Distinct Roles of Receptor Phosphorylation, G Protein Usage, and Mitogen-activated Protein Kinase Activation on Platelet Activating Factor-induced Leukotriene C4 Generation and Chemokine Production*

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Platelet activating factor (PAF) interacts with cell surface G protein-coupled receptors on leukocytes to induce degranulation, leukotriene C4 (LTC4) generation, and chemokine CCL2 production. Using a basophilic leukemia RBL-2H3 cell line expressing wild-type PAF receptor (PAFR) and a phosphorylation-deficient mutant (mPAFR), we have previously demonstrated that receptor phosphorylation mediates desensitization of PAF-induced degranulation. Here, we sought to determine the role of receptor phosphorylation on PAF-induced LTC4 generation and CCL2 production. We found that PAF caused a significantly enhanced LTC4 generation in cells expressing mPAFR when compared with PAFR cells. In contrast, PAF-induced CCL2 production was greatly reduced in mPAFR cells. Pertussis toxin and U0126, which inhibit Gi and p44/42 mitogen-activated protein kinase (ERK) activation, respectively, caused very little inhibition of PAF-induced CCL2 production (∼20% inhibition). In contrast, these inhibitors almost completely blocked both PAF-induced ERK phosphorylation and LTC4 generation in PAFR cells. However, in mPAFR cells pertussis toxin only partially inhibited PAF-induced ERK phosphorylation. A Ca2+-/calmodulin inhibitor had no effect on PAF-induced ERK phosphorylation in PAFR cells but completely blocked the response in mPAFR cells. These data demonstrate that receptor phosphorylation, which serves to desensitize PAF-induced LTC4 generation, is required for chemokine CCL2 production. They also indicate a previously unrecognized selectivity in G protein usage and ERK activation for PAF-induced responses. Whereas PAF-induced CCL2 production is, in large part, mediated independently of G1 activation or ERK phosphorylation, LTC4 generation requires ERK phosphorylation, which is mediated by different G proteins depending on the phosphorylation status of the receptor.

Platelet-activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine, PAF)1 is an important mediator of inflammation that is released from mast cells, platelets, neutrophils, monocytes, and macrophages (1, 2). PAF activates cell surface G protein-coupled receptors (GPCRs) to induce divergent biological functions (3). PAF is a potent leukocyte chemoattractant (4) that also induces degranulation (5, 6), leukotriene C4 (LTC4) generation (7, 8), and chemokine gene expression in a wide variety of cells (9–13). Although much has been learned regarding the signaling pathways involved in PAF-induced chemotaxis and degranulation (4, 6, 14), very little information is available on the mechanism by which PAF stimulates LTC4 generation and chemokine production.

Receptor phosphorylation by G protein-coupled receptor kinase (GRK) and the subsequent recruitment of β-arrestin are essential for uncoupling the receptor from G proteins (15). Thus, phosphorylation-deficient mutants of chemoattractant receptors expressed in basophilic leukemia RBL-2H3 cells respond to the ligand for enhanced G protein activation, a more sustained Ca2+ mobilization, and a greater extent of degranulation when compared with cells expressing wild-type receptors (14, 16, 17). Receptor phosphorylation and β-arrestin recruitment have recently been shown to mediate MAP kinase activation for many GPCRs (18–21). However, the chemoattractants formylpeptide and the complement component C3a stimulate ERK phosphorylation via pathways that do not require receptor phosphorylation or β-arrestin recruitment (17, 22). We have recently shown that C3a receptor phosphorylation by the G protein-coupled receptor kinase provides a stimulatory signal that synergizes with ERK activation to induce chemokine CCL2 (also known as monocytes chemoattractant protein-1 or MCP-1) production (17).

Unlike the C3a receptor, which couples to G1, the PAF receptor interacts with the G1 and Gq family of G proteins to induce distinct biological responses (4, 23). In the present study, we sought to determine the roles of receptor phosphorylation, G protein usage, and ERK phosphorylation on PAF-induced LTC4 generation and CCL2 production. For this purpose, RBL-2H3 cells expressing the wild-type PAF receptor (PAFR) and a phosphorylation-deficient mutant of PAFR (mPAFR) were used (14). Here, we demonstrate that receptor phosphorylation, which serves to desensitize PAF-induced LTC4 generation, is required for chemokine CCL2 production. Furthermore, CCL2

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1 The abbreviations used are: PAF, platelet-activating factor; GPCR, G protein-coupled receptor; LTC4, leukotriene C4; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; CCL2, CC chemokine receptor ligand 2 (formerly known as MCP-1); PAFR, PAF receptor; mPAFR, mutant PAFR; PTX, pertussis toxin; cPLA2, cytosolic phospholipase A2; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; βarr2, β-arrestin 2; GFP, green fluorescent protein.

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production is, in large part, mediated independently of G activation or ERK phosphorylation. In contrast, LTC4 generation is dependent on ERK phosphorylation, which is mediated via different mechanisms depending on the phosphorylation status of the receptor.

**EXPERIMENTAL PROCEDURES**

**Materials**—PAF, fluphenazine, Ro-31-8220, and U0126 were purchased from Calbiochem. [3H]PAF (1-O-hexadecyl-[acetyl-3H(N)]) (499.5 GBq/mmol) was obtained from PerkinElmer Life Sciences. Rabbi anti-p44/42 MAP kinase and anti-phospho-p44/42 MAP kinase antibodies were obtained from Cell Signaling Technology (Boston, MA). Anti-PAFR and mPAFR were purchased from Amersham Biosciences. A CCL2 sandwich ELISA kit was purchased from BioSource International (Camarillo, CA). Texas Red-conjugated goat anti-mouse IgG was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA).

**Cell Culture, Transfection, Ca2+ Mobilization, and Degranulation**—RBL-2H3 cells stably expressing wild-type PAFR were stimulated with different concentrations of PAF for 20 min or 6 h (A and B) or with a fixed concentration of PAF (100 nM) for the indicated time periods (C and D). LTC4 generation and CCL2 production were quantified by EIA and a sandwich ELISA, respectively. Basal values of 20.3 ± 1.2 (CCL2) and 16.8 ± 0.9 (LTC4) were subtracted from the values shown. The data shown are from one of three similar experiments.

**Results**

**FIG. 1. Dose and time dependence of PAF-induced LTC4 generation and CCL2 production.** RBL-2H3 cells stably expressing wild-type PAFR were stimulated with different concentrations of PAF for 20 min or 6 h (A and B) or with a fixed concentration of PAF (100 nM) for the indicated time periods (C and D). LTC4 generation and CCL2 production were quantified by EIA and a sandwich ELISA, respectively. Basal values of 20.3 ± 1.2 (CCL2) and 16.8 ± 0.9 (LTC4) were subtracted from the values shown. The data shown are from one of three similar experiments.

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**A)** LTC4 production in response to PAF at different concentrations over 20 min. **B)** CCL2 production in response to PAF at different concentrations over 20 min. **C)** LTC4 production in response to PAF over 6 h. **D)** CCL2 production in response to PAF over 6 h.

**Discussion**

Phosphatidylserine is known to activate PI3K/AKT signaling, which is dependent on ERK phosphorylation, which is mediated via different mechanisms depending on the phosphorylation status of the receptor.
2H3 cells (0.4 \times 10^6/well) expressing PAFR or mPAFR were cultured in complete growth medium overnight. Cells were stimulated with PAF for 6 h (CCL2) and 20 min (LTC4) unless otherwise stated. Supernatants were collected and stored frozen at -80°C until analysis. CCL2 (17) and LTC4 levels were quantified using sandwich ELISA and EIA kits, respectively, as described in the manufacturer’s protocols.

**RESULTS**

**Characterization of PAF-induced LTC4 Generation and Chemokine CCL2 Production in Transfected RBL-2H3 Cells**—We have previously shown that PAF stimulates degranulation in RBL-2H3 cells stably expressing PAFR with an EC50 value of 3 nM (6). In the present study, we stimulated these cells with different concentrations of PAF and determined LTC4 generation and chemokine CCL2 production. The data shown are from one of three similar experiments.

**Trafficking of GFP-β Arrestin by Confocal Microscopy**—Cells coexpressing hemagglutinin-tagged receptors with Barr2-GFP were plated on 35-mm glass bottom dishes (Mat Tek, Ashland, MA). The cells were stimulated with 100 nM PAF for 1 min at 37°C. The reaction was stopped by adding 3 volumes of cold phosphate-buffered saline, and the cells were then washed and fixed with 2% paraformaldehyde solution for 30 min at room temperature. To visualize cell surface receptor expression, cells were incubated with the 12CA5 antibody followed by the Texas Red-conjugated secondary antibody (Jackson ImmunoResearch). Cells were observed using a laser-scanning confocal microscope (Olympus FluoView, Olympus, Melville, NY) with a 60× lens. The GFP was excited by using a 488-nm argon/krypton laser, and Texas Red was excited at 515–540- and 570-nm band pass filters, respectively (17).

**Phosphorylation of ERK-1/ERK-2**—RBL-2H3 cells expressing PAFR or mPAFR were stimulated with PAF (100 nM) in HEPES buffered saline, and the reaction was stopped at different time periods by the addition of a 3-fold excess ice-cold phosphate-buffered saline containing 1 mM sodium orthovanadate. Cells were mixed with an equal volume of 2× SDS sample buffer and heated to 90°C for 10 min. Samples were electrophoresed in 10% SDS-polyacrylamide gels and transferred onto a nitrocellulose filter. The filter was treated with 3% nonfat milk in phosphate-buffered saline and incubated with an antibody specific for phosphorylated p44/42 MAP kinase. The reaction was detected by enhanced chemiluminescence. The membrane was stripped and reprobed with an antibody that reacts with unphosphorylated p44/42 MAP kinase (17, 26).

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**FIG. 2.** Effects of receptor phosphorylation on PAF-induced Ca2+ mobilization, cPLA2 activity, LTC4 generation, and CCL2 production in RBL-2H3 cells stably expressing PAFR and mPAFR. A, RBL-2H3 cells stably expressing wild-type PAFR or a phosphorylation-deficient mutant (mPAFR) were loaded with Indo-1 acetoxymethyl and stimulated with PAF (100 nM), and Ca2+ mobilization was determined. B, cells were stimulated with PAF (100 nM) for 2 min, and cPLA2 activity in cell lysate was determined as described in the “Experimental Procedures” section. C, cells were stimulated with PAF (100 nM) for 20 min, and the supernatants were removed and assayed for LTC4 generation by EIA. D, cells were stimulated with PAF (100 nM) for 6 h, and the supernatants were removed and assayed for CCL2 production by ELISA. Open bars, −PAF; filled bars, +PAF. The inset to panel D shows the time course of CCL2 production in response to 100 nM PAF in PAFR and mPAFR cells. The data shown are from one of three similar experiments.
Roles of Receptor Phosphorylation and β-Arrestin Recruitment on PAF-induced LTC₄ Generation and CCL2 Production—We have previously shown that receptor phosphorylation leads to the desensitization of PAF-induced degranulation in leukocytes (14). To determine the role of receptor phosphorylation on PAF-induced LTC₄ generation and CCL2 production, RBL-2H3 cells expressing wild-type PAFR and phosphorylation-deficient mutant mPAFR were used (14). PAF stimulated an equivalent Ca²⁺ mobilization in PAFR and mPAFR cells (Fig. 2A). PAF-induced LTC₄ generation requires Ca²⁺-dependent activation of cPLA₂ (27). Therefore, the ability of PAF to stimulate cPLA₂ activity in PAFR and mPAFR cells was determined. As shown in Fig. 2B, PAF caused equivalent cPLA₂ activity in PAFR and mPAFR cells. PAF also stimulated the generation of LTC₄ in PAFR and mPAFR cells to similar levels (Fig. 2C). The incubation of PAFR cells with PAF for 6 h resulted in maximal CCL2 production (Figs. 1D and 2D). Under this condition, PAF did not cause CCL2 production in mPAFR cells (Fig. 2D) despite the fact that this mutated receptor signals for Ca²⁺ mobilization, PLA₂ activity, and LTC₄ generation (Fig. 2, A–C). The possibility that the lack of CCL2 production in mPAFR cells reflects a slower rate of production is unlikely because incubation of these cells with PAF for up to 18 h failed to induce any chemokine (Fig. 2D, inset).

Receptor-ligand binding studies were performed to evaluate the number of receptors present in the cells used in the experiments described above. RBL-2H3 cells expressed 152,300 ± 2,906 (n = 3) PAFRs per cell. In contrast, mPAFR cells expressed 28,630 ± 753 (n = 3) receptors per cell. It is therefore quite possible that the inability of PAF to stimulate CCL2 production in mPAFR cells reflects the expression of lower receptor numbers than PAFR. We were previously unsuccessful in generating stable transfectants in RBL-2H3 cells expressing high levels of mPAFR. For this reason, we optimized a transient transfection procedure to express PAFR and mPAFR at similar levels. Flow cytometric analysis of receptor expression using the 12CA5 antibody is shown in Fig. 3A. Using this system, we tested the effects of PAF on LTC₄ generation, degranulation, and CCL2 production. PAF stimulated significantly enhanced LTC₄ generation and degranulation in mPAFR cells when compared with PAFR cells (Fig. 3, B and C). In contrast, the ability of PAF to induce CCL2 production in
mPAFR cells was ~60% less than that observed in PAFR cells (Fig. 3D). These data suggest that receptor phosphorylation, which desensitizes PAF-induced degranulation and LTC₄ generation, provides a stimulatory signal for CCL2 production.

Ligand-induced receptor phosphorylation is associated with the translocation of β-arrestin from the cytosol to the plasma membrane (28, 29). To determine whether β-arrestin recruitment correlates with PAF-induced responses, transient transfectants were generated in RBL-2H3 cells coexpressing PAFR or mPAFR and the β-arrestin 2/green fluorescent protein conjugate (βarr2-GFP). As shown in Fig. 4A, PAF caused the translocation of βarr2-GFP from the cytosol to the plasma membrane in PAFR cells. In contrast, PAF did not induce this response in mPAFR cells (Fig. 4B).

Roles of G Protein Usage, Phospholipase Cβ Activation, and ERK Phosphorylation on PAF-induced CCL2 Production and LTC₄ Generation—PAFR couples to G₁ in RBL-2H3 cells to induce chemotaxis (4). PAF also stimulates ERK phosphorylation in Chinese hamster ovary (CHO) cells via a PTX-sensitive G protein (30). In contrast, PAF-induced degranulation requires both G₁ and G₂-mediated activation of phospholipase Cβ, resulting in the activation of protein kinase C (PKC) and the mobilization of Ca²⁺ (4, 6). We first evaluated the role of signaling through G proteins on PAF-induced CCL2 production and LTC₄ generation in PAFR cells. Cells were cultured overnight with or without pertussis toxin (PTX, 100 ng/ml), and its effect on PAF-induced responses was determined. As shown in Fig. 5A, PTX inhibited PAF-induced CCL2 production by 27 ± 3.0%. In contrast, PAF-induced LTC₄ generation by 92.6 ± 4.6% (Fig. 5B). To determine the role of phospholipase Cβ-dependent signaling, we tested the effects of the inhibitors of protein kinase C (Ro-31-8220 and Ca²⁺/calmodulin (fluphenazine) on PAF-induced responses. Both Ro-31-8220 and fluphenazine almost completely blocked PAF-induced CCL2 production and LTC₄ generation (>90% inhibition) (Fig. 5, A and B). To test the role of p44/42 MAP kinase activation on PAF-induced responses, the effect of U0126 was tested. This MAP kinase inhibitor blocked PAF-stimulated CCL2 production by 24 ± 1.3% (Fig. 5A), but it inhibited LTC₄ generation by 95 ± 1.5% in PAFR cells (Fig. 5B). The effects of these inhibitors were also tested on PAF-induced LTC₄ generation in mPAFR cells. PTX blocked PAF-induced LTC₄ generation in mPAFR cells by 51.3 ± 3.7% (Fig 5C). This is in contrast to the situation in PAFR cells where PTX inhibited PAF-induced response by 92.6 ± 4.6% (Fig. 5B). However, as in PAFR cells, Ro-31-8220, fluphenazine, or U0126 almost completely blocked PAF-induced LTC₄ generation in mPAFR cells (>90% inhibition) (Fig. 5C).

**PAF Stimulates ERK Phosphorylation in RBL-2H3 Cells via Different Mechanisms That Depend on the Phosphorylation Status of the Receptor—** As shown above (Fig. 5, B and C), PAF-induced LTC₄ generation in PAFR and mPAFR cells appears to be mediated by different G proteins. To test the role of different G protein usage on PAF-induced ERK phosphorylation, the effects of PTX on PAFR and mPAFR responses were determined. As shown in Fig. 6A, PTX caused substantial inhibition of PAF-induced ERK phosphorylation in PAFR cells (91 ± 4.6% inhibition). In contrast, PTX was much less effective in inhibiting this response in mPAFR cells (44 ± 3.0% inhibition) (Fig. 6B). However, Ro-31-8220 caused almost complete inhibition (>90%) of PAF-induced ERK phosphorylation in both cell types (Fig. 6, A and B). Interestingly, fluphenazine had no inhibitory effect on ERK phosphorylation mediated by PAF in PAFR cells (Fig. 6A), but it inhibited the response in mPAFR cells by 95.3 ± 2.3% (Fig 6B). U0126 blocked ERK phosphorylation in response to PAF in both cell types (Fig. 6, A and B).

**DISCUSSION**

PAF plays an important role in inflammatory and cardiovascular diseases (31, 32). PAF stimulates chemotaxis and degranulation.
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PAF stimulates ERK phosphorylation in RBL-2H3 cells via different mechanisms that depend on the phosphorylation status of the receptor. RBL-2H3 cells stably expressing PAFR (A) or mPAFR (B) were incubated with medium (CON, control), PTX (100 ng/ml, overnight), Ro-31-8220 (10 μM, 10 min), fluphenazine (FLU, 30 μM, 30 min), or U0126 (1 μM, 30 min) and stimulated with PAF (100 nM) for 1 min, and ERK phosphorylation was determined by Western blotting using a phospho-ERK-specific antibody. The extent of ERK phosphorylation is expressed as percent of PAF-stimulated responses. ***,$p < 0.001$ versus the response in PAFR cells.

Fig. 6

We recently reported that complement component C3a stimulates LTC4 generation and chemokine production in a variety of cell types (7, 9–13, 27). We have previously utilized RBL-2H3 cells stably expressing PAFR and a cytoplasmic tail deletion mutant receptor (mPAFR) and demonstrated that receptor phosphorylation plays an important role in the desensitization of PAF-induced degranulation (14). The goal of the present study was to determine the role of receptor phosphorylation on PAF-induced LTC4 generation and chemokine CCL2 production. Here, we demonstrate that receptor phosphorylation desensitizes PAF-induced LTC4 generation but provides a stimulatory signal for chemokine CCL2 production. We also show distinct differences in both G protein usage and ERK phosphorylation on LTC4 generation and CCL2 production.

We have previously shown that C3a-induced chemokine production requires the interaction of two signals, one receptor phosphorylation-dependent and the other G protein-dependent (17). In contrast to the situation with PAFR, the G protein-dependent signal for the C3a receptor (C3aR) involves Gi-mediated ERK phosphorylation (17). These findings suggest that GPCR-induced chemokine production is mediated via shared (receptor phosphorylation-dependent) and distinct pathways that differ in the G protein usage of the receptor.

The demonstration in the present study that the treatment of cells with U0126 or a Ca2+/calmodulin inhibitor leads to a substantial inhibition of PAF-induced LTC4 generation is consistent with the roles of ERK phosphorylation and Ca2+ mobilization on LTC4 generation (7, 27, 37, 38). Although PAF-induced ERK phosphorylation has been studied in some detail, the mechanism of its activation has not been clearly defined (30, 39–41). The data presented herein indicate that PAF-induced ERK phosphorylation in RBL-2H3 cells is mediated by different mechanisms, depending on the phosphorylation status of the receptor. For example, PAF-induced ERK phosphorylation in PAFR cells requires activation of a PTX-sensitive G protein. In contrast, this response in mPAFR cells involves both PTX-sensitive as well as PTX-insensitive G proteins. We have previously shown that PAF-induced Ca2+/calmodulin inhibitor fluphenazine blocked ERK phosphorylation in mPAFR but not PAFR cells raises the intriguing question of whether this is due to differences in the G protein usage of the receptor.
variety of cell types (27, 34, 37, 42). Therefore, receptor–chemokine CCL2 production. The activation of many GPCRs
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reduced LTC4 generation but provides a stimulatory signal for
granulation (14). In the present study, we demonstrate that
phorylation mediates the desensitization of PAF-induced de-
possibility that receptor phosphorylation modulates the G pro-
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