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Regulation of Phosphatidylinositol 3-Kinase Activity and Phosphatidylinositol 3,4,5-Trisphosphate Accumulation by Neutrophil Priming Agents

Karen A. Cadwallader,* Alison M. Condiffe,* Alex McGregor,† Trevor R. Walker,‡ Jessica F. White,* Len R. Stephens,§ and Edwin R. Chilvers§*

Neutrophil priming by agents such as TNF-α and GM-CSF causes a dramatic increase in the response of these cells to secretagogue agonists and affects the capacity of neutrophils to induce tissue injury. In view of the central role of phosphatidylinositol 3-kinase (PI3-kinase) in regulating NADPH oxidase activity we examined the influence of priming agents on agonist-stimulated phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3) accumulation in human neutrophils. Pretreatment of neutrophils with TNF-α or GM-CSF, while not influencing fMLP-stimulated PtdIns(3,4,5)P3 accumulation at 5 s, caused a major increase in PtdIns(3,4,5)P3 at later times (10–60 s), which paralleled the augmented superoxide anion (O2−) response. The intimate relationship between PtdIns(3,4,5)P3 accumulation and O2− release was confirmed using platelet-activating factor, which caused full but transient priming of both responses. Likewise, L294002, a PI3-kinase inhibitor, and genistein, a tyrosine kinase inhibitor, caused parallel inhibition of O2− generation and PtdIns(3,4,5)P3 accumulation; in contrast, radicicol, which inhibits receptor-mediated activation of p85 PI3-kinase, had no effect on either response. Despite major increases in PI3-kinase activity observed in p85 and anti-phosphotyrosine immunoprecipitates in growth factor-stimulated smooth muscle cells, no such increase was observed in primed/stimulated neutrophils. In contrast, both fMLP and TNF-α alone caused a 3-fold increase in PI3-kinase activity in p110γ PI3-kinase immunoprecipitates. p21ras activation (an upstream regulator of PI3-kinase) was unaffected by priming. These data demonstrate that timing and magnitude of PtdIns(3,4,5)P3 accumulation in neutrophils correlate closely with O2− generation, that PI3-kinase-γ is responsible for the enhanced PtdIns(3,4,5)P3 production seen in primed cells, and that factors other than activation of p21ras underlie this response. The Journal of Immunology, 2002, 169: 3336–3344.

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4 Abbreviations used in this paper: PI3-kinase, phosphatidylinositol 3-kinase; PtdIns(3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate; O2−, superoxide anion; Ins(1,3,4,5)P4, inositol 1,3,4,5-tetrakisphosphate; GST, glutathione S-transferase; PAF, platelet-activating factor; BTSM, bovine tracheal smooth muscle; RBD, Ras binding domain; PtdIns(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PDGF, platelet-derived growth factor, EE, Glu-Glu.
While priming is an obligatory step required for full activation of the NADPH oxidase, the impact of this process on agonist-stimulated PtdIns(3,4,5)P3 accumulation and the precise PI3-kinase isoforms responsible for this response have not been defined. Our preliminary studies using [32P]P-labeled neutrophils (1) and those of Kodama et al. (15) suggest that priming may enhance fMLP-stimulated [32P]PtdIns(3,4,5)P3 accumulation, but the kinetics, magnitude, and mechanism of this effect remain to be determined. Identifying the PI3-kinase(s) involved in this response is complicated by the presence of an additional, myeloid-restricted, Gβγ-regulated PI3-kinase (p101/PI3-kinase-γ) in neutrophils and the lack of high-affinity p101 or PI3-kinase-γ Abs or PI3-kinase isoform selective inhibitors. The potential importance of PI3-kinase-γ in neutrophil activation is supported by data in p110γ knockout mice where defects in respiratory burst and migratory responses have been observed (9, 16). However, murine neutrophils have a very attenuated respiratory burst response compared with human neutrophils, and priming has not been clearly demonstrated in this species.

In the current study we have measured PtdIns(3,4,5)P3 accumulation using a recently developed mass assay (17). We show that neutrophil priming with TNF-α, GM-CSF, cytochalasin B, or platelet-activating factor (PAF) before IMPL stimulation leads to a substantially prolonged PtdIns(3,4,5)P3 signal. Manipulation of the O2− response using a number of PI3-kinase and tyrosine kinase inhibitors, and also allowing neutrophils to deprime after PAF exposure, resulted in parallel changes in both PtdIns(3,4,5)P3 accumulation and O2− release. Moreover, significant PI3-kinase activity could be detected in p110γ, but not p85 or phosphotyrosine immunoprecipitates, after TNF-α priming or IMPL stimulation. Finally, priming did not affect the extent of basal or IMPL-stimulated p21ras activity, an upstream regulator of PI3-kinase. Together, these results suggest that enhanced PI3-kinase-γ activity underlies the augmented accumulation of PtdIns(3,4,5)P3 and O2− response seen in primed neutrophils, and that a PI3-kinase regulatory protein other than p21ras controls this activity.

Materials and Methods

Materials

Percoll, dextran, [γ-32P]ATP, and the ECL detection kit were obtained from Amersham Pharmacia Biotech (Piscataway, NJ), Cytochrome c, superoxide dismutase, fMLP, LPS, and PBS (with or without CaCl2 and MgCl2) were purchased from Sigma Chemical (Poole, Dorset, U.K.). [Ino-30,31-H3]-1,3,4,5-tetrakisphosphate was supplied by NEN Life Science (Boston, MA) and [inositol-1,3,4,5-3H]-tetrakisphosphate (Ins1,3,4,5P4) was supplied by Cell Signals (Lexington, KY). Human TNF-α and GM-CSF were supplied by R&D Systems (Abingdon, U.K.). The p110γ Ab (LS1/127) was raised in mice against a Sf9 cell-expressed His-tagged full-length protein (porcine sequence); p85 and 4G10 Abs were from Upstate Biotechnology (Lake Placid, NY). PAF-18 (1-0-stearoyl-2-acetyl-sn-glycero-3-phosphocholine), LY294002, pan-Ras Ab, staurosporine, genistein, cytochalasin B, and radiocalcium were purchased from Calbiochem (La Jolla, CA). All other reagents were obtained from commercial sources and were of the highest purity available.

Preparation of human neutrophils

Human neutrophils were prepared from peripheral blood of healthy volunteers using dextran sedimentation and discontinuous plasma-Percoll gradients as previously described (18). This method routinely yielded cells that were unprimed and >95% pure with <0.1% mononuclear cell contamination.

O2− release assay

Purified neutrophils (109/ml PBS containing CaCl2 and MgCl2) were equilibrated at 37°C in a gently shaking water bath for 5 min. Priming agents were added to give final concentrations of 200 U/ml TNF-α, 100 ng/ml GM-CSF, 5 μM cytochalasin B, or 1 μM PAF. Cells were primed for the periods stated and then stimulated with IMLP (100 nM) or vehicle in the presence of prewarmed cytochrome c (final concentration 1.2 mg/ml in PBS). O2− release was calculated from the superoxide dismutase-inhibitable reduction of cytochrome c (1).

Ins[1,3,4,5]P4 isotope dilution assay

Recombinant Ins[1,3,4,5]P4 binding protein (GAPIP4BP) was purified as previously described (17, 19) and sample purity was analyzed by SDS-PAGE. Total and nonspecific binding of [3H]Ins[1,3,4,5]P4 in each preparation of GAPIP4BP was assessed by serial dilution and inclusion of 0.1 nM InsP3, respectively, and thereafter GAPIP4BP was used at a concentration to achieve a maximum binding of 20% of the total [3H]Ins[1,3,4,5]P4 input. Purified neutrophils (8 × 107) were primed with TNF-α (200 U/ml) or GM-CSF (100 ng/ml) for 30 min. Vehicle or IMLP (100 nM) was added for the indicated times and the reactions were stopped by the addition of methanol/chloroform (2:1, v/v). Lipid extractions were performed as previously described (1). After drying, the samples were processed as detailed (17) and stored at −20°C until analyzed. Immediately before the assay, samples were resuspended in dilute acetic acid to a final pH of 5. The [3H]Ins[1,3,4,5]P4 radioreceptor assay was performed as described (20) using 0.005 μCi of [3H]Ins[1,3,4,5]P4 per sample.

[3H]Thymidine incorporation

Confluent and quiescent primary bovine tracheal smooth muscle (BTSM) cells were prepared as previously detailed (21) and washed twice in serum-free DMEM, and inhibitors and mitogens were added as described. Cells were incubated for a further 24 h, with [3H]thymidine (0.1 μCi/ml) included for the final 4 h of the incubation. Cells were washed twice in PBS, trichloroacetic acid (5% w/v), and ethanol before finally being solubilized with NaOH (0.3 M). [3H]Thymidine incorporation was determined by liquid scintillation counting.

PI3-kinase activity assays

Neutrophils (20 × 106 cells) were primed and stimulated as described. After stimulation, 1 ml of ice-cold lysis buffer (10 mM Tris-HCl (pH 7.8), 1.5 mM EDTA, 10 mM KCl, 0.1% Nonidet P-40, 1 mM Na3VO4, 0.1 mM di-isopropylfluorophosphate, 0.1 mM N-α-tosyl-L-lysine chloromethyl ketone, 0.1 mM DTT, 10 μg/ml aprotinin, 10 μg/ml pepstatin, and 10 μg/ml leupeptin) was added and the samples were left on ice for 20 min with intermittent vortex mixing. Samples were sonicated (2 × 10 s at maximum setting) and then centrifuged. The supernatants were collected and incubated with p85, 4G10, and p110γ Ab overnight and protein A Sepharose beads were added for a further 2 h. The beads were collected and washed once with lysis buffer, once with 100 mM Tris/500 mM LiCl (pH 7.6), and twice with 200 mM HEPES, 40 mM MgCl2, 600 mM NaCl (pH 7.4). The washed beads were then incubated for 10 min at 30°C in HEPES buffer (200 mM HEPES, 40 mM MgCl2, 600 mM NaCl (pH 7.4)) together with phosphatidylinositol/phosphatidylinerine (3:1, 0.2 mg/ml) vesicles, 10 μCi [32P]ATP, and 50 μM unlabeled ATP. Lipid extractions were performed as previously described (22) and the lower organic phase was dried in an Eppendorf concentrator, redissolved in chloroform, and spotted onto silica gel 60 TLC plates. The plates were run in a solvent system containing chloroform/methanol/ammonia/water (20:14:3:5, v/v/v/v). The 32P-labeled phosphoinositide 3-phosphate band was detected by autoradiography and [32P] incorporation measured by liquid scintillation counting. In experiments characterizing the mouse p110γ mAb (LS1/127), PI3-kinase activity was assayed as described by Stephens et al. (12) with the lipid products deacylated before resolution by TLC.

Transfection of COS7 cells with EE-tagged p110γ

Rapidly growing COS7 cells were cultured to 40–60% confluence in DMEM containing 10% FBS, washed twice in serum-free DMEM buffered with 10 mM HEPES (pH 7.3), and incubated with 1 μg Glu-Glu (EE-tagged) p110γ DNA/0.5 ml serum-free DMEM together with 125 μg DEAE-dextran (23). The cells were incubated for 90 min at 37°C with the DNA solution followed by 5 h with DMEM containing 100 μM chloroquine. Cells were used 24–48 h after transfection. Cell lysis, protein immunoprecipitation (using LS1/127 Ab-coupled protein G-Sepharose beads), SDS-PAGE, and Western blotting were performed as previously detailed (24).

Measurement of activated p21ras using Raf1 Ras binding domain (RBD)

pGex-KG-RBD (encoding a fusion protein of glutathione S-transferase (GST) and residues 1–149 of human cRaf1 RBD) was used to measure activation of p21ras using the method described by de Rooij and Bos (25).
Neutrophils (1.5 x 10^7/sample in 80 µl PBS with divalent cations) were primed (200 U/ml TNF-α, 100 ng/ml GM-CSF, or appropriate diluent) for 30 min at 37°C and stimulated with 100 nM fMLP or buffer for 10–300 s. Reactions were stopped with ice-cold lysis buffer (100 mM NaCl, 50 mM HEPES (pH 7.5), 1% Triton X-100, 1 mM EDTA (pH 8), 5 mM NaF, 12 mM MgCl2, 1 mM Na3VO4, and 10 µg/ml apro tinin, pepstatin, leupeptin, and antipain). Nuclei were removed by centrifugation and pre-coupled GST-RBD beads added to the lysates before incubation at 4°C for 30 min. Beads were washed three times with cold PBS containing 5 mM NaCl and 0.1% Triton X-100, and subsequently boiled in Laemmli sample buffer. Protein samples were separated by SDS-PAGE and Western blotted with a pan-Ras Ab. Blots were developed using ECL Plus detection reagents.

Statistics
Data are presented as the mean ± SEM of n separate experiments. Data were analyzed using the Student t test or ANOVA followed by the Student-Kewan-Keuls post-test. Results were considered to be significant with p < 0.05. The Kd and maximal binding capacity of [3H]Ins(1,3,4,5)P4 were determined by Scatchard analysis of [3H]Ins(1,3,4,5)P4 displacement with unlabeled d-Ins(1,3,4,5)P4 after correction for isotopic dilution and non-specific binding.

Results
PtdIns(3,4,5)P3 mass assay
PtdIns(3,4,5)P3 has been proposed to be an important second messenger in O2− generation in phagocytic cells (8, 26). Traditional methods of measuring PtdIns(3,4,5)P3 accumulation involve metabolic labeling of cells with [32P]PO4 followed by HPLC analysis of individual sample extracts. Moreover, the incubation times and conditions required to achieve true isotopic equilibrium labeling of the agonist-sensitive PtdIns(4,5)P2 pool in neutrophils invariably result in significant basal priming. These factors have precluded a detailed analysis of the effects of cytokine-mediated priming on agonist-stimulated PtdIns(3,4,5)P3 accumulation in neutrophils.

To address these problems we have used a recently described PtdIns(3,4,5)P3 mass assay (17) based on the ability of boiling KOH to convert, in a quantitative manner, cell-extracted PtdIns(3,4,5)P3 to its water-soluble counterpart, Ins(1,3,4,5)P4. This product can be measured by competitive radioreceptor assay using a bacterially expressed GST-tagged GAP-Ins(1,3,4,5)P4

FIGURE 1. Displacement of [3H]Ins(1,3,4,5)P4 by authentic Ins(1,3,4,5)P4 and inositol 3,4,5-trisphosphate using recombinant GAP IP4BP. a, The displacement of [3H]Ins(1,3,4,5)P4 by Ins(1,3,4,5)P4 ( ■) and inositol 3,4,5-trisphosphate ( ●) at the indicated concentrations was measured in a final assay volume of 0.4 ml after a 30-min incubation on ice with recombinant GAP IP4BP. Each point represents a determination in triplicate ± SEM of three independent experiments. b and d, O2− generation in human neutrophils. Cells were primed with 200 U/ml TNF-α ( b) or 100 ng/ml GM-CSF ( d) for 30 min and then stimulated with 100 nM fMLP for 10 min in the presence of cytochrome c. O2− release was measured by the superoxide dismutase-inhibitable reduction of cytochrome c. Data points represent mean ± SEM of three separate experiments, each performed in triplicate. c and e, The corresponding time courses of PtdIns(3,4,5)P3 accumulation in primed and stimulated neutrophils. For PtdIns(3,4,5)P3 measurements, cells were primed with 200 U/ml TNF-α ( c) or 100 ng/ml GM-CSF ( e) for 30 min and then stimulated with 100 nM fMLP for various times. ○, Control; ●, TNF-α or GM-CSF; ▲, fMLP; ▲, TNF-α or GM-CSF followed by fMLP. Reactions were stopped by the addition of MeOH/CHCl3 (2:1 v/v), lipids were extracted, and the Ins(1,3,4,5)P4 mass assay was performed as described. Results are mean ± SEM of duplicate samples from three independent experiments ( d) or the means ± SD of duplicate samples from one experiment representative of three independent experiments ( e).
binding protein (GAPIP4BP). A typical calibration curve for the displacement of [3H]Ins(1,3,4,5)P4 from the GAPIP4BP by unlabeled Ins(1,3,4,5)P4 is shown in Fig. 1a (Kd 3.9 ± 0.38 nM; n = 5). The specificity of the assay for Ins(1,3,4,5)P4 was confirmed in this system using authentic Ins(1,4,5)P3. As shown in Fig. 1a, no significant displacement of [3H]Ins(1,3,4,5)P4 binding was observed with up to 120 pmol of Ins(1,4,5)P3. The recovery of Ins(1,3,4,5)P4 during the extraction process (determined using spiked [3H]Ins(1,3,4,5)P4) was 93% (data not shown). Assay values for each sample were corrected for these losses and the efficiency of PtdIns(3,4,5)P3 to Ins(1,3,4,5)P4 conversion (62%) and expressed as pmol PtdIns(3,4,5)P3/8 × 10^6 neutrophils.

Effect of neutrophil priming on fMLP-stimulated PtdIns(3,4,5)P3 accumulation

As previously demonstrated, quiescent neutrophils or those treated with TNF-α (200 U/ml) or fMLP (100 nM) alone released minimal O2−, whereas cells primed with TNF-α and subsequently stimulated with fMLP showed greatly enhanced O2− release (Fig. 1b).

A detailed time course of fMLP-stimulated PtdIns(3,4,5)P3 mass accumulation was undertaken in TNF-α-primed or unprimed neutrophils. Cells exposed to TNF-α or vehicle alone showed minimal PtdIns(3,4,5)P3 accumulation compared with values obtained in freshly isolated cells (Fig. 1c). fMLP stimulation of unprimed cells resulted in a rapid but transient increase in PtdIns(3,4,5)P3 accumulation with peak values between 5 and 15 s (Fig. 1c). In contrast, cells primed with TNF-α and then stimulated with fMLP showed a greater increase in PtdIns(3,4,5)P3 accumulation at early times and a sustained elevation in PtdIns(3,4,5)P3 levels lasting for at least 60 s (Fig. 1c). Similar data were obtained with the alternative priming agent GM-CSF (Fig. 1, d and e), suggesting that the ability of TNF-α to enhance fMLP-stimulated PtdIns(3,4,5)P3 accumulation is a “class effect” and is shared by other priming agents.

To test this hypothesis further, the effects of an alternative non-receptor-acting priming agent, cytochalasin B, were also examined. Table I shows that cytochalasin B significantly increased both O2− generation and PtdIns(3,4,5)P3 accumulation in a similar manner to TNF-α and GM-CSF, suggesting that a broad array of priming agents activate these responses through the same signaling pathways.

PtdIns(3,4,5)P3 and O2− responses in neutrophils after transient priming with PAF

We have demonstrated previously that neutrophils primed with PAF spontaneously “deprime” over a period of 120 min even if left continuously exposed to PAF (7). Fig. 2a confirms that the ability of PAF to enhance fMLP-stimulated O2− generation in neutrophils is transient, with the priming effect completely lost by 120 min. Previous studies have shown that cells exposed to PAF in this way remain viable throughout this incubation period, revert to a fully depolarized state with normal CD11b expression, and indeed are capable of being “reprimed” by a second priming agent such as TNF-α (7).

To compare PtdIns(3,4,5)P3 responses in primed and deprimed cells, neutrophils were incubated with PAF for 10 or 120 min before stimulation with fMLP for 45 s. This latter time point was chosen for this and subsequent experiments because it gave the greatest discrimination between PtdIns(3,4,5)P3 responses in acutely primed and unprimed cells (Fig. 1, c and e). In agreement with the previous data with TNF-α and GM-CSF, cells treated with PAF for 10 min and then stimulated with fMLP showed a large increase in O2− generation and PtdIns(3,4,5)P3 accumulation compared with unprimed, fMLP-stimulated cells (Fig. 2). In contrast, cells treated with PAF for 120 min displayed O2− and PtdIns(3,4,5)P3 responses identical to unprimed cells (Fig. 2). Time-controlled matches were included where cells were incubated for 2 h before the addition of PAF, showing that the O2− and

![FIGURE 2. Neutrophil depriming of both O2− and PtdIns(3,4,5)P3 accumulation with PAF. a. Cells were incubated with 1 μM PAF for 10 min (hatched bars) or 120 min (open bars) and then stimulated with fMLP (100 nM) for 10 min in the presence of cytochrome c. Another set of samples was incubated for 120 min before priming and stimulation (filled bars). O2− release was measured by the superoxide dismutase-inhibitable reduction of cytochrome c. Data points represent mean ± SEM of three separate experiments, each performed in triplicate. b. PtdIns(3,4,5)P3 measurements under the same conditions as in a. Cells were incubated with 1 μM PAF for 10 min (hatched bars) or 120 min (open bars) and then stimulated with fMLP (100 nM) for 45 s. Other cells were incubated for 120 min before PAF priming and fMLP stimulation (filled bars). Reactions were stopped by the addition of MeOH/CHCl3 (2:1 v/v), lipids were extracted, and the assay was performed as described. Results are the means of duplicates ± SEM of three independent experiments.](http://www.jimmunol.org/)

Table 1. Priming effects of TNF-α and cytochalasin B on O2− generation and PtdIns(3,4,5)P3 accumulation

<table>
<thead>
<tr>
<th></th>
<th>O2− Generation (nmol/106 cells)</th>
<th>PtdIns(3,4,5)P3 Accumulation (pmol/8 × 106 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.08 ± 0.04</td>
<td>0.55 ± 0.52</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.43 ± 0.24</td>
<td>0.3 ± 0.17</td>
</tr>
<tr>
<td>fMLP</td>
<td>1.1 ± 0.39</td>
<td>3.54 ± 1.76</td>
</tr>
<tr>
<td>TNF-α + fMLP</td>
<td>14.63 ± 2.85</td>
<td>21.09 ± 5.59</td>
</tr>
<tr>
<td>Cytochalasin B</td>
<td>0.46 ± 0.11</td>
<td>0.23 ± 0.1</td>
</tr>
<tr>
<td>Cytochalasin B + fMLP</td>
<td>18.29 ± 3.65</td>
<td>12.62 ± 4.35</td>
</tr>
</tbody>
</table>

* Neutrophils were prepared and incubated as described. Cells were primed with TNF-α (200 U/ml) for 30 min or cytochalasin B (5 μM) for 5 min prior to fMLP stimulation (100 nM) for 10 min (O2− generation) or 45 s (PtdIns(3,4,5)P3 accumulation). Samples were assayed as described. Values are means ± SEM of three independent experiments performed in duplicate.
PtdIns(3,4,5)P₃ responses of these cells were identical to those seen in freshly isolated cells. These results demonstrate that the enhanced fMLP-stimulated PtdIns(3,4,5)P₃ accumulation seen in acutely primed cells is lost when the cells are deprimed and that this effect parallels O₂− responsiveness and does not reflect changes in cell integrity or metabolic status.

Effects of PI3-kinase and protein kinase inhibitors on O₂− and PtdIns(3,4,5)P₃ generation

Consistent with previous results, LY294002, a well-characterized and highly selective inhibitor of PI3-kinase activity, inhibited fMLP-stimulated O₂− generation and PtdIns(3,4,5)P₃ accumulation in both primed and unprimed cells (Fig. 3, a and c). Identical data were obtained with the alternative PI3-kinase inhibitor wortmannin (data not shown). These results confirm the involvement of a type I or III PI3-kinase in O₂− generation and PtdIns(3,4,5)P₃ accumulation in human neutrophils.

To explore the involvement of a tyrosine kinase-regulated PI3-kinase in the priming-associated increase in O₂− and PtdIns(3,4,5)P₃ responses in neutrophils, the effects of a group of protein kinase inhibitors with variable tyrosine kinase activity were examined. Genistein is a protein tyrosine kinase inhibitor that, at high concentrations, has a broader spectrum of activity reflecting its ability to act as a competitive inhibitor of ATP. Pretreatment of neutrophils with genistein resulted in a concentration-dependent inhibition of fMLP-stimulated O₂− generation (Fig. 3b) and a matched inhibition of PtdIns(3,4,5)P₃ accumulation (Fig. 3f). Genistein was effective only at concentrations >10 μM, suggesting that its effect may be due to inhibition of alternative (non-tyrosine) kinases.

Staurosporine is a broad-spectrum serine/threonine protein kinase and protein kinase C (PKC) inhibitor that has the paradoxical ability to augment rather than inhibit agonist-stimulated respiratory burst activity in neutrophils (27). Fig. 3, c and g, demonstrates the ability of staurosporine to increase both fMLP-stimulated O₂− generation and PtdIns(3,4,5)P₃ accumulation in primed and unprimed cells. This again demonstrates the close association between these two events and the potential involvement of a PKC-dependent pathway in inhibiting PtdIns(3,4,5)P₃ accumulation.

The most discriminating inhibitor used in this set of experiments was radicicol, which is an antifungal antibiotic with selective anti-tyrosine kinase properties that has been reported to block Lyn-dependent activation of p85 PI3-kinase and yet has no direct inhibitory effect on the Gβγ-regulated p101/p110γ (28). Importantly, radicicol was found to have no effect on fMLP-stimulated O₂− generation or PtdIns(3,4,5)P₃ accumulation in primed or unprimed cells (Fig. 3, d and h), despite causing a concentration-dependent and complete inhibition of platelet-derived growth factor (PDGF)-stimulated [3 H]thymidine incorporation in BTSM (Fig. 3i); we have previously shown this latter response to reflect activation of a tyrosine kinase-dependent p85 PI3-kinase pathway.
Radicicol was also found to inhibit thrombin-stimulated DNA synthesis in BTSM cells (data not shown). These results in primed, deprimed, and kinase inhibitor-manipulated cells underscore the close correlation between PtdIns(3,4,5)P3 accumulation and O2− generation in neutrophils. The radicicol data in particular also suggest the involvement of the Gβγ-regulated p101/p110γ PI3-kinase in the augmented PtdIns(3,4,5)P3 and O2− responses seen under TNF-α-primed conditions.

**Determination of which PI3-kinase isoforms are responsible for enhanced PtdIns(3,4,5)P3 accumulation under primed/stimulated conditions**

To determine more precisely which PI3-kinase isoforms are responsible for the enhanced PtdIns(3,4,5)P3 production observed in primed neutrophils, selective immunoprecipitation-based PI3-kinase assays were performed. Positive control data for the experiments involving 4G10 and p85 immunoprecipitations were obtained using primary BTSM cells stimulated with PDGF (20 ng/ml for 10 min). Fig. 4a demonstrates the substantial increase in PI3-kinase activity associated with 4G10 anti-phosphotyrosine immunoprecipitates in BTSM cells after stimulation with PDGF. In contrast, 4G10 (Fig. 4b) and p85 (Fig. 4d) immunoprecipitates prepared from primed and fMLP-stimulated (45 s) neutrophils showed no increase in PI3-kinase activity over control levels. Of note, wortmannin was able to fully inhibit p85-associated PI3-kinase activity (Fig. 4d), confirming the specificity of this assay. These results concur with the data obtained using radicicol and imply that the increases observed in PtdIns(3,4,5)P3 accumulation under primed/stimulated conditions do not reflect activation of a tyrosine kinase-linked p85/p110α, β, or δ PI3-kinase.

To detect changes in the Gβγ-regulated isoform of PI3-kinase (p101/p110γ) activity under primed/activated conditions, a mouse mAb (LS1/127, IgM isotype-μ chain) was raised against Histagged p110γ expressed in S9 insect cells (29). The LS1/127 Ab was able to immunoprecipitate EE-tagged p110γ from transfected COS7 cells with a similar efficiency to EE beads (Fig. 5a) and identify specific cytoplasmic staining in Myc-p110γ transfected COS7 cells (data not shown). LS1/127 was also able to immunoprecipitate p110γ activity from porcine neutrophils (Fig. 5b) (but no other porcine tissues; data not shown) and from EE-p110γ transfected COS7 cells (Fig. 5b).

Use of this Ab demonstrated significant increases in PI3-kinase activity in p110γ immunoprecipitates prepared from cells treated with TNF-α (200 U/ml for 30 min) or fMLP (100 nM for 45 s) alone and under primed/stimulated conditions compared with control levels (Fig. 5c). Of note, Western blot analysis confirmed that an identical amount of p110γ was immunoprecipitated from neutrophil lysates irrespective of the treatment conditions (data not shown). This result further supports the suggestion that priming-induced increases in fMLP-stimulated PtdIns(3,4,5)P3 accumulation are mediated via enhanced activation of PI3-kinase-γ.

**Effect of priming on fMLP-stimulated p21ras activation**

In view of these data and the previous demonstration that priming up-regulates mitogen-activated protein kinase activity (15), we investigated the effect of priming on p21ras activation, an upstream regulator of both PI3-kinase and MEK1/2 responses. Human neutrophils primed with GM-CSF or TNF-α were stimulated with fMLP, and p21ras activation was determined. While no GTP-bound p21ras was detected in either control cells or cells treated with TNF-α alone (Fig. 6, upper panel), GM-CSF induced a slight increase in detectable GTP-Ras (Fig. 6, lower panel). Stimulation with fMLP alone induced dramatic activation of p21ras, maximal at 30–60 s and diminishing to near baseline by 5 min (Fig. 6). Priming with TNF-α did not augment this response. The minor
increase in the fMLP-stimulated p21ras signal seen in GM-CSF-pretreated cells was purely additive when quantified by densitometry (data not shown). Thus, increased availability of GTP-bound p21ras does not appear to underlie the enhancement of PtdIns(3,4,5)P3 generation seen in primed/stimulated neutrophils.

Discussion

The PI3-kinase family plays a critical role in a variety of cellular functions, including survival, mitogenesis, vesicular trafficking, chemotaxis, and oxidative burst activity (11). It would seem likely that many of the more universal cellular processes are controlled by the ubiquitous PI3-kinase isoforms, such as PI3-kinase-α and -β, while specialized leukocyte functions are the province of the predominantly leukocyte-restricted PI3-kinase-δ and -γ isoforms. While defects in chemotaxis and oxygen radical formation seen in neutrophils derived from animals lacking PI3-kinase-γ support this premise, these results cannot be extrapolated to human neutrophils, which undergo a far more intense and primeable O2− response compared with their murine counterparts. While Naccache et al. (30) have also demonstrated PI3-kinase activity in p110γ but not p85 immunoprecipitates in fMLP-stimulated neutrophils, the impact of priming, which is an essential regulator of O2− generation, was not studied.

The main aims of this research were to extend the observation that PtdIns(3,4,5)P3 accumulation correlates with, and may signal for, O2− generation, and to determine which PI3-kinase isoforms are responsible for the increases in fMLP-stimulated PtdIns(3,4,5)P3 mass observed in primed cells. Because there is a lack of reagents, both Abs and inhibitors specific for the p110/p101 PI3-kinase-γ isoform, several different experimental strategies were used to address this issue.

A radioligand displacement assay was used to measure total PtdIns(3,4,5)P3 mass accumulation in primed and unprimed neutrophils after fMLP stimulation. This assay relies on the quantitative conversion of PtdIns(3,4,5)P3 to Ins(1,3,4,5)P4 under strong alkaline hydrolysis conditions and assay of the Ins(1,3,4,5)P4 generated using a specific radioreceptor assay incorporating the GAPPI4BP. As previously reported (30, 31), fMLP alone led to a rapid but transient increase in PtdIns(3,4,5)P3 accumulation maximal at 5–15 s. However, after priming with either TNF-α or GM-CSF, fMLP resulted in a more robust and prolonged increase in PtdIns(3,4,5)P3 accumulation, with PtdIns(3,4,5)P3 levels remaining elevated for up to 2 min. Our earlier studies in metabolically labeled cells failed to detect any defect in the metabolism of...
PtdIns(3,4,5)P₃ to PtdIns(3,4)P₂ in these cells (1), suggesting that the augmented accumulation of PtdIns(3,4,5)P₃ reflects enhanced production rather than inhibition of metabolism. Moreover, the reported ability of the NADPH oxidase to activate the PtdIns(3,4,5)P₃-5 phosphatase Src homology 2 domain-containing inositol phosphatase-1 via a Lyn-dependent mechanism would predict that the metabolism of PtdIns(3,4,5)P₃ would be enhanced rather than inhibited after fMLP stimulation (32). These data provide the first evidence of a difference in PtdIns(3,4,5)P₃ mass accumulation between fMLP-stimulated and primed/stimulated neutrophils. We also found that, while TNF-α treatment alone had no effect on PtdIns(3,4,5)P₃ levels at 30 min, GM-CSF incubation over the same period resulted in a small but consistent increase in PtdIns(3,4,5)P₃ accumulation. This agrees with previous work using metabolically labeled cells and estimates of [³²P]PtdIns(3,4,5)P₃ accumulation (30, 33). Of note, the priming status of the cells used in these experiments was confirmed by performing O₂⁻ release assays alongside each PtdIns(3,4,5)P₃ mass and immunoprecipitation assay to ensure that no biochemical analysis was undertaken in cells that had been inadvertently primed during cell isolation. Hence, any cells showing evidence of basal priming (i.e., having a ratio between TNF-α/fMLP and fMLP O₂⁻ generation of <4) were discarded.

Other priming agents were also examined to determine whether all such agents caused similar increases in O₂⁻ and PtdIns(3,4,5)P₃ responses. Cytochalasin B was found to prime both O₂⁻ generation and PtdIns(3,4,5)P₃ accumulation after 45 s of fMLP stimulation in a similar manner to that observed with TNF-α. Cytochalasin B is a non-G protein receptor-mediated agonist that has been widely used to induce neutrophil priming; however, its exact mode of action is not known and may involve other pathways besides PI3-kinase. The effects of LPS were also examined and, while both O₂⁻ and PtdIns(3,4,5)P₃ responses were increased, in the absence of serum the effects were much less dramatic than those observed with the other priming agents used (data not shown).

The ability of neutrophils to spontaneously “deprime” after exposure to PAF for 120 min was also investigated. Previous work from our group has shown that full priming (equivalent to that seen with TNF-α or GM-CSF at 30 min) is observed 10 min after PAF addition; thereafter the cells regain their nonpolarized rounded morphology, and CD11b/18 expression and function and O₂⁻ responses return to normal (7). Our results confirm normalization of the O₂⁻ responses after a 120-min incubation with PAF and show a parallel decline in fMLP-stimulated PtdIns(3,4,5)P₃ accumulation at a time point (45 s) that coincides with maximal respiratory burst activity (1).

Using a variety of PI3-kinase and other kinase inhibitors we have been able to show a remarkably similar response profile with respect to O₂⁻ generation and PtdIns(3,4,5)P₃ accumulation. Both PtdIns(3,4,5)P₃ and O₂⁻ generation was severely inhibited by LY294002, confirming the involvement of a PI3-kinase in both of these responses. The tyrosine kinase inhibitor genistein was also found to inhibit both PtdIns(3,4,5)P₃ and O₂⁻ generation, albeit at relatively high concentrations. Stauorosporine, a serine/threonine protein kinase and PKC inhibitor, was found to increase both fMLP-stimulated O₂⁻ generation and PtdIns(3,4,5)P₃ accumulation to levels equal to or higher than those observed with TNF-α priming. This rather surprising “priming” effect of stauorosporine has also been observed by other workers (27); however, the mechanism remains to be defined. More importantly, the lack of inhibition observed with radicicol, a selective tyrosine kinase inhibitor that lacks activity against the Gβγ-regulated PI3-kinase (28) but abolished p85-regulated PI3-kinase activity in BTSM cells (Fig. 3i and Ref. 21), suggested that the enhanced O₂⁻ and PtdIns(3,4,5)P₃ signal observed under priming conditions occurs via the p101/ p110γ PI3-kinase rather than through a tyrosine kinase-regulated process. However, it is possible that a component of the fMLP-stimulated PtdIns(3,4,5)P₃ response observed in unprimed cells originates from an alternative PI3-kinase; the data of Ptasznik et al. (28), who have shown that radicicol can inhibit fMLP-stimulated PtdIns(3,4,5)P₃ production in unprimed cells, support this conclusion.

Using more traditional lipid kinase assays to measure PI3-kinase activity, we found that fMLP was unable to stimulate PI3-kinase activity in either p85 or p40 immunoprecipitates, irrespective of the priming status of these cells. These data were obtained in the face of clearly positive signals in PDGF-stimulated BTSM cells and again point to the lack of involvement of a tyrosine kinase-regulated form of PI3-kinase in the PtdIns(3,4,5)P₃ responses observed at 45 s post-agonist addition. While such data agrees with that obtained by Naccache et al. (30), Stephens et al. (10), and Vlahos et al. (34), other groups have reported enhanced PI3-kinase activity in p85 immunoprecipitates from neutrophils (35, 36). However, in the latter study (36), lysates were prepared from adherent cells treated with TNF-α and it is possible that this, as well as the use of different primary Abs, may underlie such differences.

In agreement with Naccache et al. (30), we were able to show significant involvement of the p110γ subunit in neutrophil PI3-kinase activity after fMLP treatment. However, the increased PI3-kinase activity observed in p110γ immunoprecipitates prepared from cells treated with TNF-α alone was surprising, because there was no accompanying increase in PtdIns(3,4,5)P₃ accumulation. While it is possible that TNF-α enhances PtdIns(3,4,5)P₃ degradation (and this would concur with the small increases in [³²P]PtdIns3P accumulation in TNF-α-treated neutrophils previously reported (1)), effects of priming agents on Src homology 2 domain-containing inositol phosphatase-1 and the PtdIns(3,4,5)P₃ phosphatases are not described. It is also possible that the enzyme has limited access to PtdIns(4,5)P₂ or, more likely, that the absence of activated Gβγ subunits precludes PI3-kinase-γ activation in the intact cell. Similar explanations could pertain to the lack of difference in PI3-kinase-γ activity observed between unprimed and primed cells treated with fMLP. New insights into how 3-phosphorylated lipids might control the assembly and hence activity of the neutrophil NADPH oxidase complex have come from recent work demonstrating that components of the enzyme complex, namely p47(phox) and p40(phox), bind PtdIns(3,4)P₂ (both breakdown products of PtdIns(3,4,5)P₃), respectively, via PX domains (14). Therefore, it seems likely that PtdIns(3,4,5)P₃ degrading enzymes may be intimately involved in the control of the oxidase response.

The p85 and Gβγ-regulated forms of PI3-kinase have both been shown to bind p21ras, and indeed ras is thought to be an important upstream regulator of PI3-kinase. Additional data suggest that p21ras can also be a target of PI3-kinase (37). Ras also regulates the activity of the MEK1/2 pathway, which is also known to be up-regulated after priming (15). Despite this, we demonstrated that TNF-α had no effect on the amount of active, GTP-bound p21ras present in neutrophils and failed to influence the strength of the fMLP-stimulated signal. Likewise, fMLP alone caused robust activation of p21ras, with GM-CSF having an intermediate effect. While we have not excluded the possibility that priming agents alter the activity of p21ras within the detergent-insoluble neutrophil fraction, our data concur with those reported by Coffer et al. (35) and suggest a dissociation between p21ras activity and both O₂⁻ release and PtdIns(3,4,5)P₃ accumulation. Thus, these data indicate that priming agents enhance PI3-kinase-γ activity by factors other than p21ras. Because previous studies have shown that priming-mediated effects on receptor expression are temporally
dissociated from the up-regulation of secretory responses and that MLPL-stimulated Ins(1,4,5)P₃ accumulation is unaffected by priming (1), other mechanisms, including the regulation of Gₛ subunit expression (38) or factors affecting PI3-kinase localization or substrate access, need to be considered.

In conclusion, priming represents a major checkpoint controlling the functional responsiveness of neutrophils to secretagogue agonists and, as such, their capacity to induce tissue damage in vivo. We have demonstrated the close relationship between MLPL-stimulated PtdIns(3,4,5)P₃ accumulation and O₂⁻ generation under conditions of (TNF-α, GM-CSF, cytochalasin B, and PAF-induced) priming, depriming, and kinase inhibition and present evidence to suggest that the Gβγ-regulated PI3-kinase (P101/p110γ) is the dominant PI3-kinase involved in this response.

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References


