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Abstract

Platelet activating factor (PAF) interacts with cell surface G protein-coupled receptors on leukocytes to induce degranulation, leukotriene C_4 (LTC_4) 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 LTC_4 generation and CCL2 production. We found that PAF caused a significantly enhanced LTC_4 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 G_i 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 LTC_4 generation in PAFR cells. However, in mPAFR cells pertussis toxin only partially inhibited PAF-induced ERK phosphorylation. A Ca^{2+}/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 LTC_4 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 G_i activation or ERK phosphorylation, LTC_4 generation requires ERK phosphorylation, which is mediated by different G proteins depending on the phosphorylation status of the receptor.

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Distinct Roles of Receptor Phosphorylation, G Protein Usage, and Mitogen-activated Protein Kinase Activation on Platelet Activating Factor-induced Leukotriene C_4 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 C_4 (LTC_4) 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 LTC_4 generation and CCL2 production. We found that PAF caused a significantly enhanced LTC_4 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 G_i 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 LTC_4 generation in PAFR cells. However, in mPAFR cells pertussis toxin only partially inhibited PAF-induced ERK phosphorylation. A Ca^{2+}/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 LTC_4 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 G_i activation or ERK phosphorylation, LTC_4 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 C_4 (LTC_4) 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 LTC_4 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 Ca^{2+} 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 G_i, the PAF receptor interacts with the G_i and G_q 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 LTC_4 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 LTC_4 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; LTC_4, leukotriene C_4; 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; $cPLA_2$, cytosolic phospholipase A_2; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; βarr2, β-arrestin 2; GFP, green fluorescent protein.

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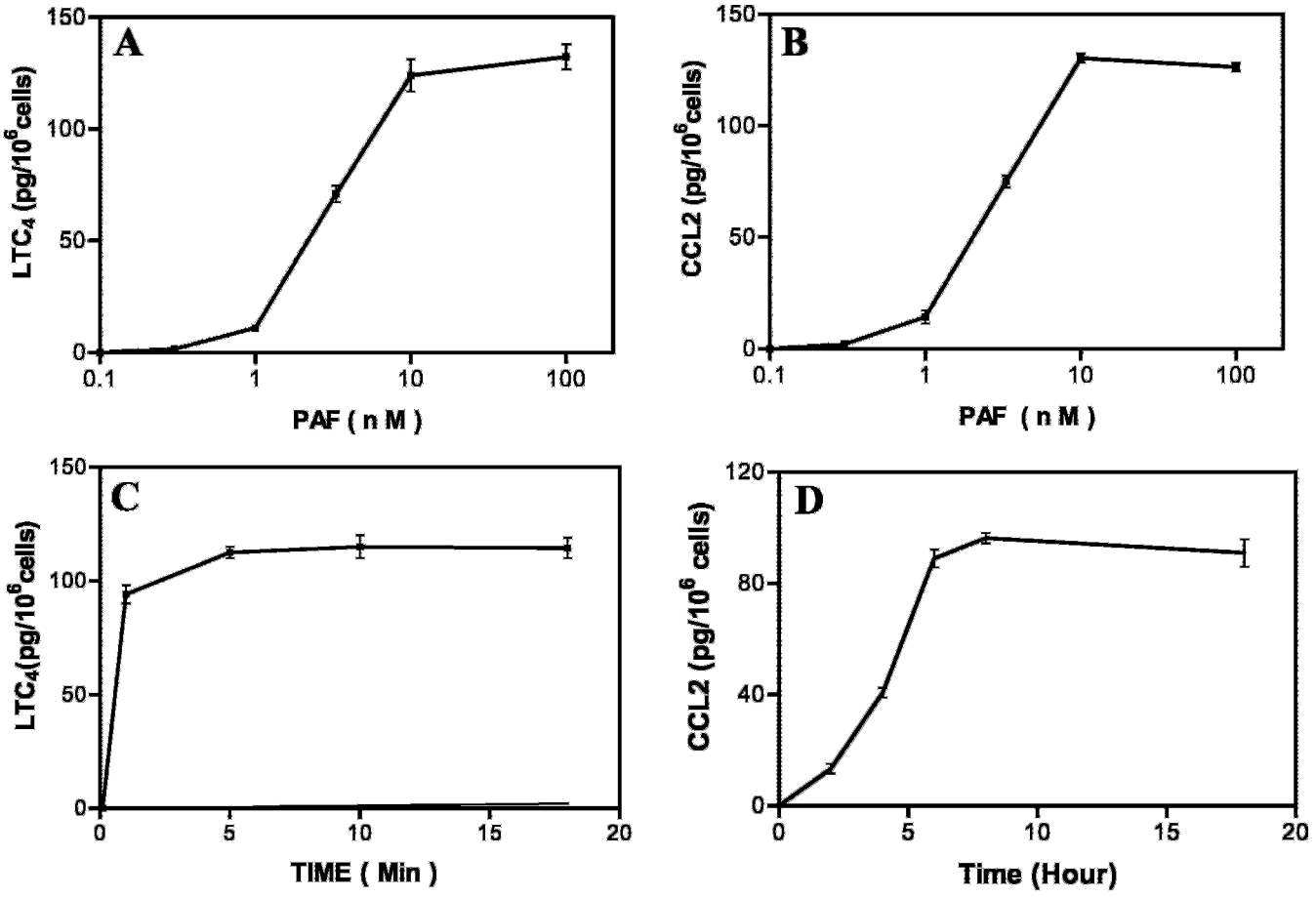

FIG. 1. **Dose and time dependence of PAF-induced LTC_4 generation and CCL2 production.** RBL-2H3 cells stably expressing wild-type PAFR were stimulated with different concentrations of PAF for 20 min or 6 h (*A* and *B*) or with a fixed concentration of PAF (100 nM) for the indicated time periods (*C* and *D*). LTC_4 generation and CCL2 production were quantified by EIA and a sandwich ELISA, respectively. Basal values of 20.3 ± 1.2 (CCL2) and 16.6 ± 0.9 (LTC_4) were subtracted from the values shown. The data shown are from one of three similar experiments.

production is, in large part, mediated independently of G_i activation or ERK phosphorylation. In contrast, LTC_4 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. [^{3}H]PAF (1-*O*-hexadecyl-[acetyl-^{3}H(N)]) (499.5 GBq/mmol) was obtained from PerkinElmer Life Sciences. Rabbit anti-p44/42 MAP kinase and anti-phospho-p44/42 MAP kinase antibodies were obtained from New England Biolabs (Beverly, MA). 12CA5 and anti-mouse IgG-R-phycoerythrin antibodies were obtained from Roche Molecular Biochemicals and Southern Biotechnology Associates (Birmingham, AL), respectively. Pertussis toxin (PTX) and all tissue culture reagents were purchased from Invitrogen. Indo-1 acetoxymethyl and Pluronic F-127 were from Molecular Probes (Eugene, OR). A $cPLA_2$ assay kit was purchased from Cayman Chemicals (Ann Arbor, MI). LTC_4 sandwich EIA and ECL Western blotting analysis kits 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, Ca^{2+} Mobilization, and Degranulation—RBL-2H3 cells stably expressing hemaglutinin-tagged PAFR and mPAFR were used in this study (6, 14). The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum, glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml) (6, 24). To express equivalent receptors, 7 μg of cDNA encoding PAFR and 25 μg cDNA encoding mPAFR were used for transient transfection by electroporation. For studies with β-arrestin, 20 μg of total cDNA in the ratio of 1:4 for PAFR/mPAFR and βarr2-GFP (17) were used. Cells were cultured in complete growth medium, and experiments were performed 16–18 h after transfection. Cell surface receptor expression was determined by incubating cells with 12CA5 or an isotope-matched antibody followed by phycoerythrin-conjugated mouse IgG and analyzed on a FACStar^{PLUS} flow cytometer (BD Biosciences, Mountain View, CA). For Ca^{2+} mobilization, cells (3×10^6) were loaded with 1 μM Indo-1 acetoxymethyl in the presence of 1 μM Pluronic F127 for 30 min at room temperature. Cells were washed, resuspended in 1.5 ml of HEPES-buffered saline, and intracellular Ca^{2+} mobilization was determined as described previously (6). For degranulation, cells (5×10^4 cells/well) were cultured overnight in a 96-well tissue culture plate. Cells were washed with HEPES-buffered saline, stimulated with PAF, and the extent of degranulation was determined by measuring the release of β-hexosaminidase (6, 24).

PAF Binding—Binding studies were performed to evaluate the number of receptors present in PAFR and mPAFR cells as described by Carlson *et al.* (25). Briefly, RBL-2H3 cells (2×10^5/well) stably expressing PAFR and mPAFR were plated in 24-well dishes. Cells were washed twice with ice-cold buffer and then resuspended in 0.5 ml of the same buffer containing 10 nM [^{3}H]PAF alone or with 10 μM unlabeled PAF. Cells were then incubated for 4 h at 4 °C. Cells were washed with same buffer three times and lysed with 0.5 ml of 0.1% Triton X-100. Bound radioactivity was determined by scintillation counting of the cell lysates. B_{max} values were normalized on the basis of cell number by counting the number of cells in three individual wells.

$cPLA_2$ Enzyme Activity—RBL-2H3 cells (2×10^6/ml) were stimulated with 100 nM PAF for 2 min at 37 °C. The reaction was stopped by adding 3 volumes of ice-cold buffer. Cell pellets were resuspended in 50 μl of phosphate-buffered saline containing protease inhibitors, homogenized briefly in a microhomogenizer (0.2 ml), and centrifuged at 12,000 × *g* at 4 °C for 15 min. PLA_2 activity of the cell free lysate was determined using a $cPLA_2$ assay kit as described in the manufacturer's protocol (Cayman Chemicals).

Assay of Chemokine CCL2 Production and LTC_4 Generation—RBL-

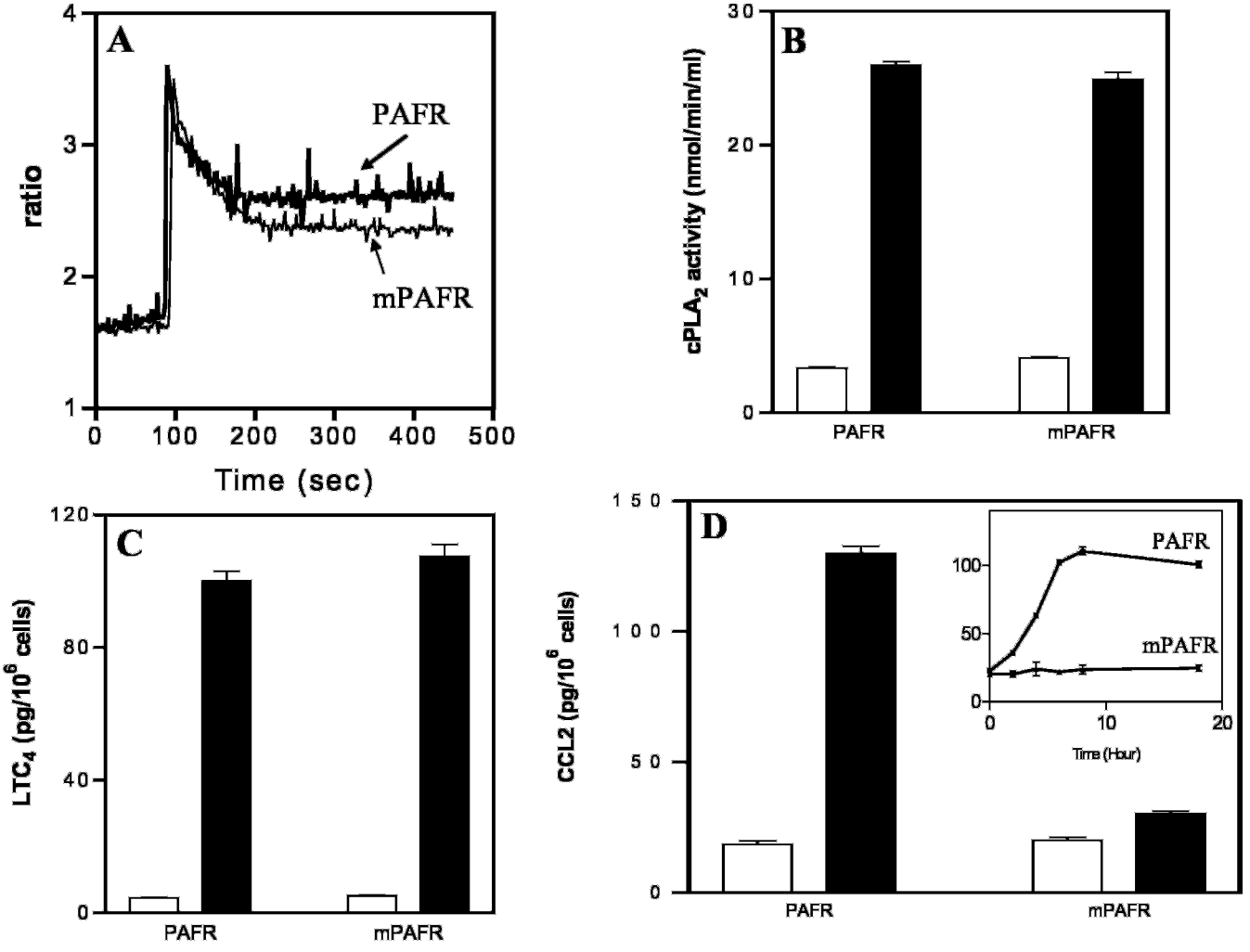

FIG. 2. **Effects of receptor phosphorylation on PAF-induced Ca^{2+} mobilization, $cPLA_2$ activity, LTC_4 generation, and CCL2 production in RBL-2H3 cells stably expressing PAFR and mPAFR.** *A*, RBL-2H3 cells stably expressing wild-type PAFR or a phosphorylation-deficient mutant (mPAFR) were loaded with Indo-1 acetoxymethyl and stimulated with PAF (100 nM), and Ca^{2+} mobilization was determined. *B*, cells were stimulated with PAF (100 nM) for 2 min, and $cPLA_2$ 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 LTC_4 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.

2H3 cells (0.4×10^6/well) expressing PAFR or mPAFR were cultured in complete growth medium overnight. Cells were stimulated with PAF for 6 h (CCL2) and 20 min (LTC_4) unless otherwise stated. Supernatants were collected and stored frozen at −80 °C until analysis. CCL2 (17) and LTC_4 levels were quantified using sandwich ELISA and EIA kits, respectively, as described in the manufacturer's protocols.

Trafficking of GFP-β Arrestin by Confocal Microscopy—Cells coexpressing hemagglutinin-tagged receptors with βarr2-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).

RESULTS

Characterization of PAF-induced LTC_4 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 EC_{50} value of 3 nM (6). In the present study, we stimulated these cells with different concentrations of PAF and determined LTC_4 generation and chemokine CCL2 production. As for degranulation, PAF stimulated both LTC_4 generation and CCL2 production with an EC_{50} of ~3 nM (Fig. 1, *A* and *B*). However, there were remarkable differences in the time course of these responses. For example, LTC_4 generation was essentially complete within 1 min after stimulation (Fig. 1*C*). In contrast, CCL2 production was not evident until 2 h, reached a peak at ~6 h, and remained elevated for up to 18 h after stimulation (Fig. 1*D*).

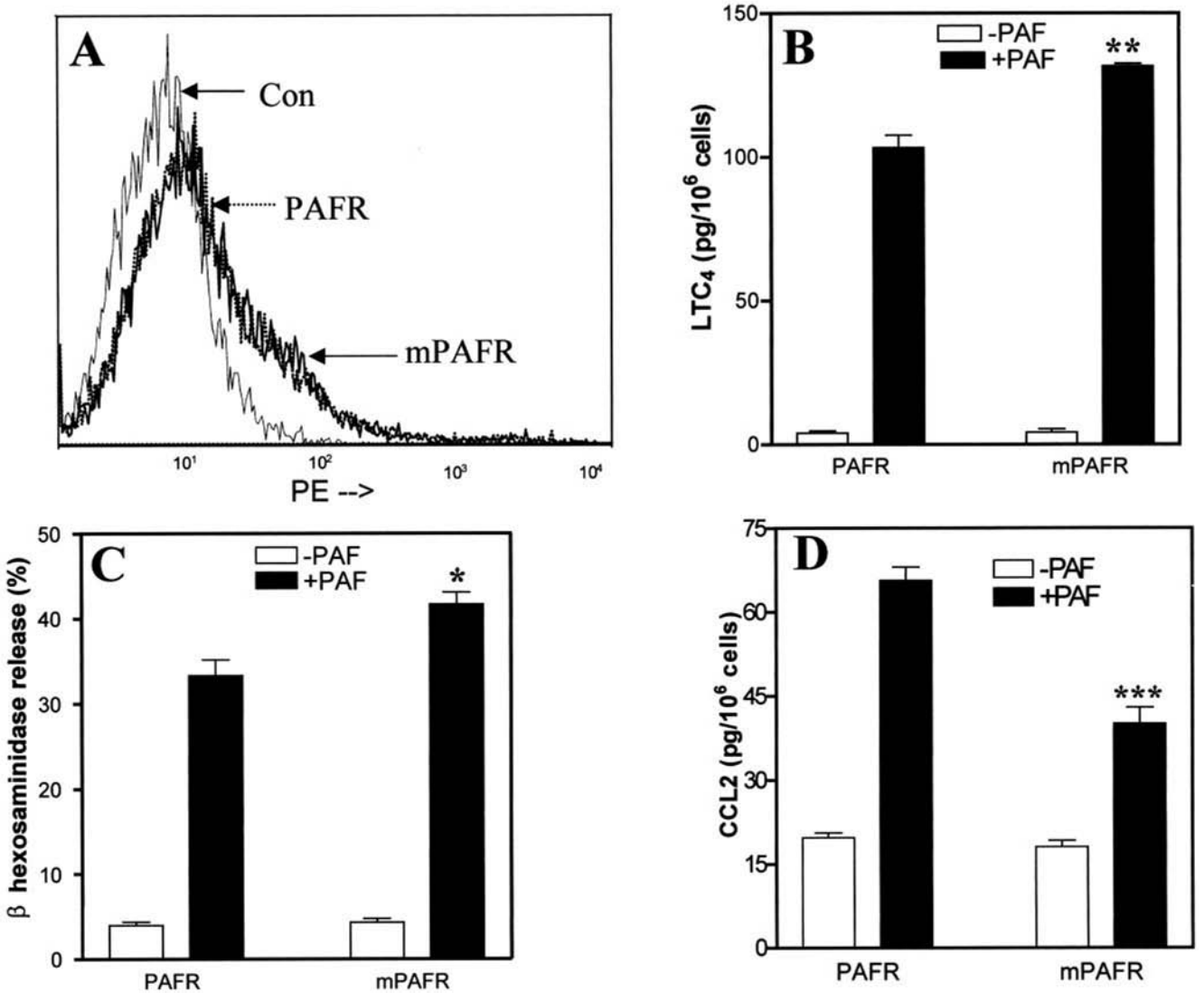

FIG. 3. **Effects of receptor phosphorylation on PAF-induced LTC_4 generation, degranulation, and CCL2 production in RBL-2H3 cells transiently expressing PAFR and mPAFR.** Transient transfectants were generated in RBL-2H3 cells expressing hemagglutinin-tagged PAFR or mPAFR. Cell surface receptor expression was determined by flow cytometry using the 12CA5 antibody (*A*). Cells were stimulated with PAF (100 nM) for 20 min, and the supernatants were removed and assayed for LTC_4 generation (*B*) and β-hexosaminidase release (*C*). Cells were stimulated with PAF (100 nM) for 6 h; the supernatants were removed and assayed for CCL2 production by ELISA (*D*). *Con*, control; *, $p < 0.05$; **, <0.01; ***, <0.001 *versus* the response in PAFR cells.

Roles of Receptor Phosphorylation and β-Arrestin Recruitment on PAF-induced LTC_4 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 LTC_4 generation and CCL2 production, RBL-2H3 cells expressing wild-type PAFR and phosphorylation-deficient mutant mPAFR were used (14). PAF stimulated an equivalent Ca^{2+} mobilization in PAFR and mPAFR cells (Fig. 2*A*). PAF-induced LTC_4 generation requires Ca^{2+}-dependent activation of $cPLA_2$ (27). Therefore, the ability of PAF to stimulate $cPLA_2$ activity in PAFR and mPAFR cells was determined. As shown in Fig. 2*B*, PAF caused equivalent $cPLA_2$ activity in PAFR and mPAFR cells. PAF also stimulated the generation of LTC_4 in PAFR and mPAFR cells to similar levels (Fig. 2*C*). The incubation of PAFR cells with PAF for 6 h resulted in maximal CCL2 production (Figs. 1*D* and 2*D*). Under this condition, PAF did not cause CCL2 production in mPAFR cells (Fig. 2*D*) despite the fact that this mutated receptor signals for Ca^{2+} mobilization, PLA_2 activity, and LTC_4 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. 2*D*, *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. 3*A*. Using this system, we tested the effects of PAF on LTC_4 generation, degranulation, and CCL2 production. PAF stimulated significantly enhanced LTC_4 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

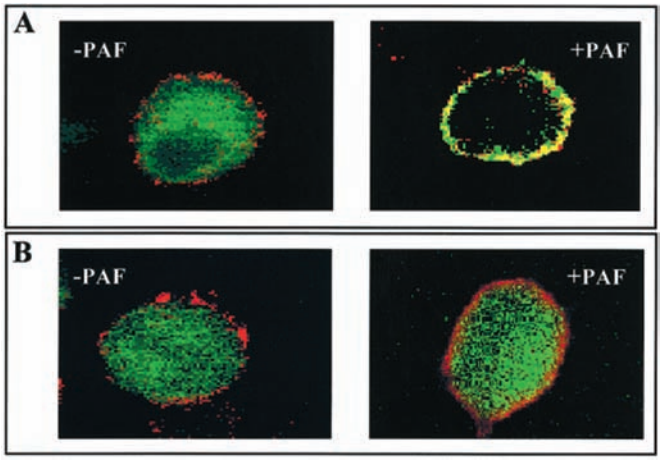

FIG. 4. **PAF causes translocation of βarr2-GFP from the cytoplasm to the plasma membrane in PAFR but not in mPAFR cells.** RBL-2H3 cells transiently coexpressing PAFR and βarr2-GFP (*A*) or mPAFR and βarr2-GFP (*B*) were stimulated with PAF (100 nM) for 1 min, and the translocation of βarr2-GFP was determined by confocal microscopy. The data shown are representative of three similar experiments.

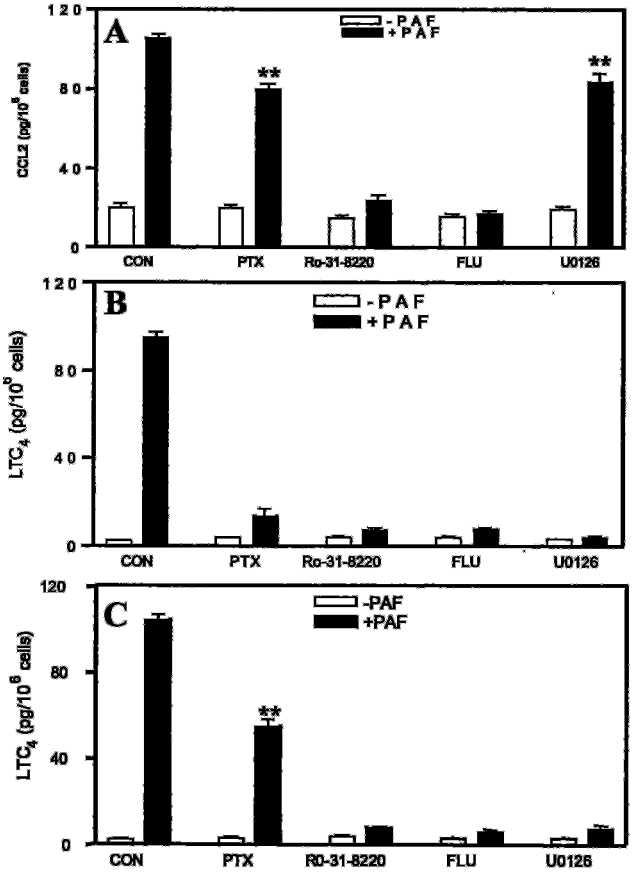

FIG. 5. **Effects of inhibitors on PAF-stimulated CCL2 production and LTC_4 generation.** RBL-2H3 cells stably expressing PAFR (*A* and *B*) or mPAFR (*C*) were preincubated with PTX (100 ng/ml, overnight), Ro-31-8220 (10 μM, 10 min), fluphenazine (*FLU*; 30 μM, 30 min) or U0126 (1 μM, 30 min), and PAF (100 nM)-induced CCL2 production in PAFR (*A*) and LTC_4 generation in PAFR (*B*) and mPAFR (*C*) cells were determined. *CON*, control; **, $p < 0.01$ *versus* control.

mPAFR cells was ~60% less than that observed in PAFR cells (Fig. 3*D*). These data suggest that receptor phosphorylation, which desensitizes PAF-induced degranulation and LTC_4 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. 4*A*, 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. 4*B*).

Roles of G Protein Usage, Phospholipase Cβ Activation, and ERK Phosphorylation on PAF-induced CCL2 Production and LTC_4 Generation—PAFR couples to G_i 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 G_i and G_q-mediated activation of phospholipase Cβ, resulting in the activation of protein kinase C (PKC) and the mobilization of Ca^{2+} (4, 6). We first evaluated the role of signaling through G proteins on PAF-induced CCL2 production and LTC_4 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. 5*A*, PTX inhibited PAF-induced CCL2 production by 27 ± 3.0%. In contrast, PTX blocked LTC_4 generation by 92.6 ± 4.6% (Fig. 5*B*). To determine the role of phospholipase Cβ-dependent signaling, we tested the effects of the inhibitors of protein kinase C (Ro-31-8220) and Ca^{2+}/calmodulin (fluphenazine) on PAF-induced responses. Both Ro-31-8220 and fluphenazine almost completely blocked PAF-induced CCL2 production and LTC_4 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. 5*A*), but it inhibited LTC_4 generation by 95 ± 1.5% in PAFR cells (Fig. 5*B*). The effects of these inhibitors were also tested on PAF-induced LTC_4 generation in mPAFR cells. PTX blocked PAF-induced LTC_4 generation in mPAFR cells by 51.3 ± 3.7% (Fig 5*C*). This is in contrast to the situation in PAFR cells where PTX inhibited PAF-induced response by 92.6 ± 4.6% (Fig. 5*B*). However, as in PAFR cells, Ro-31-8220, fluphenazine, or U0126 almost completely blocked PAF-induced LTC_4 generation in mPAFR cells (>90% inhibition) (Fig. 5*C*).

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 LTC_4 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. 6*A*, 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. 6*B*). 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. 6*A*), but it inhibited the response in mPAFR cells by 95.3 ± 2.3% (Fig 6*B*). 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 degran-

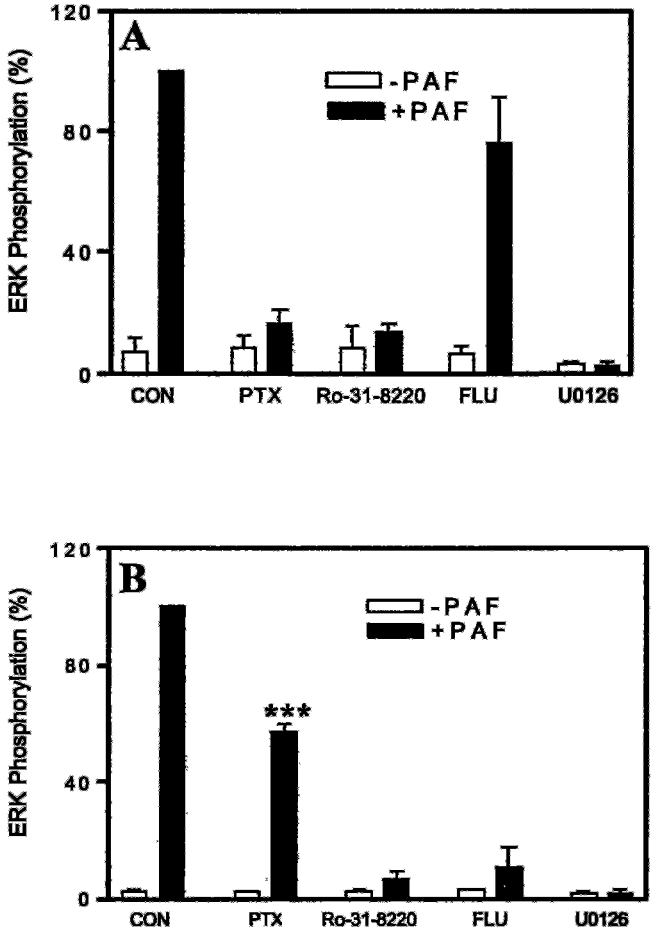

FIG. 6. **PAF stimulates ERK phosphorylation in RBL-2H3 cells via different mechanisms that depend on the phosphorylation status of the receptor.** RBL-2H3 cells stably expressing PAFR (*A*) or mPAFR (*B*) were incubated with medium (*CON*, control), PTX (100 ng/ml, overnight), Ro-31-8220 (10 μM, 10 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.

ulation in leukocytes (6, 33). It also causes LTC_4 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 LTC_4 generation and chemokine CCL2 production. Here, we demonstrate that receptor phosphorylation desensitizes PAF-induced LTC_4 generation but provides a stimulatory signal for chemokine CCL2 production. We also show distinct differences in both G protein usage and ERK phosphorylation on LTC_4 generation and CCL2 production.

We recently reported that complement component C3a stimulates CCL2 production via a pathway that requires receptor phosphorylation (17). Furthermore, Schwarz and Murphy (34) showed that Kaposi's sarcoma-associated herpes virus stimulates chemokine gene expression via the activation of a GPCR. However, truncation of the final five amino acids in the cytoplasmic tail of the receptor, which contains one serine and two threonine residues, resulted in a significant decrease in chemokine production. These findings suggest that receptor phosphorylation likely provides a general mechanism for stimulating GPCR-induced chemokine gene expression. The receptor phosphorylation-dependent signal that mediates chemokine production is not known. A substantial and growing body of evidence suggests that the interaction of phosphorylated receptors with the adapter molecule β-arrestin leads to the formation of a scaffold in the cytoplasm of cells. This complex directly interacts with Src, raf-1, ERK, c-Jun amino terminal kinase 3 (JNK-3), and a small GTP-binding protein, ADP-ribosylation factor 6 (ARF-6), to induce their activation (20, 21, 35). These findings suggest that β-arrestin could be involved in PAF-induced CCL2 production. The demonstration that PAF caused the recruitment of β-arrestin in cells expressing PAFR but not mPAFR is consistent with this notion. It is, however, important to note that the ligand-induced phosphorylation of PAFR and the interaction of the phosphorylated receptor with β-arrestin do not require G protein activation (36). Furthermore, PTX, which had no effect on ligand-induced PAFR phosphorylation (4), caused a substantial inhibition of PAF-induced ERK phosphorylation but had very little effect on CCL2 production. In addition, U0126, which completely blocked PAF-induced ERK phosphorylation, did not cause a substantial inhibition of CCL2 production. These findings suggest that if β-arrestin mediates PAF-induced CCL2 production, it does so via the activation of a pathway that is mostly independent of G protein activation and ERK phosphorylation.

We have shown in the present study that when PAFR and mPAFR were expressed at similar levels in RBL-2H3 cells, PAF was able to induce CCL2 production in mPAFR cells but at lower level (Fig. 3). Schwarz and Murphy (34) also made a similar observation for a wild-type and a mutant GPCR for a Kaposi's sarcoma-associated herpes virus lacking serine and threonine residues at its carboxyl terminus. These findings suggest that receptor phosphorylation alone does not provide a full signal for chemokine production. This contention is supported by the finding that the inhibition of $G\alpha_q$-mediated responses such as Ca^{2+} mobilization and PKC activation leads to the inhibition of PAF-induced CCL2 production in PAFR cells. 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 G_i-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 Ca^{2+}/calmodulin inhibitor leads to a substantial inhibition of PAF-induced LTC_4 generation is consistent with the roles of ERK phosphorylation and Ca^{2+} mobilization on LTC_4 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 Ca^{2+} mobilization depends on the activation of $G\alpha_q$ (6). The demonstration that the Ca^{2+}/calmodulin inhibitor fluphenazine blocked ERK phosphorylation in mPAFR but not PAFR cells raises the intriguing

possibility that receptor phosphorylation modulates the G protein-coupling specificity of PAF-induced ERK phosphorylation.

In summary, we have previously shown that receptor phosphorylation mediates the desensitization of PAF-induced degranulation (14). In the present study, we demonstrate that receptor phosphorylation also serves to desensitize PAF-induced LTC_4 generation but provides a stimulatory signal for chemokine CCL2 production. The activation of many GPCRs leads to LTC_4 generation and chemokine gene expression in a variety of cell types (27, 34, 37, 42–46). Therefore, receptor phosphorylation is likely to have a greater impact on cellular functions than previously recognized.

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