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Platelet-activating Factor-induced Chemokine Gene Expression Requires NF-κB Activation and Ca^{2+}/Calcineurin Signaling Pathways

INHIBITION BY RECEPTOR PHOSPHORYLATION AND β-ARRESTIN RECRUITMENT*

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Previously, we reported that platelet-activating factor (PAF) stimulates higher G protein activation and a more robust Ca^{2+} mobilization in RBL-2H3 cells expressing carboxyl terminus deletion, phosphorylation-deficient mutant of PAF receptor (mPAFR) when compared with the wild-type receptor (PAFR). However, PAF did not provide sufficient signal for CC chemokine receptor ligand 2 (CCL2) production in cells expressing mPAFR. Based on these findings, we hypothesized that receptor phosphorylation provides a G protein-independent signal that synergizes with Ca^{2+} mobilization to induce CCL2 production. Here, we show that a mutant of PAFR (D289A), which does not couple to G proteins, was resistant to agonist-induced receptor phosphorylation. Unexpectedly, we found that when this mutant was coexpressed with mPAFR, it restored NF-κB activation and CCL2 production. PAF caused translocation of β-arrestin from the cytoplasm to the membrane in cells expressing PAFR but not a phosphorylation-deficient mutant in which all Ser/Thr residues were replaced with Ala (ΔST-PAFR). Interestingly, PAF induced significantly higher NF-κB and nuclear factor of activated T cells (NFAT)-luciferase activity as well as CCL2 production in cells expressing ΔST-PAFR than those expressing PAFR. Furthermore, a Ca^{2+}/calcineurin inhibitor completely inhibited PAF-induced NFAT activation and CCL2 production but not NF-κB activation. These findings suggest that the carboxyl terminus of PAFR provides a G protein-independent signal for NF-κB activation, which synergizes with G protein-mediated Ca^{2+}/calcineurin activation to induce CCL2 production. However, receptor phosphorylation and β-arrestin recruitment inhibit CCL2 production by blocking both NF-κB activation and Ca^{2+}/calcineurin-dependent signaling pathways.

Platelet-activating factor (PAF)[1] (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) 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 (3–6). PAF is a potent leukocyte chemoattractant (7), and it also induces degranulation (8, 9), leukotriene C_4 generation (10, 11), and chemokine gene expression in a wide variety of cells (12–17). Although PAF-induced chemokine production appears to depend on the activation of transcription factor NF-κB, a crucial transcription factor regulating the expression of many proinflammatory cytokines and immunoregulatory molecules (12, 16, 18, 19), the early receptor-mediated signaling pathway that initiates this response has not been determined.

Receptor phosphorylation by G protein-coupled receptor kinase and the subsequent recruitment of β-arrestin are essential for uncoupling the receptor from G proteins (20). β-Arrestin also acts as an adapter molecule, leading to the formation of a scaffold in the cytoplasm of cells. This complex directly interacts with Src, Raf-1, extracellular signal-regulated kinase, c-Jun amino-terminal kinase-3, and a small GTP-binding protein, ADP-ribosylation factor 6 and the oncoprotein Mdm-2 to induce their activation (21–24). At a functional level, β-arrestin signaling is required for GPCR-induced chemotaxis in lymphocytes, lymphoid enhancer factor transcriptional activity in transfected cell lines and development of allergic asthma *in vivo* (25–28). Recently, β-arrestin has shown to inhibit NF-κB activation in response to some but not all GPCRs (29, 30). Although β-arrestin mediates agonist-induced internalization of PAFR (31–33), the role of this adapter molecule on PAFR signaling and biological responses has not been determined.

We have recently shown that PAF-induced chemokine CCL2 production in transfected RBL-2H3 cells requires G-protein-dependent Ca^{2+} mobilization and protein kinase C activation (31). We also reported the surprising observation that a carboxyl terminus deletion, phosphorylation-deficient mutant of PAFR (mPAFR), which couples to G protein more efficiently than the wild type receptor, did not provide sufficient signal for chemokine CCL2 production. Based on these findings, we hypothesized that PAF-induced CCL2 production requires the synergistic interac-

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[1] The abbreviations used are: PAF, platelet-activating factor; PAFR, PAF receptor; mPAFR, mutant of PAF receptor; GPCR, G protein-coupled receptor; CCL2, CC chemokine receptor ligand 2 (formerly known as MCP-1); GFP, green fluorescent protein; βarr2-GFP, β-arrestin 2-green fluorescent protein conjugate; NFAT, nuclear factor of activated T cells; ELISA, enzyme-linked immunosorbent assay; MEF, mouse embryo fibroblast.

tion of G protein-independent and G protein-dependent signals (31). In the present study, we show that G protein-independent signaling is mediated via the carboxyl terminus of the receptor without involving receptor phosphorylation. We also demonstrate that receptor phosphorylation and the subsequent β-arrestin recruitment inhibits PAF-induced NF-κB activation, Ca^{2+}/calcineurin-mediated nuclear factor of activated T cells (NFAT) activation, and CCL2 production.

EXPERIMENTAL PROCEDURES

Materials—Platelet-activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) and cyclosporin A were purchased from Calbiochem. 12CA5 and anti-mouse IgG-RPE antibodies were obtained from Roche Applied Science and Southern Biotechnology Associates (Birmingham, AL), respectively. All tissue culture reagents were purchased from Invitrogen. The CCL2 sandwich ELISA kit was purchased from BioSource International (Camarillo, CA). pNF-κB-Luc and pNFAT-luc plasmid were purchased from Stratagene (La Jolla, CA). pRL *Renilla* control luciferase vector (pRL-SV40) and dual luciferase reporter assay system were obtained from Promega (Madison, WI). [^{32}P]Orthophosphate (8500–9120 Ci/mmol) was obtained from PerkinElmer Life Sciences.

Generation of PAF Receptor Mutants—A hemagglutinin-tagged human PAFR cloned in pRK-5 was restriction-digested with HindIII and BamHI and inserted into pcDNA3.1 (+) vector. Mutants of PAFR (D289A and Y293A) were generated by site-directed mutagenesis using the QuikChange mutagenesis kit (Stratagene). A phosphorylation-deficient mutant of PAFR (ΔST-PAFR) was constructed by the PCR method using the 5′ oligonucleotide (5′-TGC AAG CTT GCA ATG TAC CCA TAC GAC GTC CCA GAC TAC GCT GAG CCA CAT GAC TCC TCC CAC ATG GAC-3′) and a 3′ oligonucleotide complementary to the PAFR tail replacing all serine and threonine residues with alanine (5′-GCAT GGATCC CT AAT TTT TGA GGG CAT TGC CAG GGA TCT GGT TGA ATG GCA CAA CCA CTT CAG CGA CCG CAT CCG CGG CGG CCC GGG CGC ATT TCC GGG CAG CGC GCA TGG CGT AGA ACT TTT CGG CGA GGT GCT T-3′). Mutants were cloned into pcDNA3 vector as described (31) and confirmed by sequencing.

Cell Culture, Transfection, Receptor Phosphorylation, and Degranulation—RBL-2H3 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) (9, 34). Mouse embryonic fibroblast cell lines (MEFs) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) (35). Transient transfection of RBL-2H3 cells and MEFs were performed as described previously (31, 36). Briefly, cells (2×10^7) were mixed with appropriate ratios of cDNA constructs in a total volume of 200 μl of transfection medium. Cells were then electroporated with a single pulse using a Bio-Rad gene pulser. Cells were cultured in complete growth medium, and experiments were performed 16–18 h after transfection.

To determine cell surface receptor expression, cells (1×10^6) were incubated with 12CA5 or isotope-matched antibody, followed by incubation for 1 h with a secondary antibody conjugated with phycoerythrin and analyzed on a FACStar^{PLUS} flow cytometer (BD Biosciences) (31, 36). Cell surface receptor expression was also determined by ELISA using 12CA5 antibody, as described previously (37). Receptor phosphorylation was determined as described previously (9). 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 and stimulated with PAF, and the extent of degranulation was determined by measuring the release of β-hexosaminidase (9, 34).

Confocal Microscopy—Cells expressing hemagglutinin-tagged receptors and β-arrestin 2/green fluorescent protein conjugate (β-arr2-GFP) were plated on coverslips in 24-well plates. The cells were stimulated with 100 nM PAF for 5 min at 37 °C. The reaction was stopped by adding 3 volumes of cold phosphate-buffered saline, and cells were fixed with 4% paraformaldehyde solution for 30 min at room temperature. The cells were permeabilized by 0.1% saponin (Sigma) for 20 min at room temperature. To visualize receptor expression, cells were incubated with 12CA5 antibody followed by biotin-labeled anti-mouse IgG and streptavidin Cy5 (Jackson ImmunoResearch). Cells were observed using a laser-scanning confocal microscope (Bio-Rad Radiance 2100) with a × 100 lens. The GFP was excited using a 488-nm argon laser and detected at emission 515 ± 30 nm, and Cy5 was excited at 647 nm and detected at 660 long pass.

Assay of NF-κB Luciferase and NFAT Luciferase Activity—RBL-2H3 and MEF cells were transiently co-transfected with 22.5 μg of PAFR constructs, 7.5 μg of pNF-κB-Luc, or pNFAT-luc plasmid and 0.5 μg of pRL-SV40 *Renilla* plasmid by electroporation. The following day, cells were serum-starved for 2–4 h and stimulated with 10 nM PAF for another 6 h. Reactions were stopped by washing cells with ice-cold phosphate-buffered saline. Cells were then lysed with 100 μl of lysis buffer, and luciferase activity was determined using the dual luciferase reporter assay system (Promega). The firefly luciferase activities were normalized to *Renilla* luciferase activity.

Assay of Chemokine (CCL2) Production—RBL-2H3 cells (0.25×10^6/well) were cultured in complete growth medium overnight. Cells were serum-starved for 2–4 h and stimulated with PAF to a final concentration of 10 nM for 6 h. Supernatants were collected and stored frozen at −80 °C until analysis. CCL2 levels were quantified using sandwich ELISA as described previously (31, 36).

RESULTS

We have previously shown that agonist-induced phosphorylation of PAFR does not require G protein activation (9). To determine the role of receptor phosphorylation and G protein-independent signaling on PAF-induced transcription factor NF-κB activation and CCL2 production, we generated two previously characterized mutants of PAFR that do not couple to G proteins (38, 39). These mutants were constructed by making single substitutions in the putative internalization motif DPXXY in the seventh transmembrane domain of the receptor. Changing aspartate 289 into alanine (D289A) blocks agonist-induced β-arrestin binding and receptor internalization (38). In contrast, replacing the distal tyrosine residue with alanine (Y293A) abolishes G protein coupling but does not interfere with its ability to interact with β-arrestin or to undergo internalization in transfected COS cells (38, 39). We first sought to characterize the properties of D289A and Y293A in transiently transfected basophilic leukemia RBL-2H3, a cell line that we have extensively utilized as a model to study functional regulation of leukocyte chemoattractant receptors (9, 40, 41). RBL-2H3 cells transiently transfected with PAFR, and its D289A or Y293A mutants were labeled with [^{32}P]orthophosphate and exposed to a concentration of PAF (100 nM) that is 10 times higher than required for optimal receptor phosphorylation (9). We found that PAF caused robust phosphorylation of PAFR and Y293A but not D289A mutant (Fig. 1*A*). The resistance of D289A to undergo agonist-induced phosphorylation is not due to inefficient transfection or unequal protein loading as receptor expression for each transfectants was carefully monitored and protein concentration was adjusted to assure that equal numbers of receptor were used for each experiment.

To determine the role of receptor phosphorylation on NF-κB activation and chemokine CCL2 production, we generated transient transfectants coexpressing PAFR or its mutants with NF-κB luciferase constructs. As shown in Fig. 1, *B* and *C*, PAF stimulated NF-κB reporter activity and CCL2 production in cells expressing PAFR but not its D289A or Y293A mutant.

To determine the role of carboxyl terminus of PAFR and G protein-independent signaling on PAF-induced responses, we used RBL-2H3 cells stably expressing mPAFR (31) and transiently expressed D289A (Fig. 2*A*). D289A had no effect on PAF-induced degranulation (Fig. 2*B*) but significantly enhanced mPAFR-induced NF-κB luciferase activity (Fig. 2*C*) and CCL2 production (Fig. 2*D*) and restored them to levels similar to those observed in cells expressing the wild-type receptor (see Fig. 1). These findings demonstrate that the carboxyl terminus of PAFR does not regulate degranulation but is required for CCL2 gene expression.

It is important to note that mPAFR used in our previous studies was generated by deleting 30 amino acids from the carboxyl terminus of PAFR, of which eight are serine and threonine residues (31, 42). To determine, specifically, the role of receptor phosphorylation on PAF-induced responses, we con-

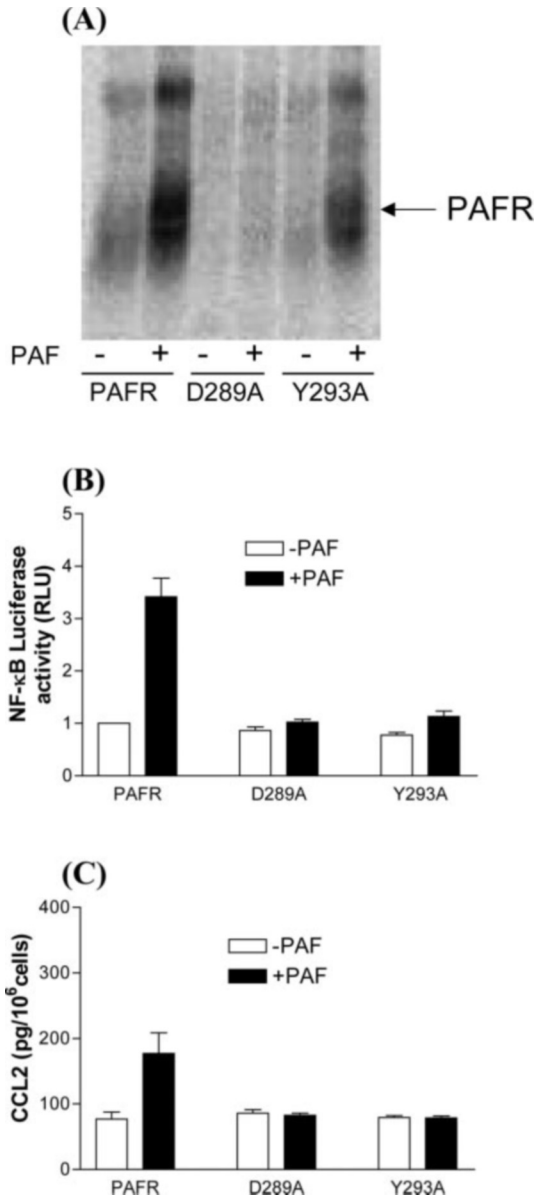

FIG. 1. **Receptor phosphorylation does not mediate PAF-induced NF-κB activation and CCL2 production.** *A*, transient transfectants were generated in RBL-2H3 cells expressing equal numbers of PAFR, D289A, or Y293A. Cells were labeled with [^{32}P]orthophosphate and exposed to PAF (100 nM for 5 min), and receptor phosphorylation was determined. *B*, RBL-2H3 cells coexpressing PAFR, D289A, or Y293A with NF-κB luciferase and *Renilla* luciferase plasmids were stimulated with PAF (10 nM) for 6 h, and NF-κB luciferase activity was determined in cell lysate. Data presented are relative luciferase activity normalized to *Renilla* luciferase activity as relative luciferase units (*RLU*). *C*, cells expressing PAFR, D289A, or Y293A were stimulated with PAF (10 nM) for 6 h, and CCL2 production was determined by ELISA. The data in *A* are representative of three similar experiments, and the data presented in *B* and *C* are mean ± S.E. of three separate experiments performed in triplicate.

structed a substitution mutant in which all Ser/Thr residues on the carboxyl terminus of the receptor were replaced with alanine residues, ΔST-PAFR (Table I). We found that PAF caused phosphorylation of PAFR but not ΔST-PAFR (Fig. 3*A*). Although the carboxyl terminus of PAFR is required for β-arrestin recruitment (31, 39), whether receptor phosphorylation mediates this process is not known. We therefore transfected RBL-2H3 cells expressing PAFR or ΔST-PAFR with βarr2-GFP. As shown in Fig. 3*B*, PAF caused translocation of βarr2-GFP from the cytosol to the membrane in PAFR cells. In contrast, PAF did not induce this response in ΔST-PAFR cells.

To determine the role of receptor phosphorylation on PAF-induced responses, we generated transient transfectants in RBL-2H3 cells expressing equivalent PAFR and ΔST-PAFR (Fig. 4*A*). As shown in Fig. 4*B*, PAF caused ~2-fold greater degranulation in cells expressing ΔST-PAFR than PAFR. This finding is similar to our previous report with mPAFR and suggests that phosphorylation sites within the carboxyl terminus of PAFR are responsible for desensitization of degranulation. Surprisingly, we found that unlike mPAFR (Fig. 2), PAF caused ~5-fold greater NF-κB activation in cells expressing ΔST-PAFR when compared with PAFR (Fig. 4*C*). PAF also caused enhanced CCL2 production in ΔST-PAFR cells (Fig. 4*D*). These findings suggest that the carboxyl terminus of PAFR is required for PAF-induced NF-κB activation and CCL2 production and that receptor phosphorylation serves to inhibit these responses.

The demonstration that PAF causes greater NF-κB activation and CCL2 production in cells expressing ΔST-PAFR than PAFR (Fig. 4) raises the interesting possibility that β-arrestin could actually provide an inhibitory signal for NF-κB activation and CCL2 production. A constitutively active mutant of β-arrestin (βarr-R169E) has been shown to associate with phosphorylation-deficient mutants of a number of G protein-coupled receptors (43–45). To determine the role of β-arrestin on PAF-induced responses, transient transfectants were generated in RBL-2H3 cells coexpressing ΔST-PAFR with βarr-R169E/green fluorescent protein conjugate (GFP-βarr-R169E). As shown in Fig. 5*A*, GFP-βarr-R169E inhibited PAF-induced degranulation by 52.8 ± 3%. Interestingly, GFP-βarr-R169E blocked PAF-induced NF-κB activation and CCL2 production by 82.3 ± 3 and 88.5 ± 2%, respectively (Fig. 5, *B* and *C*). These inhibitory effects were specific for βarr-R169E, since GFP or GFP-βarr had no effect on PAF-induced responses.

To determine the roles of β-arrestin on PAF-induced NF-κB activation further, we generated transient transfectants in wild-type mouse embryonic cell line (MEF) and a mutant cell line deficient in both β-arrestin 1 and β-arrestin 2 (35). As shown in Fig. 6*A*, PAFRs were expressed at equal levels in both cell types. Furthermore, PAF caused internalization of its receptor in wild-type but not in β-arrestin knockout MEFs (Fig. 6*B*). However, PAF stimulated a significantly higher NF-κB activation in β-arrestin knockout MEFs when compared with wild-type cells (Fig. 6*C*).

We have previously shown that PAF-induced chemokine CCL2 production in RBL-2H3 cells requires sustained Ca^{2+} mobilization (31). Calcinuerin is a Ca^{2+}/calmodulin-dependent phosphatase that dephosphorylates the transcription factor, NFAT, allowing it to translocate to the nucleus, where it combines with the AP-1 complex to regulate the transcription of early cytokine genes (46, 47). Calcineurin has been shown to promote the expression of CCL2 in vascular myocytes and to mediate vascular inflammation (48). The immunosuppressive drug cyclosporin A inhibits cytokine gene expression by blocking calcineurin-mediated NFAT activation (46, 49). To determine the role of carboxyl terminus of PAFR and receptor phosphorylation on PAF-induced calcineurin activation, we coexpressed PAFR, mPAFR, or ΔST-PAFR with NFAT and NF-κB luciferase constructs. As shown in Fig. 7*A*, PAF caused significantly higher NFAT activation in cells expressing mPAFR and ΔST-PAFR when compared with PAFR. Despite this difference, mPAFR was the least susceptible for PAF-induced NF-κB activation and CCL2 production (Fig. 7, *B* and *C*). Cyclosporin A completely inhibited PAF-induced NFAT activation and CCL2 production but only partially blocked NF-κB activation.

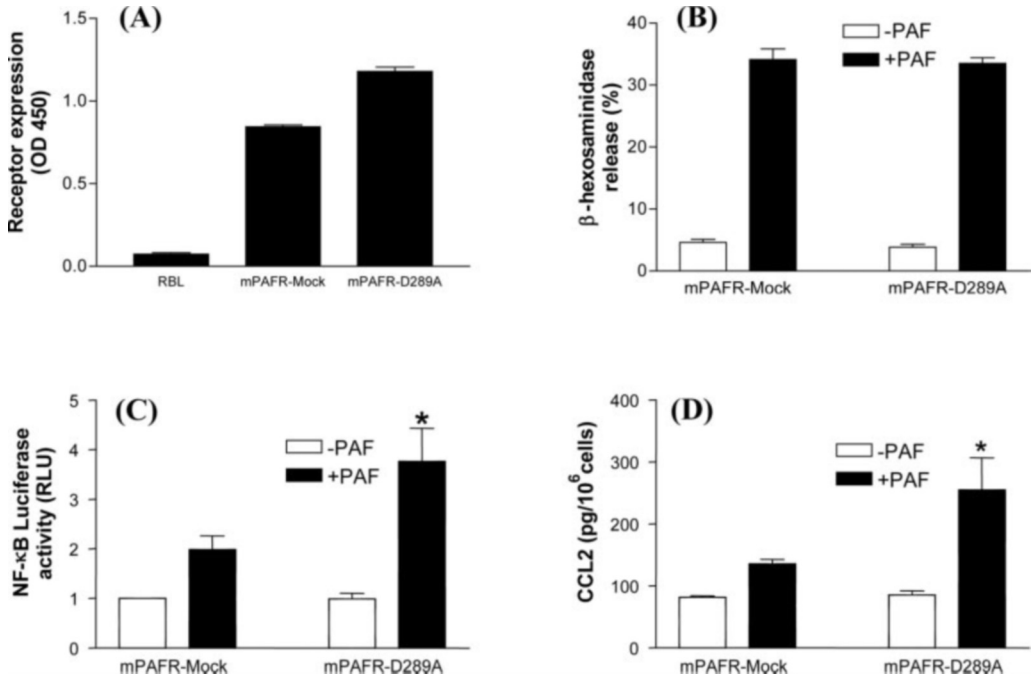

FIG. 2. **D289A mutant of PAFR does not desensitize PAF-induced degranulation but enhances NF-κB activation and CCL2 production.** RBL-2H3 cells stably expressing mPAFR were transiently transfected with control vector pcDNA3.1 (mPAFR-Mock) or D289A (mPAFR-D289A) in the presence of NF-κB luciferase and *Renilla* luciferase plasmids. *A*, cell surface receptor expression was measured by ELISA. *B*, cells were stimulated with PAF (10 nM) for 20 min, and supernatant was assayed for β-hexosaminidase release. Cells were also stimulated with PAF (10 nM) for 6 h, and NF-κB-luciferase activity was measured from the cell lysate (*C*), and CCL2 production was determined in supernatant by ELISA (*D*). The data presented are mean ± S.E. of four experiments performed in triplicate. *, $p < 0.05$ in mPAFR-mock *versus* mPAFR-D289A.

TABLE I

Amino acid sequences of the carboxyl terminus of PAFR, a deletion mutant (mPAFR), and phosphorylation-deficient mutant (ΔST-PAFR), in which all Ser and Thr residues were replaced with Ala

PAFR	Sequence
	298 ▼ 342 ▼
PAFR	KKFRKHL**T**EKFY**S**MR**SS**RKC**S**RA**TT**D**T**V**T**EVVVPFNQIPGN**S**LKN
mPAFR	KKFRKHL**A**EKFY**A**M
ΔST-PAFR	KKFRKHL**A**EKFY**A**MR**AA**RKC**A**RA**AA**D**A**V**A**EVVVPFNQIPGN**A**LKN

DISCUSSION

In the present study, we demonstrate the novel finding that PAF-induced CCL2 gene expression requires the interaction of two signaling pathways. One is G protein-independent, requires the carboxyl terminus of PAFR, and mediates NF-κB activation. The other is G protein-dependent, requires Ca^{2+} mobilization, and involves the activation of calcineurin-mediated NFAT activation. Surprisingly, we found that phosphorylation of PAFR at Ser/Thr residues within its carboxyl terminus and subsequent β-arrestin recruitment blocks CCL2 gene expression via inhibition of both NF-κB and Ca^{2+}/calcineurin-mediated signaling pathways.

We have previously shown that PAF stimulates higher G protein activation and a more robust Ca^{2+} mobilization in RBL-2H3 cells expressing carboxyl terminus deletion, phosphorylation-deficient mutant, mPAFR when compared with the wild-type receptor (PAFR) (31). However, PAF did not provide sufficient signal for CCL2 production in cells expressing mPAFR. Based on these findings, we hypothesized that receptor phosphorylation provides a G protein-independent signal that synergizes with Ca^{2+} mobilization to induce CCL2 production. PAFR mutant D289A, containing a single substitution in the putative internalization motif DPXXY in its seventh transmembrane domain does not interact with β-arrestin but signals via a G protein-independent pathway (38, 39). We found that D289A was resistant to agonist-induced receptor phosphoryla-

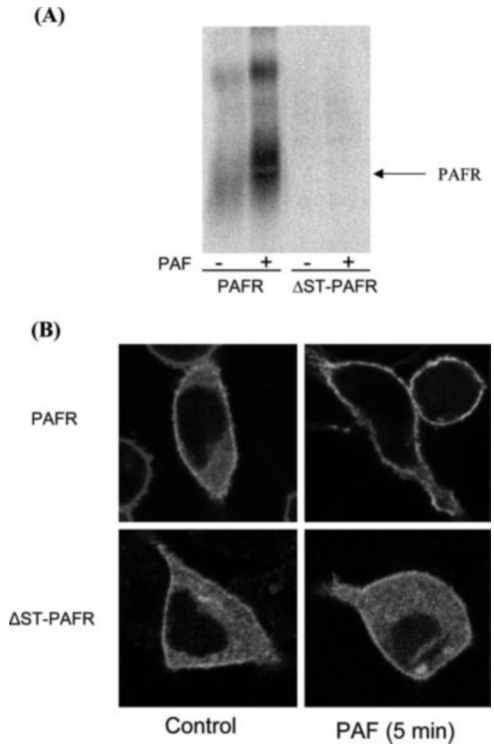

FIG. 3. **Phosphorylation-deficient mutant ΔST-PAFR does not interact with β-arrestin.** *A*, RBL-2H3 cells expressing equal numbers of PAFR and ΔST-PAFR, were labeled with [^{32}P]orthophosphate exposed to PAF (100 nM for 5 min), and receptor phosphorylation was determined. *B*, RBL-2H3 cells expressing PAFR and ΔST-PAFR were transfected with β-arrestin 2-GFP and stimulated with PAF (100 nM) for 5 min, fixed in paraformaldehyde, and permeabilized in saponin. The cells were incubated with 12CA5 antibody followed by biotin-labeled anti-mouse IgG and streptavidin-Cy5. The interaction between receptor and β-arrestin 2-GFP was detected by confocal microscopy. The data shown are representative of three similar experiments.

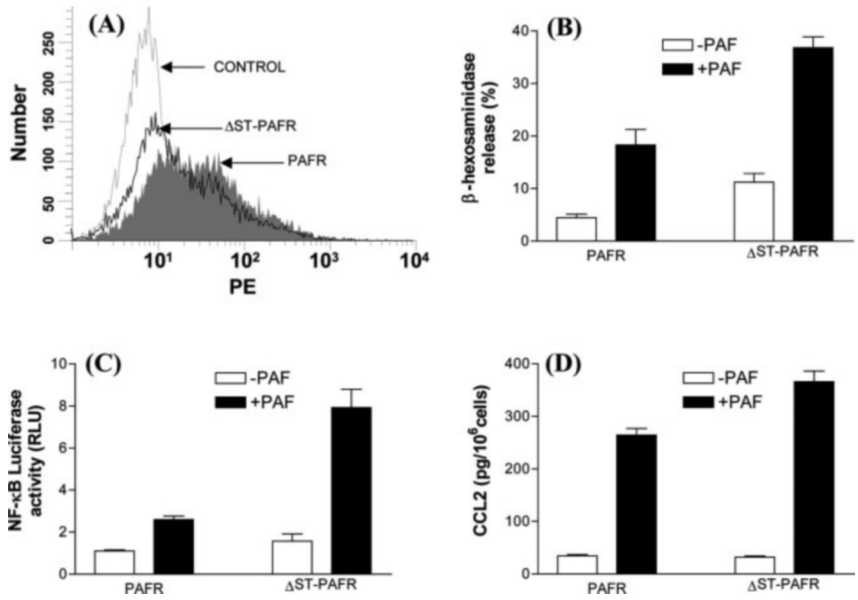

FIG. 4. **Receptor phosphorylation plays an inhibitory role on PAF-induced degranulation, NF-κB activation, and CCL2 production.** Transient transfectants were generated in RBL-2H3 cells expressing PAFR or ΔST-PAFR. *A*, cell surface receptor expression was determined by fluorescence-activated cell sorting analysis. Mock-transfected cells were used as a control. *B*, cells expressing PAFR or ΔST-PAFR were incubated in the absence and presence of PAF (10 nM for 20 min), and the release of β-hexosaminidase was determined. Cells were incubated with or without PAF (10 nM) for 6 h, and NF-κB luciferase activity was determined in cell lysate (*C*), and CCL2 production was determined in supernatants by ELISA (*D*). The data shown in *A* is representative of three similar experiments, and data in *B–D* are mean ± S.E. of three separate experiments performed in triplicate.

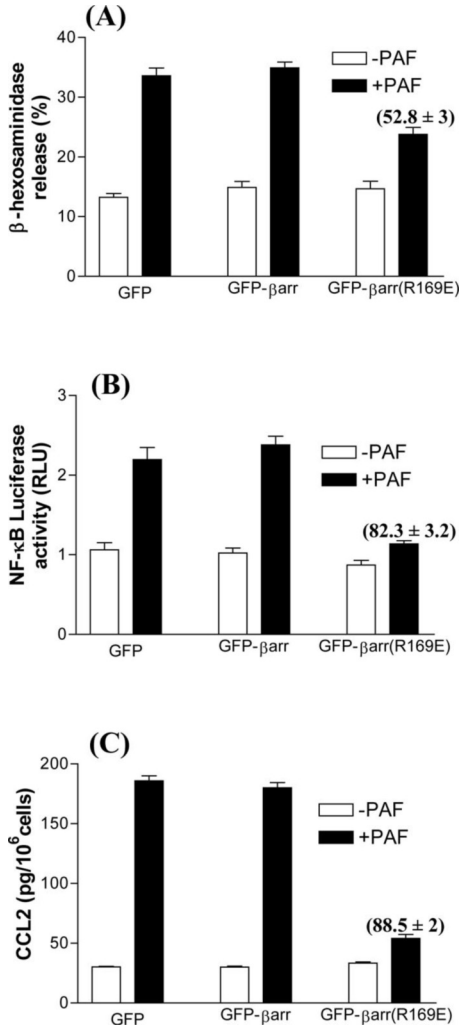

FIG. 5. **Constitutively active mutant of β-arrestin inhibits PAF-induced responses in RBL-2H3 cells expressing ΔST-PAFR.** RBL-2H3 cells coexpressing ΔST-PAFR with GFP, βarr-GFP, or R169E-βarr-GFP were stimulated with PAF (10 nM) for 20 min, and β-hexosaminidase release was determined (*A*). Cells were stimulated with PAF for 6 h, and NF-κB luciferase activity (*B*) and CCL2 production (*C*) were determined as described under "Experimental Procedures." The data presented are mean ± S.E. of three separate experiments performed in triplicate. Percentage inhibitions are shown in *parentheses*.

tion. Furthermore, PAF did not induce NF-κB activation or CCL2 production in cells expressing D289A. Our expectation was that when D289A is coexpressed with mPAFR, it would have little or no effect on PAF-induced CCL2 gene expression. Surprisingly, we found that D289A restored PAF-induced NF-κB activation and CCL2 production. These findings suggest that the carboxyl terminus of PAFR, but not receptor phosphorylation, mediates G protein-independent signal for NF-κB activation, which synergizes with Ca^{2+} mobilization to induce CCL2 gene expression.

The role of NF-κB activation on chemokine CCL2 production is well established (19, 50, 51). However, Satonaka *et al.* (48), recently demonstrated that CCL2 gene expression in vascular myocytes requires Ca^{2+}/calmodulin-mediated calcineurin activation. The ability of D289A to restore PAF-induced CCL2 production in mPAFR cells provided us with an important tool to delineate the roles of distinct signaling pathways on PAF-induced responses. An interesting finding of the present study was that while PAF caused significantly lower NF-κB activation in cells expressing mPAFR when compared with PAFR, the reverse was the case for NFAT activation (see Fig. 7). Despite the fact that PAF stimulated a robust calcineurin-mediated NFAT activation in mPAFR cells, a G protein-independent signal for NF-κB activation by D289A was required to restore CCL2 production. These findings suggest that the carboxyl terminus of PAFR provides a G protein-independent signaling for NF-κB activation, which synergizes with mPAFR-induced Ca^{2+}/calcineurin-mediated signaling pathway to induce CCL2 gene expression. This contention is supported by the finding that a Ca^{2+}/calcineurin inhibitor completely blocked PAF-induced NFAT activation and CCL2 production but only partially inhibited NF-κB activation.

An interesting finding of the present study was that although the carboxyl terminus of PAFR is required for PAF-induced CCL2 gene expression, phosphorylation of the receptor at Ser/Thr residues within this domain and the subsequent β-arrestin recruitment inhibits this response. This contention is supported by the following observations. First, PAFR but not ΔST-PAFR interacts with β-arrestin in response to PAF. Second, PAF was more active in stimulating CCL2 gene expression in RBL-2H3 cells expressing ΔST-PAFR when compared with PAFR. Third, a constitutively active mutant of β-arrestin (R169E) caused a substantial inhibition of PAF-induced CCL2 gene expression. Fourth, PAF-induced NF-κB activation was

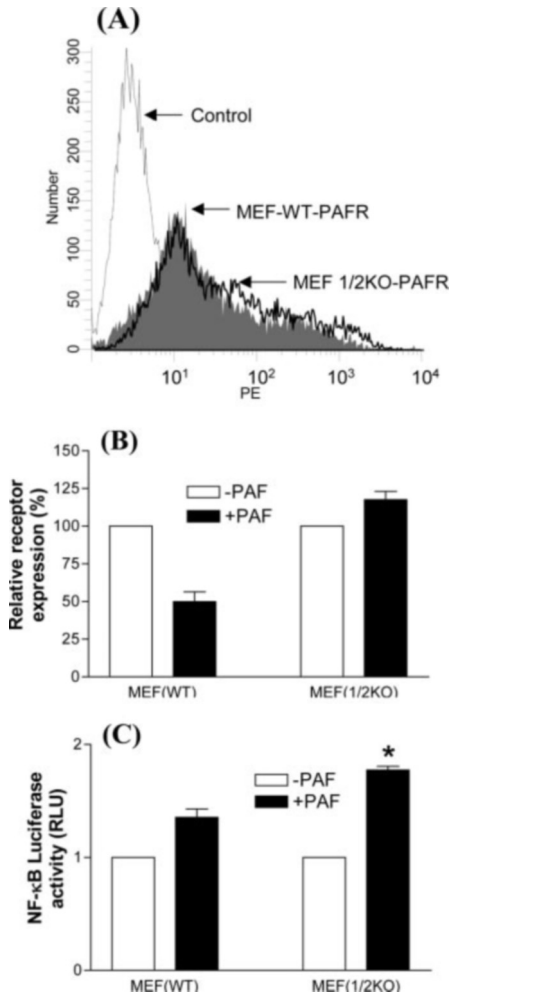

FIG. 6. ***β*-Arrestin provides an inhibitory signal for PAF-induced NF-κB activation.** Mouse embryonic fibroblasts from knockout mice that lack both β-arrestin 1 and β-arrestin 2 (*MEF 1/2KO*) and its wild-type littermate (MEF-WT) were transiently co-transfected with PAFR and NF-κB/*Renilla* luciferase constructs by electroporation. *A*, cell surface receptor expression was determined by flow cytometry. *B*, cells were also stimulated with PAF (10 nM) for 20 min, and the cell surface receptor expression was determined by flow cytometry. The data are expressed as percentage of receptors that are present on the cell surface in the absence of agonist stimulation (− *PAF*; 100%). *C*, cells were serum-starved for 4 h and stimulated with PAF (10 nM) for another 6 h. Cell lysates were used to determine NF-κB luciferase activity. The data shown in *A* is representative of three similar experiments, and data in *B* and *C* are mean ± S.E. of three separate experiments performed in triplicate. *, $p < 0.05$ in wild-type *versus* β-arrestin deficient cells.

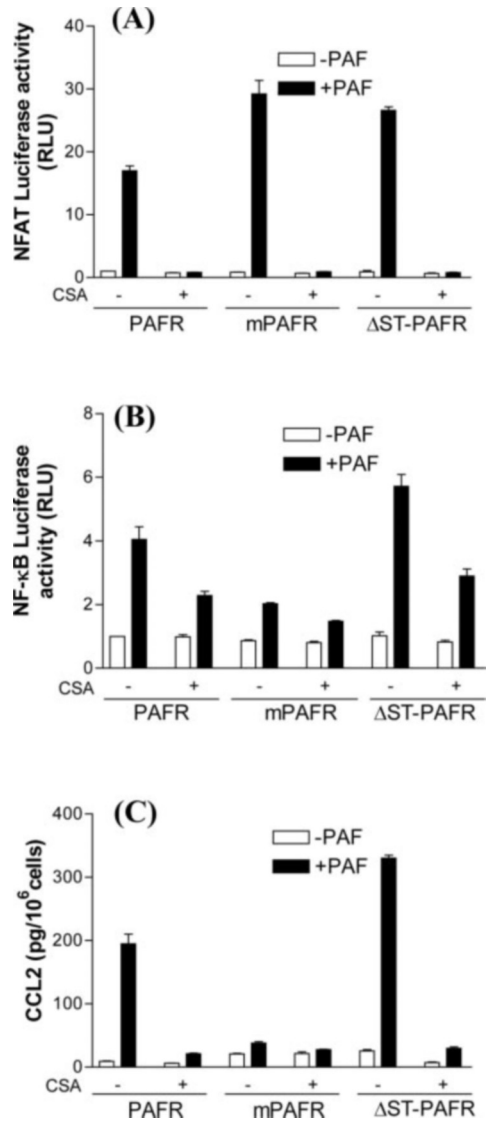

FIG. 7. **Cyclosporin A completely inhibits PAF-induced NFAT activation and CCL2 production but only partially inhibits NF-κB activation.** RBL-2H3 cells transiently expressing PAFR, mPAFR, and ΔST-PAFR were treated with and without cyclosporin A (100 nM) for 30 min and stimulated with PAF (10 nM) for 6 h. *A*, PAF-induced NFAT-luciferase activity; *B*, NF-κB-luciferase activity measured from cell lysate; *C*, CCL2 production measured by ELISA. The data presented are mean ± S.E. of three experiments performed in triplicate.

significantly enhanced in β-arrestin knockout MEFs when compared with wild-type MEFs.

The mechanism by which receptor phosphorylation and β-arrestin recruitment inhibit PAF-induced CCL2 gene expression is not known. Recently, it has been shown that interaction of β-arrestin with IκBα or its upstream kinases inhibit NF-κB activation stimulated by some but not all GPCRs (29, 30). In the present study, we have shown that receptor phosphorylation and β-arrestin recruitment inhibits PAF-induced NF-κB activation. We further demonstrated that PAF-induced NF-κB activation is enhanced in β-arrestin knockout MEFs when compared with wild-type cells. These findings are consistent with the idea that β-arrestin modifies IκB or its upstream kinases to inhibit PAF-induced CCL2 production in RBL-2H3 cells. We also showed that PAF-induced NFAT activation was enhanced in cells expressing ΔST-PAFR when compared with PAFR and that this Ca^{2+}/calcineurin-mediated response is essential for CCL2 production. This finding suggests that the ability of receptor phosphorylation and β-arrestin recruitment to inhibit CCL2 production involves the modification of at least two components in the PAFR signaling pathway. One involves the inhibition of NF-κB, and the other involves modulation of Ca^{2+}/calcineurin-mediated signaling pathway.

In summary, we have shown that PAF-induced CCL2 gene expression requires the carboxyl terminus of PAFR and involves a complex interaction of G protein-dependent and -independent signaling pathways. However, phosphorylation of Ser/Thr residues within this region blocks gene expression via the modification of multiple signaling pathways. It is noteworthy that PAFR is not the only GPCR that requires its carboxyl terminus for the induction of NF-κB activation and chemokine production. Schwarz *et al.* (52) showed that Kaposi's sarcoma-associated herpesvirus stimulates NF-κB activation and CCL2 production and that deletion of the terminal five amino acids on the carboxyl terminus of its GPCR resulted in substantial

inhibition of these responses. Most interestingly, Hernandez *et al.* recently showed that WHIM syndrome, an immunodeficiency disease characterized by neutropenia, is associated with deletion of the carboxyl terminus of the G protein-coupled chemokine receptor 4 (53). Therefore, the mechanism described in the present study for PAF-induced chemokine production is likely to be a general mechanism by which functions of different G protein-coupled receptors are regulated.

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