoform involved in fMLP-elicited NADPH oxidase activation. Extensive cross talk exists between different kinases and may complicate the interpretation of our experimental data. The lack of highly specific inhibitors for the individual PKC isoforms also hinders studies using neutrophils that are not genetically amenable. Published reports suggest that fMLP can activate PKCβII in differentiated HL-60 cells (39) and PKCζ in human neutrophils (40). Based on these observations, it is likely that the fMLP-induced response requires more than one PKC isoform for optimal $O_2^-$ production. This possibility, as well as the relative position of PKCδ in the FPR-mediated signaling cascade, will need to be investigated in future studies.

The development of COS-phox-based reconstitution assay has enabled us to assess the roles of individual signaling molecules in NADPH oxidase activation. These signaling molecules can be divided into two groups. The first group consists of signaling molecules immediately downstream of the activated FPR. Our results demonstrated that Gαi2 and Gαi3 are equally capable of mediating fMLP-induced $O_2^-$ generation in intact cells. Exogenous expression of Gαi2 or Gαi3 potentiates $O_2^-$ production in response to fMLP, suggesting that the endogenous Gαi proteins became a limiting factor when FPR is overexpressed. With the development of the COS-phox-based reconstitution assay, it is now possible to examine other G proteins for their potential involvement in G protein coupled receptor-mediated $O_2^-$ production. PLCβ2 is a downstream effector of G proteins. Our experimental data indicate that exogenous expression of PLCβ2 is necessary for optimal reconstitution of fMLP-elicited $O_2^-$ production, but significant amount of $O_2^-$ can still be generated in the absence of PLCβ2 (Fig. 4). This result suggests that FPR is able to use the endogenous PLCβ3 in COS-7 cells for downstream signaling. PLCβ activation is triggered by the G protein βγ subunits that become available after the activation of Gαi, which serves to link the agonist-occupied receptor with sec-

Another group of proteins studied in the COS-phox cells includes PKC, Rac, and p40phox, factors that directly participate in the modification and assembly of the phox proteins. In addition to demonstrating a role of PKCδ in the fMLP-elicited response, we have shown that the FPR-mediated signaling pathway is able to trigger activation of endogenous Rac1. Exogenous expression of Rac1 further increased the level of Rac activation as well as $O_2^-$ production, suggesting that Rac1 is one of the limiting factors in this pathway. This finding does not conflict with previous observations that Rac2 plays an important role in NADPH oxidase activation in human neutrophils, but suggest that FPR-mediated signaling pathway can lead to Rac1 activation in an epithelial cell line. fMLP may stimulate Rac1 dissociation from Rho GDP-dissociation inhibitor, as seen in primary human monocytes (41), thereby promoting NADPH oxidase activation. We have further demonstrated the ability of fMLP to induce Rac2 activation and to potentiate $O_2^-$ generation in the reconstituted COS-phox cells expressing human Rac2. This finding suggests that Rac2 activation is not an exclusive property of hemopoietic cells where it is naturally expressed; instead, it can be achieved by the guanine nucleotide factor(s) present in epithelial cells such as COS-7. Further studies will be necessary to compare the different Rac guanine nucleotide exchange factors for their ability to mediate fMLP-induced $O_2^-$ production.

In addition to the four essential phox proteins and Rac, recent studies suggest that p40phox may be involved in regulating NADPH oxidase activation. Published results indicate that p40phox can enhance $O_2^-$ generation in cell-free assays (42–44) and in K562 cells (45). The PX domain of p40phox is considered important in the interaction with the lipid products of PI3Ks (44, 46, 47), which may be one of the mechanisms for the potentiation of $O_2^-$ production. However, there are also published reports indicating that p40phox is an inhibitory molecule for NADPH oxidase activation, in both K562 cells (48) and cell-free assays (49, 50). In light of the controversy surrounding the exact role of p40phox in NADPH oxidase activation, we sought to examine this cytosolic protein, which is absent from the epithelial COS-phox cells. By exogenous expression, we observed that p40phox enhances the level of fMLP-induced $O_2^-$ production in the reconstituted COS-phox cell, suggesting that p40phox can be stimulatory for chemoattractant-induced NADPH oxidase activation in intact cells. Expression of p40phox does not alter the kinetics of fMLP-induced $O_2^-$ production, because the initial rise to peak and subsequent fall of $O_2^-$ levels follow a similar pattern in the presence or absence of p40phox (Fig. 6B). Overexpression of p40phox affects the basal level of NADPH oxidase activity as well. Because COS-phox cells can be readily transfected with DNA expression constructs, the reconstitution system is suitable for further delineation of the mechanism of p40phox action through analysis of mutants and chimeric proteins.

In summary, the FPR-reconstituted COS-phox cells share many functional features with neutrophils and provide a genetically amenable system for characterization of the individual signaling components and phox proteins. Using the COS-phox reconstitution system, we have identified PKCδ as an important kinase for fMLP-induced $O_2^-$ production, demonstrated the ability of FPR to activate both Rac1 and Rac2 in a nonhemopoietic cell line, and confirmed the requirement of PLCβ2 and PI3Kγ for optimal NADPH oxidase activity. We further demonstrated that p40phox is a positive regulator of fMLP-induced $O_2^-$ production. This whole-cell-based reconstitution system is expected to complement the existing cell-free system for more detailed analysis of receptor-mediated signaling pathways leading to $O_2^-$ generation.

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References

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