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Cross-Desensitization Among Receptors for Platelet Activating Factor and Peptide Chemoattractants: Evidence for Independent Regulatory Pathways

Abstract

Cross-desensitization among receptors for peptide chemoattractants have been shown to involve two independent processes, receptor phosphorylation and inhibition of phospholipase C (PLC) activation. Receptors for lipid chemoattractants, i.e. platelet activating factor (PAF) and leukotriene B_4, did not inhibit the responses of peptide chemoattractant receptors, suggesting distinct signaling pathways. To examine cross-desensitization between receptors for lipid and peptide chemoattractants, cDNA encoding the PAF receptor (PAFR) was co-expressed into RBL-2H3 cells with cDNAs encoding receptors for either formylated peptides (FR), a product of the fifth component of complement (C5aR) or interleukin-8 A (IL-8RA). PAFR was homologously phosphorylated and desensitized by PAF, and cross-phosphorylated and cross-desensitized by fMet-Leu-Phe, C5a, and IL-8. In contrast, the receptors for peptide chemoattractants were neither cross-phosphorylated nor cross-desensitized by PAF. Staurosporine blocked cross-phosphorylation and cross-desensitization of the PAFR by peptide chemoattractants. Truncation of the cytoplasmic tail of PAFR (mPAFR) abolished its homologous and cross-phosphorylation. mPAFR was also resistant to cross-desensitization by peptide chemoattractants at the level of PLC activation. Interestingly, mPAFR mediated a sustained Ca^{2+} mobilization in response to PAF and was more active in inducing GTPase activity, phosphoinositide hydrolysis, secretion, and phospholipase D activation than the wild type PAFR. In contrast to PAFR, stimulation of the mPAFR cross-phosphorylated and cross-desensitized responses to IL-8RA. As expected, FR, which is resistant to cross-phosphorylation by C5aR and IL-8RA, was not phosphorylated by mPAFR. However, unlike C5aR and IL-8RA, mPAFR did not inhibit the ability of FR to activate PLC. Blocking Ca^{2+} influx inhibited mPAFR-mediated sustained Ca^{2+} response, phospholipase D activation and secretion, but not phosphoinositide hydrolysis and cross-phosphorylation and cross-desensitization of IL-8RA. The data herein suggest that cross-desensitization of PAFR by peptide chemoattractants is solely due to receptor phosphorylation. The PAFR and the peptide chemoattractant receptors do not cross-regulate each other at the level of PLC, suggesting distinct regulatory pathways.

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Cross-desensitization Among Receptors for Platelet Activating Factor and Peptide Chemoattractants

EVIDENCE FOR INDEPENDENT REGULATORY PATHWAYS*

(Received for publication, May 3, 1996, and in revised form, July 23, 1996)

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Cross-desensitization among receptors for peptide chemoattractants have been shown to involve two independent processes, receptor phosphorylation and inhibition of phospholipase C (PLC) activation. Receptors for lipid chemoattractants, *i.e.* platelet activating factor (PAF) and leukotriene B_4, did not inhibit the responses of peptide chemoattractant receptors, suggesting distinct signaling pathways. To examine cross-desensitization between receptors for lipid and peptide chemoattractants, cDNA encoding the PAF receptor (PAFR) was co-expressed into RBL-2H3 cells with cDNAs encoding receptors for either formylated peptides (FR), a product of the fifth component of complement (C5aR) or interleukin-8 A (IL-8RA). PAFR was homologously phosphorylated and desensitized by PAF, and cross-phosphorylated and cross-desensitized by fMet-Leu-Phe, C5a, and IL-8. In contrast, the receptors for peptide chemoattractants were neither cross-phosphorylated nor cross-desensitized by PAF. Staurosporine blocked cross-phosphorylation and cross-desensitization of the PAFR by peptide chemoattractants. Truncation of the cytoplasmic tail of PAFR (mPAFR) abolished its homologous and cross-phosphorylation. mPAFR was also resistant to cross-desensitization by peptide chemoattractants at the level of PLC activation. Interestingly, mPAFR mediated a sustained Ca^{2+} mobilization in response to PAF and was more active in inducing GTPase activity, phosphoinositide hydrolysis, secretion, and phospholipase D activation than the wild type PAFR. In contrast to PAFR, stimulation of the mPAFR cross-phosphorylated and cross-desensitized responses to IL-8RA. As expected, FR, which is resistant to cross-phosphorylation by C5aR and IL-8RA, was not phosphorylated by mPAFR. However, unlike C5aR and IL-8RA, mPAFR did not inhibit the ability of FR to activate PLC. Blocking Ca^{2+} influx inhibited mPAFR-mediated sustained Ca^{2+} response, phospholipase D activation and secretion, but not phosphoinositide hydrolysis and cross-phosphorylation and cross-desensitization of IL-8RA. The data herein suggest that cross-desensitization of PAFR by peptide chemoattractants is solely due to receptor phosphorylation. The PAFR and the peptide chemoattractant receptors do not cross-regulate each other at the level of PLC, suggesting distinct regulatory pathways.

Phagocytic leukocyte accumulation and activation are regulated in part by chemoattractants released by bacteria or produced by the host. Chemoattractants include products of bacterial protein synthesis (*n*-formylated peptides, *i.e.* fMLP),[1] a cleavage product of the fifth component of complement (C5a), interleukin-8 (IL-8); and the lipid chemoattractants, platelet activating factor (PAF) and leukotriene B_4 (1). Chemoattractants activate leukocytes via cell surface receptors which are coupled to guanine nucleotide regulatory proteins (G protein) to activate phospholipase C (PLC) (1–3). Like other members of this family, chemoattractant receptors become desensitized upon agonist exposure, resulting in a loss of cellular responsiveness (1, 4). Receptor desensitization has long been categorized as homologous or heterologous, the former being restricted to the agonist-occupied form of the receptor, mediated by phosphorylation by a receptor-specific kinase (GRK) (4–6). Heterologous desensitization occurs independently of receptor occupancy by ligand and involves phosphorylation of the receptor by second messenger activated kinases such as protein kinase C (PKC) (5) or protein kinase A (PKA) (4, 5). Chemoattractant-mediated leukocyte functions have been shown to be also regulated by a third type of desensitization designated as "class desensitization" (7, 8). This type of desensitization was demonstrated among receptors for peptide chemoattractants (FR, C5aR, and IL-8RA) (9), which utilize the same G protein ($G_i\alpha 2$) to activate a common pool of phospholipase C ($PLC\beta_2$) (10, 11). This form of cross-desensitization is less specific than homologous (involves unoccupied receptor) but more specific than heterologous desensitization in that only a class of chemoattractant receptors appeared to be involved (8, 9). Studies in neutrophils also demonstrated that whereas the responses to the lipid chemoattractants PAF and leukotriene B_4 were cross-desensitized by all the peptide chemoattractants, the lipid chemoattractants did not cross-desensitize responses to any of the peptide chemoattractants (8). This finding further supported the notion of distinct classes of chemoattractant receptors with different mechanisms of regulation.

To better define the role of desensitization in the regulation of the inflammatory response, this laboratory has developed a model system using a rat basophilic leukemia cell line (RBL-2H3) into which multiple chemoattractant receptors can be expressed and stimulated to elicit cellular responses similar to those observed in neutrophils (9, 12–14). Using this system, receptors for peptide chemoattractants were co-expressed and

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[1] The abbreviations used are: fMLP, formylmethionylleucylphenylalanine; ET-FR, epitope-tagged fMLP receptor; C5a, peptide from the fifth component of complement; ET-C5aR, epitope-tagged C5a receptor; IL-8, interleukin-8; ET-IL-8R, epitope-tagged IL-8 receptor; GTPγS, guanosine 5′-3′-*O*-(thio)triphosphate; G protein, GTP-regulatory protein; PAF, platelet activating factor; PAFR, PAF receptor; PLC, phospholipase C; PKC, protein kinase c: FR, formylated peptide; RBL, rat basophilic leukemia; PMA, phorbol 12-myristate 13-acetate.

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it was recently demonstrated that in addition to receptor phosphorylation and uncoupling of receptors from G protein activation, inhibition of downstream effector activity, presumably phospholipase C, plays an important role in the class desensitization of peptide chemoattractants (9).

In the present work, the nature of cross-regulation among peptide and lipid chemoattractant receptors was investigated. To this end, receptors for PAFR were co-expressed in RBL-2H3 cells with those for FR, C5aR, or IL-8RA to study cross-desensitization among receptor classes. A PAFR mutated to express a truncated and phosphorylation deficient carboxyl terminus was also co-expressed with the peptide chemoattractant receptors in these cells. The data presented in this work show that cross-desensitization of PAFR by the peptide chemoattractants is a consequence of PAFR cross-phosphorylation and does not involve inhibitory effects on the activation of PLC.

EXPERIMENTAL PROCEDURES

Materials—[^{32}P]Orthophosphate (8,500–9,120 Ci/mmol), *myo*-[2-^{3}H]inositol (24.4 C_i/mmol), [^{3}H]myristic acid (11.2 Ci/mmol), [γ-^{32}P]GTP (6000 Ci/mmol), and [^{35}S]GTPγS (1300 Ci/mmol) were purchased from DuPont NEN. ^{125}I-IL-8 was from Amersham. IL-8 (monocyte derived) was purchased from Genzyme. Geneticin (G418) and all tissue culture reagents were purchased from Life Technologies, Inc. Monoclonal 12CA5 antibody, protein G-agarose, and proteases inhibitors were from Boehringer Manheim. fMLP, Indo-1 acetoxymethyl ester, phorbol 12-myristate 13-acetate (PMA), and pluronic acid were from Molecular Probes. PAF was from Calbiochem. C5a, GDP, GTP, GTPγS, and ATP were purchased from Sigma. All other reagents were from commercial sources.

Construction of Epitope-tagged Receptors and PAFR Deletion Mutant—Nucleotides encoding a 9-amino acid epitope sequence (YPYDVPDYA) was inserted between the NH_2-terminal initiator methionine and the second amino acid of each cDNA by polymerase chain reaction as described previously (12–14). Polymerase chain reaction was also used to construct a carboxyl-terminal truncated epitope-tagged PAFR mutant. The 5′-oligonucleotide corresponding to the epitope-tagged PAFR was used with a 3′-oligonucleotide complementary to amino acids 302–312 in PAFR. The 3′-oligonucleotide was altered to convert amino acids Thr^{305} and Ser^{310} to alanine and glycine, respectively, and a stop codon following amino acid 312. The resulting polymerase chain reaction product was cloned into the eukaryotic expression vector pcDNA3 and the entire receptor was sequenced to confirm the intended mutations and lack of secondary mutations.

Cell Culture and Transfection—RBL-2H3 cells were maintained as monolayer cultures in Earle's modified Eagle's medium supplemented with 15% fetal bovine serum, 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 mg/ml) (12). Cells (1×10^7/250 μl) were transfected by electroporation with either pRK5 or pcDNA3 vector containing the receptor cDNAs (20 μg). Geneticin-resistant cells were selected by subculturing the transfected cells in growth medium supplemented with geneticin (1 mg/ml). Cell surface expression of the receptors were monitored by fluorescence-activated cell sorter analysis as described previously (11–13). For the generation of doubly transfected cell lines, RBL-2H3 cells were transfected with cDNAs encoding both receptors. Clones were expanded from a single cell and those clones responding to both ligands were used.

Phosphorylation of the Epitope-tagged (ET) Receptors—Phosphorylation of ET receptors was performed as described previously (10, 12–14). Briefly, RBL-2H3 cells (3×10^6) expressing a combination of receptors were subcultured overnight in 60-mm tissue culture dishes. The following day, the cells were rinsed twice with 5 ml of phosphate-free Dulbecco's modified Eagle's medium and incubated in the same medium supplemented with [^{32}P]orthophosphate (150 μCi/dish) for 90 min in order to metabolically label the intracellular ATP pool. The labeled cells were then stimulated with or without agonists (IL-8, 100 nM; C5a, 100 nM; or fMLP, 1 μM) for 5–7 min at 37 °C. The phosphorylated receptors were immunoprecipitated from cell lysate with the 12CA5 antibody, analyzed by SDS electrophoresis and visualized by autoradiography.

GTPγS Binding and GTPase Activity—Cells were treated with appropriate concentrations of stimulants and membranes were prepared as already described (12). [^{35}S]GTPγS binding and GTPase activity using 10–20 μg of membrane preparations were carried out as described previously (14).

Calcium Measurement—Cells (3×10^6) were washed with HEPES-buffered saline and loaded with 1 μM Indo I-AM in the presence of 1 μM pluronic acid for 30 min at room temperature. The cells were washed and resuspended in 1.5 ml of buffer. Intracellular calcium increase in the presence of different ligands at the indicated doses (lowest EC_{100} for Ca^{2+} mobilization: fMLP, 100 nM; C5a, 10 nM; IL-8, 10 nM; and PAF, 10 nM) were measured as described (12).

Phosphoinositide Hydrolysis—RBL-2H3 cells were subcultured overnight in 96-well culture plates (5×10^4 cells/well) in inositol-free medium supplemented with 10% dialyzed fetal bovine serum and 1 μCi/ml [^{3}H]inositol. The cells were then washed with HEPES-buffered saline containing 10 mM LiCl and 0.1% bovine serum albumin and incubated in the same buffer with and without stimulants. Reactions were stopped by adding 200 μl of chloroform, methanol, 4 N HCl (100:200:2). The generation of [^{3}H]inositol phosphates was determined as reported (13, 14).

Secretion of β-Hexosaminidase—RBL-2H3 cells were subcultured overnight in 96-well culture plates (5×10^4 cells/well). Cells were washed with HEPES-buffered saline containing 0.1% bovine serum albumin. Cells were then treated with and without agonist and β-hexosaminidase release was assessed as previously reported (12).

Phospholipase D Activation Assay—Transfected RBL-2H3 cells were subcultured in 24-well plates (2.5×10^5 per well) overnight and labeled with [^{3}H]myristic acid (2 μCi/well) in HEPES-buffered saline supplemented with 0.1% bovine serum albumin for 90 min. Cells were then washed and stimulated with an EC_{100} dose of either PAF (100 nM) or IL-8 (100 nM) for 5 min. Reactions were terminated by adding 750 μl of chloroform, methanol, 4 N HCl (100:200:5) to form a monophasic mixture. This was separated into two phases by further addition of chloroform (250 μl) and 0.1 N HCl (250 μl). The upper aqueous phase was discarded and 10 μl of the lower organic phase was removed and counted to determine total ^{3}H incorporation. An aliquot (200 μl) was evaporated to dryness under nitrogen and resuspended in 25 μl of chloroform:methanol (2:1). Phospholipids were separated by TLC using chloroform:methanol:acetic acid (65:15:2) as the mobile phase. Unlabeled phosphatidylethanol (500 pmol) was used as a standard. Phosphatidylethanol was visualized with iodine vapor, and the corresponding spot was excised and counted in a liquid scintillation counter to determine the formation of phosphatidylethanol (8).

RESULTS

Phosphorylation and Immunoprecipitation of Epitope-tagged Chemoattractant Receptors in RBL-2H3 Cells—RBL-2H3 cells expressing epitope-tagged FR and PAFR (FR-PAFR), C5aR and PAFR (C5aR-PAFR), and IL-8RA and PAFR (IL8R-PAFR) were labeled with ^{32}P, treated with different ligands (100 nM PAF, 1 μM fMLP, 100 nM C5a, or 100 nM IL-8) and cells lysates were immunoprecipitated with 12CA5 antibody, which specifically bind to the epitope tag. The identity of the phosphorylated bands for the respective receptors (FR ~ 65 kDa, C5aR ~ 45 kDa, IL-8RA ~ 70 kDa, and PAFR ~ 42 kDa) have been previously demonstrated by immunoprecipitation of iodinated and phosphorylated receptors in the presence and absence of the epitope tag peptide (12–14). As shown in Fig. 1*A*, PAFR (~42 kDa) was phosphorylated by PAF (*lanes 2, 5,* and *8*) and cross-phosphorylated by fMLP (*lane 3*), C5a (*lane 6* and see next paragraph), and IL-8 (*lane 9*). FR (~65 kDa) (*lane 3*), C5aR (~45 kDa) (*lane 6*), and IL-8RA (~70 kDa) (*lane 9*) were homologously phosphorylated by their respective ligands, but were resistant to cross-phosphorylation by PAF stimulation.

Since PAFR (~42 kDa) and C5aR (~45 kDa) migrate as broad overlapping bands in SDS gels, double transfected RBL-2H3 cell lines with one native and the other epitope-tagged receptors were prepared. This allowed clear resolution of the nature of cross-phosphorylation of these two receptors, as only the ET receptors are immunoprecipitated with the 12CA5 antibody. In RBL-2H3 cells expressing wild type C5aR and ET-PAFR (**C5aR*-PAFR) or wild type PAFR and ET-C5aR (**PAFR*-C5aR), activation of the C5aR resulted in cross-phosphorylation of the PAFR but not vice versa (Fig. 1*B, lanes 11* and *14 versus lanes 12* and *15*).

Effect of Staurosporine on Peptide Chemoattractant-medi-

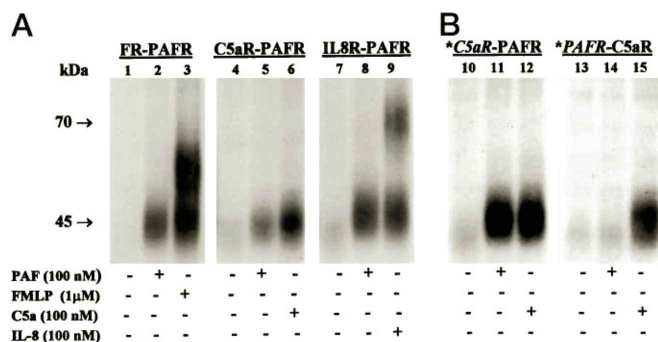

FIG. 1. **Phosphorylation of epitope-tagged chemoattractant receptors expressed in RBL-2H3 cells.** *A*, ^{32}P-labeled double transfected RBL-2H3 cells (3×10^6/60-mm plate) expressing epitope-tagged receptors for either fMLP and PAF (FR-PAFR), C5a and PAF (C5aR-PAFR), or IL-8 and PAF (IL8R-PAFR) were incubated for 5 min with (*lanes 2, 3, 5, 6, 8* and *9*) or without (*lanes 1, 4*, and *7*) stimulants. Cells were lysed, immunoprecipitated with 12CA5 antibody, and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. This experiment has been repeated 3 times with similar results. *B*, RBL-2H3 cells expressing either epitope-tagged PAFR and native C5aR (**C5aR-PAFR*) (*lanes 10–12*) or epitope-tagged C5aR and native PAFR (**PAFR-C5aR*) (*lanes 13–15*) were stimulated with or without PAF and C5a and receptor phosphorylation were assessed as described above. This experiment has been repeated twice with similar results.

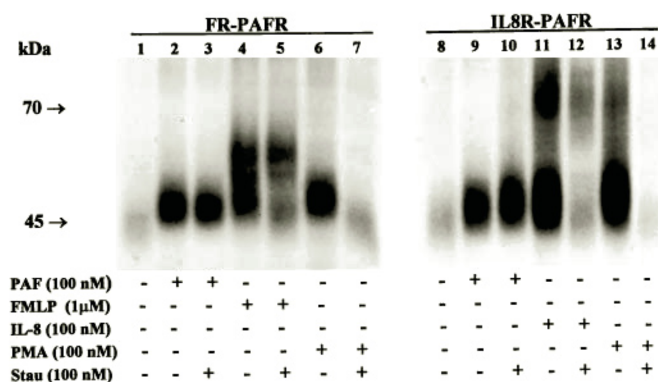

FIG. 2. **Effect of staurosporine on fMLP, IL-8, and PMA-induced PAFR cross-phosphorylation.** ^{32}P-Labeled double transfected RBL-2H3 cells (3×10^6/60-mm plate) expressing either FR-PAFR or IL8R-PAFR were preincubated for 3 min with (*lanes 3, 5, 7, 10, 12*, and *14*) or without (*lanes 1, 2, 4, 6, 8, 9, 11*, and *13*) staurosporine (100 nM) and stimulated with fMLP, IL-8, or PMA for 5 min. Cells were lysed, immunoprecipitated with 12CA5 antibody, and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The results are from one of two representative experiments.

ated Cross-phosphorylation of PAFR—The involvement of PKC on fMLP and IL-8-mediated cross-phosphorylation of PAFR was studied. ^{32}P-Labeled RBL-2H3 cells expressing FR-PAFR and IL8R-PAFR were preincubated with or without the PKC inhibitor staurosporine (100 nM) for 3 min and then stimulated with PAF (100 nM), fMLP (1 μM), IL-8 (100 nM), or PMA (100 nM). Whereas PAF-induced phosphorylation of ET-PAFR was not affected by staurosporine (Fig. 2, *lanes 2, 9, 3,* and *10*), PAFR cross-phosphorylation by either fMLP or IL-8 were markedly inhibited (*lanes 4, 5, 11*, and *12*). PMA-induced phosphorylation of PAFR was also blocked by staurosporine (*lanes 6, 7, 13,* and *14*).

GTPγS Binding in Cross-phosphorylated Membranes—To determine the effect of cross-phosphorylation on PAFR function, agonist-stimulated [^{35}S]GTPγS binding was measured in membranes prepared from double transfected RBL-2H3 cells expressing PAFR and C5aR, and pretreated with either PAF (100 nM) or C5a (100 nM). As shown in Fig. 3, pretreatment of cells with either PAF or C5a resulted in homologous desensi-

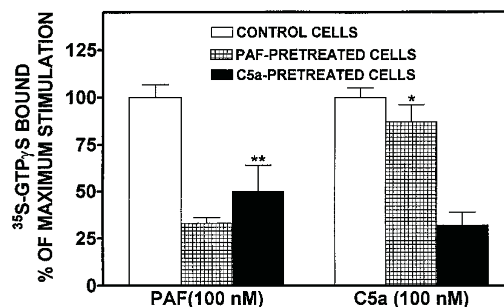

FIG. 3. **Homologous and cross-desensitization of PAFR stimulated [^{35}S]GTPγS binding.** Double transfected RBL-2H3 cells expressing C5aR and PAFR were treated with either C5a (100 nM) or PAF (100 nM) for 5 min, membranes were prepared and assayed for agonist-stimulated [^{35}S]GTPγS binding. The data shown are the means of three different experiments performed in triplicate. The values are represented as percentage of maximum stimulation, which is defined as the maximal increase above basal [^{35}S]GTPγS bound to control membranes (untreated cells) after 10 min of reaction. Basal activities were 0.17 ± 0.02 pmol of [^{35}S]GTPγS bound/mg of protein. Maximum net stimulation was 0.114 ± 0.006 (C5a) and 0.122 ± 0.008 (PAF) pmol of [^{35}S]GTPγS bound/mg of protein for untreated cells. *, $p < 0.05$ and **, $p < 0.01$ compared to control.

TABLE I
Cross-desensitization of PAFR-mediated Ca^{2+} mobilization by fMLP, C5a, and IL-8

RBL-2H3 cells (3×10^6 cells/assay) expressing FR-PAFR, C5aR-PAFR, or IL8R-PAFR were loaded with Indo-1 and stimulated with either fMLP (100 nM), PAF (10 nM), C5a (10 nM), or IL-8 (10 nM). Cells were rechallenged 3 min later with a second dose of ligand and peak intracellular Ca^{2+} mobilization was determined. Values are represented as percentage of inhibition (cross-desensitization) of the Ca^{2+} response elicited in the absence of pretreatment (PAF, 582 ± 19 nM; fMLP, 628 ± 32 nM; C5a, 684 ± 16 nM, IL-8, 530 ± 21 nM). Data are the mean ± S.E. of three different experiments.

Cells	Agonists	% Cross-desensitization
FR-PAFR	fMLP → PAF	47 ± 12.3[a]
	PAF → fMLP	−6 ± 7
C5aR-PAFR	C5a → PAF	80 ± 7.9[b]
	PAF → C5a	3 ± 2.7
IL8R-PAFR	IL-8 → PAF	31 ± 14[a]
	PAF → IL-8	1 ± 3.7

[a] $p < 0.05$.
[b] $p < 0.01$.

tization (~70%) of agonist-induced [^{35}S]GTPγS binding to membranes. Membranes from cells pretreated with C5a showed a ~50% decrease in PAF-stimulated [^{35}S]GTPγS binding. Only a ~13% desensitization was observed for C5a-stimulation in cells pretreated with PAF.

Cross-desensitization of Receptor-induced Ca^{2+} Mobilization—Ca^{2+} mobilization was measured to further determine the relationship between receptor cross-phosphorylation and cross-desensitization. Ca^{2+} mobilization in response to an EC_{100} dose of fMLP (100 nM), C5a (10 nM), IL-8 (10 nM), or PAF (10 nM) was homologously desensitized by a first dose of the same ligand (data not shown, see Refs. 12–14). PAF-induced Ca^{2+} mobilization was cross-desensitized by pretreatment of the cells with a first dose of fMLP, C5a, or IL-8 (Table I). In contrast, pretreatment of the cells with an EC_{100} first dose of PAF had no effect on Ca^{2+} mobilization induced by any of the peptide chemoattractants (Table I).

Co-expression and Characterization of a Mutant PAFR in RBL-2H3 Cells—In order to assess the role of phosphorylation in the cross-desensitization of PAFR by the peptide chemoattractant receptors, a phosphorylation deficient PAFR mutant (mPAFR) was constructed (Table II). The mPAFR was cotransfected into RBL-2H3 cells with pcDNA3 plasmid containing either FR or IL-8RA. Stable transfectants were generated and

TABLE II

Amino acid sequences of the carboxyl-terminal tails of the wild type PAF receptor (PAFR) and the mutant PAF receptor (mPAFR) construct

Bold and underlined serines and threonines residues are potential phosphorylation sites of the wild type receptor which have been either removed by deletion or substituted by alanine or glycine in the mutant PAF receptor.

	298 ↓	342 ↓
C-tail PAFR	KKFRKHL**T**EKFY**S**MR**SS**RKC**S**RA**TT**D**T**V**T**EVVVPFNQIPGN**S**LKN	
mPAFR	KKFRKHL**A**EKFY**G**MR	

single cell cloning was utilized to isolate double transfectants. Competition binding using [^{3}H]WEB 2086 and Scatchard analysis of wild type *versus* the mutant indicated that deletion of the carboxyl tail had no significant effect on the affinity of the receptor (K_d ~10 and ~19 nM for wild type and mutant PAFR, respectively). Clones expressing similar receptor numbers (2.8–3.3 × 10^3 receptors/cell) were utilized to determine the functional properties of the mutant PAFR *versus* the wild type receptor.

Time course of both PAFR- and mPAFR-mediated [^{35}S]GTPγS binding in membranes were similar, with half-maximal binding at ~4 min (data not shown). Maximum binding after 20 min of reactions were 0.13.± 0.02 and 0.12 ± 0.016 pmol of [^{35}S]GTPγS bound/mg of protein for mPAFR and PAFR, respectively. However, GTPase activity in membranes (Fig. 4*A*), phosphoinositide hydrolysis (Fig. 4*B*), and β-hexosaminidase release (Fig. 4*C*) in intact cells revealed that mPAFR was more active than the wild type PAFR in mediating cellular responses. Peaks of intracellular Ca^{2+} mobilization in response to PAF (10 nM) were similar for both, wild type (598 nM) and mutant PAFR (624 nM) (Fig. 4*D*). However, a more sustained response was obtained with mPAFR as compared to the wild type receptor (Fig. 4*D*).

The ability of mPAFR and wild type PAFR to stimulate PLD activation was also determined and compared to that of IL-8RA in RBL-2H3 cells co-expressing IL-8RA and either PAFR (IL8R-PAFR) or mPAFR (IL8R-mPAFR). As shown in Fig. 5, IL-8-induced PLD activation was similar in both cell lines, 0.61 ± 0.014- and 0.53 ± 0.04-fold over basal, for IL8R-PAFR and IL8R-mPAFR cells, respectively (Fig. 5). In contrast, PAF-induced PLD activity was ~5-fold greater in cells expressing IL8R-mPAFR (1.43 ± 0.07) than IL8R-PAFR (0.26 ± 0.02).

Cross-phosphorylation of mPAFR by fMLP and IL-8—^{32}P-Labeled RBL-2H3 cells co-expressing the epitope-tagged mPAFR and either FR (FR-mPAFR), or IL-8RA (IL8R-mPAFR) were treated with different ligands (PAF, 100 nM; fMLP, 1 μM; or IL-8, 100 nM) and immunoprecipitated with 12CA5 antibody. As shown in Fig. 6, mPAFR (~42 kDa) was resistant to phosphorylation by PAF (*lanes 2* and *5*) and cross-phosphorylation by either fMLP (*lane 3*) or IL-8 (*lane 6*). FR (~65 kDa) (*lane 3*) and IL-8RA (~70 kDa) (*lane 6*) were homologously phosphorylated by fMLP and IL-8, respectively. While FR was resistant to cross-phosphorylation, stimulation of the mutant PAFR resulted in IL-8RA cross-phosphorylation (*lane 5*). The extent of mPAFR-mediated cross-phosphorylation of IL-8RA was similar to the extent of phosphorylation of IL-8RA by PMA (Fig. 2, *lane 13*). In addition, IL-8RA cross-phosphorylation by mPAFR was inhibited by staurosporine (100 nM) (data not shown).

Effect of mPAFR-mediated Cross-phosphorylation of IL-8RA on Receptor/G Protein Coupling—The ability of IL-8RA to stimulate [^{35}S]GTPγS binding in membranes from cells expressing IL8-RA and mPAFR was determined. As shown in Fig. 7, pretreatment of the cells with IL-8 caused ~67% homologous desensitization of IL-8-induced response compared to control (untreated cells). There was no effect on mPAFR. Membranes from cells pretreated with PAF (100 nM) was resistant to homologous desensitization but showed a ~50% decrease of the response to IL-8RA. Simultaneous pretreatment of the cells with staurosporine (100 nM) blocked significantly both the homologous (~40%) and cross- (66%) desensitization of IL-8RA-induced [^{35}S]GTPγS binding.

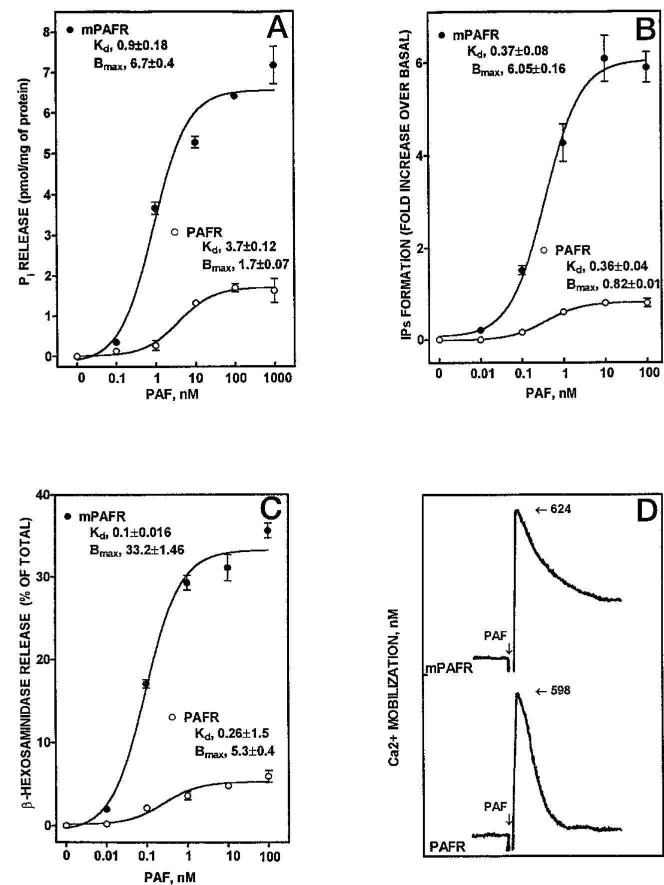

FIG. 4. **Functional characterization of phosphorylation-deficient PAF receptor mutant (*mPAFR*) *versus* PAFR.** *A*, for GTPase activity, membranes were prepared from RBL-2H3 cells expressing either the mutant PAFR or the wild type PAFR and assayed at different concentrations of PAF. Data shown are representative of one of three experiments performed in triplicate. *B,* for the generation of inositol phosphates, cells were cultured overnight in the presence of [^{3}H]inositol (1 μCi/ml). Cells were preincubated (10 min, 37 °C) with a HEPES-buffered saline containing 10 mM LiCl in a total volume of 200 μl and stimulated with different concentrations of PAF for 10 min. [^{3}H]Inositol phosphates release was determined. Data are represented as fold stimulation over basal, which was 475 ± 30 cpm for mPAFR and 347 ± 49 for PAFR. The experiment was repeated three times with similar results. *C,* cells were treated with different concentrations of PAF and β-hexosaminidase release in the medium was assessed. Data are represented as percentage of total which were 0.56 ± 0.003 (PAFR) and 0.47 ± 0.006 (mPAFR). The experiment was repeated twice with similar results. *D,* RBL-2H3 cells (3 × 10^6 cells/assay) were loaded with Indo-1 and PAF (10 nM)-induced Ca^{2+} mobilization was measured.

Cross-desensitization of mPAFR-induced Ca^{2+} Mobilization by fMLP and IL-8—It was determined whether the Ca^{2+} mobilization in response to PAF was cross-desensitized by either fMLP or IL-8 in RBL-2H3 cells expressing FR-mPAFR or IL8R-mPAFR. PAF-induced Ca^{2+} mobilization was resistant to cross-desensitization by pretreatment of the cells with either

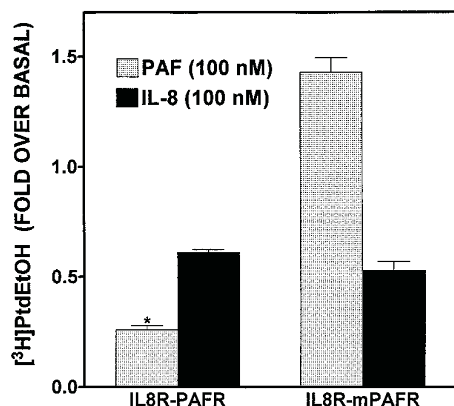

FIG. 5. **PAFR- and mPAFR-mediated PLD activation.** RBL-2H3 cells expressing IL8R-PAFR or IL8R-mPAFR were labeled with [^{3}H]myristic acid and stimulated with or without agonists. Formation of phosphatidylethanol (*PtdEtOH*) was measured as described under "Experimental Procedures." Data are represented as fold increase over basal and are from one of three representative experiments. Basal values were 0.99 ± 0.08 (IL8R-PAFR) and 0.97 ± 0.09 (IL8R-mPAFR). Similar results were obtained in three separate experiments. *, $p < 0.01$ as compared to mPAFR.

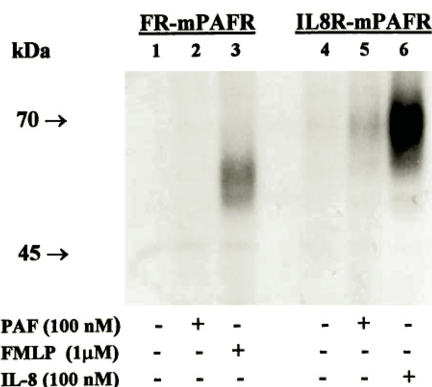

FIG. 6. **Cross-phosphorylation of mPAFR by FR and IL-8R.** RBL-2H3 cells co-expressing FR-mPAFR or IL8R-mPAFR were ^{32}P-labeled and stimulated for 5 min with (*lanes 2, 3, 5,* and *6*) or without (*lanes 1* and *4*) agonists. Receptor phosphorylation was assessed as described in the legend to Fig. 1. This experiment has been repeated three times with similar results.

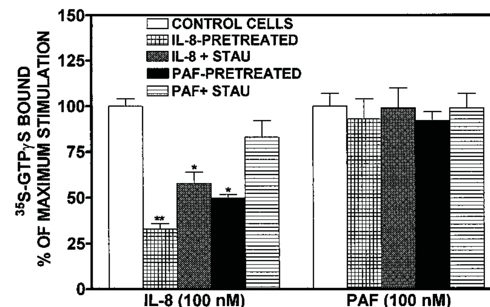

FIG. 7. **Cross-desensitization of IL-8RA-stimulated [^{35}S]GTPγS binding by mPAFR.** Double transfected RBL-2H3 cells expressing IL-8RA and mPAFR were treated with either IL-8 (100 nM) or PAF (100 nM) in the presence or absence of staurosporine (100 μM) for 5 min, membranes were prepared and assayed for agonist-stimulated [^{35}S]GTPγS binding. The data shown are the means of three different experiments performed in triplicate. The values are represented as percentage of maximum stimulation, which is defined as the maximal increase above basal [^{35}S]GTPγS bound to control membranes (untreated cells) after 10 min of reaction. Basal activities were 0.2 ± 0.015 pmol of [^{35}S]GTPγS bound/mg of protein. Maximum net stimulation were 0.16 ± 0.07 (IL-8) and 0.14 ± 0.03 (PAF) pmol of [^{35}S]GTPγS bound/mg of protein for untreated cells. *, $p < 0.05$; and **, $p < 0.01$ compared to control.

TABLE III
Cross-desensitization of Ca^{2+} mobilization in cells expressing FR-mPAFR and IL8R-mPAFR

RBL-2H3 cells (3 × 10^6 cells/assay) expressing FR-mPAFR or IL8R-mPAFR and, for comparison, cells expressing FR-C5aR were loaded with Indo-1 and stimulated with PAF (10 nM), fMLP (100 nM), IL-8 (10 nM), or C5a (10 nM). Cells were rechallenged 3 min later with a second dose of the indicated ligand and peak of intracellular Ca^{2+} mobilization was determined. Values are represented as percentage of inhibition (cross-desensitization) of the Ca^{2+} response elicited in the absence of pretreatment (PAF, 595 ± 41 nM, fMLP, 532 ± 28 nM, IL-8, 533 ± 17 nM, and C5a, 487 ± 17 nM). Data are the means ± S.E. of three different experiments.

Cells	Agonists	% Cross-desensitization
FR-mPAFR	fMLP → PAF	7 ± 0.3
	PAF → fMLP	2 ± 0.26
IL8R-mPAFR	IL-8 → PAF	−8 ± 1
	PAF → IL-8	61 ± 4.7^a
FR-C5aR	fMLP → C5a	60 ± 9^a
	C5a → fMLP	50 ± 10^a

^{a} $p < 0.05$.

fMLP or IL-8 (Table III). However, while pretreatment of cells with a first dose of PAF had no effect on fMLP-induced Ca^{2+} mobilization, response to IL-8 was desensitized by ~60% (Table III). In contrast to PAFR and mPAFR, Ca^{2+} mobilization in response to FR was cross-desensitized by both C5aR (Table III) and IL-8RA (data not shown) despite FR resistance to cross-phosphorylation (9).

Effect of Extracellular Calcium on mPAFR-mediated Signal Transduction—The effect of Ca^{2+} influx on mPAFR-mediated cellular responses as well as cross-phosphorylation and cross-desensitization of IL-8RA was assessed using the cation chelator EGTA to block extracellular calcium influx. EGTA had no effect in mPAFR-mediated PI hydrolysis (Fig. 8*A*) However, EGTA blocked mPAFR-induced PLD activation (Fig. 8*B*), secretion (Fig. 8*C*), and the sustained Ca^{2+} mobilization in response to PAF (Fig. 8, *D* and *E*). EGTA had no significant effect on mPAFR-mediated cross-desensitization of IL-8RA induced Ca^{2+} response (Fig. 8, *D* and *E*) and cross-phosphorylation of IL-8RA (Fig. 9).

DISCUSSION

Previous studies have indicated that chemoattractant receptors trigger cellular activities by two related but distinguishable pathways. The first via the heterotrimeric G protein-mediated activation of PLC, the second via activation of PLD (1). Whereas all chemoattractants (peptides and lipids) initiate the former equally well, peptide chemoattractants are far more active in stimulating PLD, which is required for the cytotoxic actions of phagocytes (*i.e.* respiratory burst and exocytosis) (1, 15–17). While chemoattractant receptors for peptides and lipids all activate PLC via G proteins, the difference in their regulation has been suggested to be a consequence of their utilizing different G proteins and PLC isozymes (10, 18–20). This laboratory has been investigating the regulation and cross-regulation of leukocyte chemoattractant receptors and has developed cellular models allowing genetic analysis of receptor functions. Using these strategies, it has been shown that peptide chemoattractant receptor desensitization occurs at two sites, the first involving receptor phosphorylation leading to diminished G protein activation. The second involves the inhibition of the activation of PLC (9). Data in neutrophils demonstrated differences in cross-regulation among receptors for peptide *versus* lipid chemoattractants (8), suggesting disparate signaling and regulatory pathways. However, the molecular mechanism underlying this phenomenon of cross-regulation among different classes of receptors has not been studied.

A goal of this study was to better define the regulation and

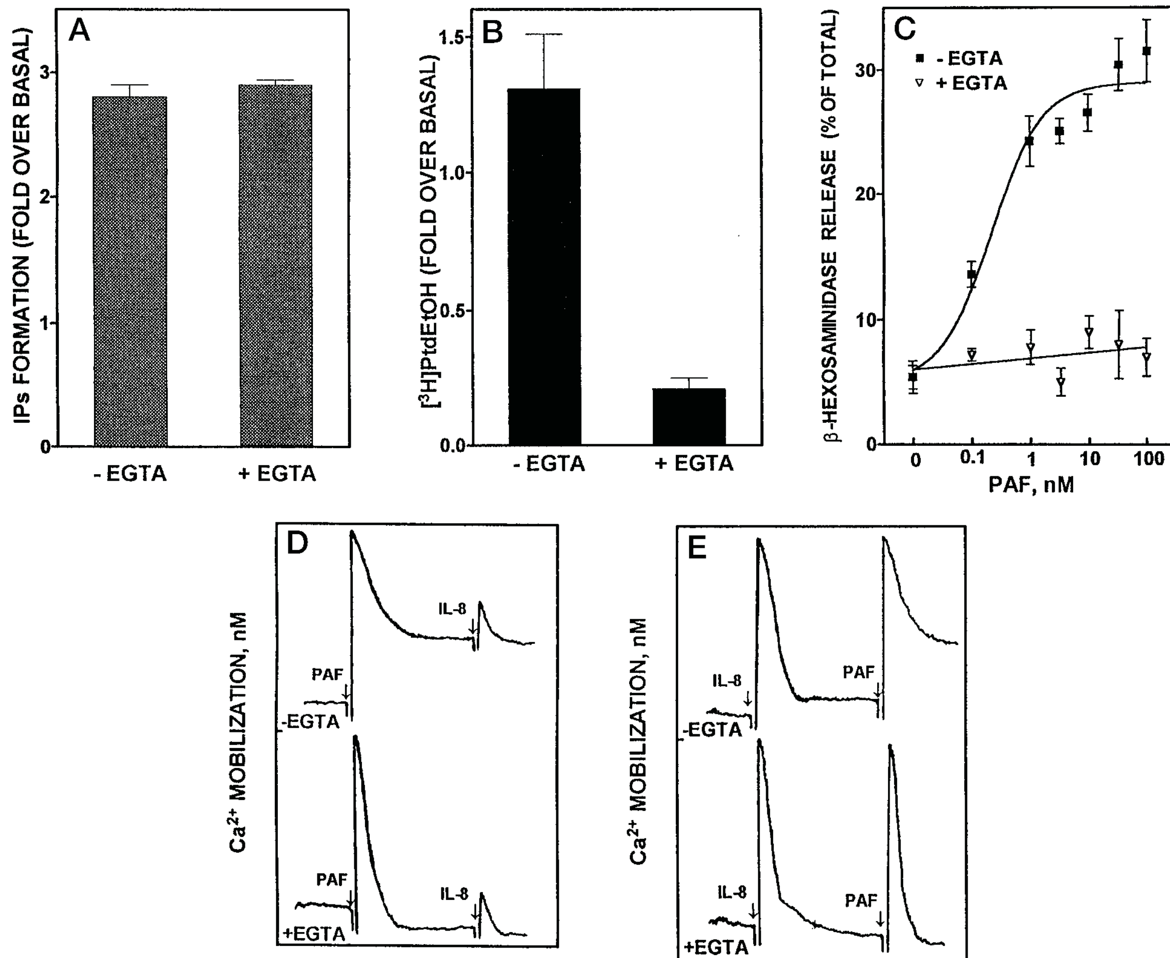

FIG. 8. **Effect of EGTA on mPAFR-mediated cellular responses.** *A*, inositol phosphates (*IP*) generation; *B*, phospholipase D activation; *C*, β-hexosaminidase release; and *D* and *E*, Ca^{2+} mobilization in the presence and absence of EGTA (5 mM) were determined as described in the legend to Fig. 4 and Table I. The experiments were repeated twice with similar results.

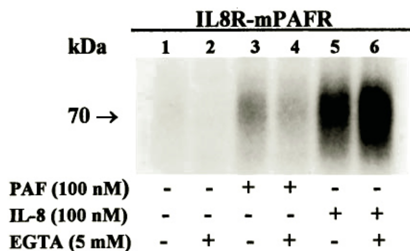

FIG. 9. **Effect of EGTA on mPAFR-mediated cross-phosphorylation of IL-8RA.** RBL-2H3 cells expressing IL8R-mPAFR were ^{32}P-labeled and stimulated for 5 min with (*lanes 3–6*) or without (*lanes 1* and *2*) agonists in the presence (*lanes 2, 4,* and *6*) and absence (*lanes 1, 3,* and *5*) of 5 mM EGTA. Receptor phosphorylation was assessed as described in the legend to Fig. 1. This experiment has been repeated three times with similar results.

cross-regulation of the PAF receptor. The approach undertaken was to co-express, in RBL-2H3 cells, PAFR, or a phosphorylation deficient PAFR with the receptors for fMLP, C5a, or IL-8. The data presented herein demonstrate that PAFR was cross-phosphorylated upon activation of all three peptide chemoattractant receptors (Fig. 1) and that this correlated with the cross-desensitization of the receptor as measured by GTPγS binding in membranes (Fig. 3) and Ca^{2+} mobilization in intact cells (Table I). The results suggest a role for phosphorylation mediated by PKC in PAFR cross-desensitization since both peptide chemoattractants- and PMA-mediated phosphorylation were inhibited by the PKC inhibitor staurosporine (Fig. 2).

Like many members of the G-protein coupled receptor family, PAFR possesses a serine/threonine-rich cytoplasmic tail (5 serines and 6 threonines), which contains potential sites for agonist-dependent and -independent phosphorylation (21, 22). The carboxyl terminus plays a key role in the desensitization and regulation of several G-protein-coupled receptors (23–28). Elimination of phosphorylatable residues in the carboxyl terminus of the guinea pig PAFR, by either alanine substitution or truncation, diminished agonist-induced receptor desensitization (29). Based on these observations and, in an attempt to assess the role of receptor phosphorylation on PAFR regulation, a phosphorylation deficient human PAFR mutant containing a truncated carboxyl terminus was constructed (Table II). When co-expressed with FR and IL-8RA, this mutant was resistant to cross-phosphorylation and, importantly, cross-desensitization both at the level of G protein activation and Ca^{2+} mobilization (Fig. 6 and Table III). This indicates that, unlike the peptide chemoattractant receptors, cross-desensitization of PAFR by the peptide chemoattractants is solely due to phosphorylation of the receptor by a PKC-dependent process and does not involve the direct downstream inhibition of PLC activation. This latter observation further supports the concept of class desensitization as a form of cellular regulation involving group of receptors sharing similar activation pathways (7–9) and suggests that receptors for lipid chemoattractants are regulated differently.

Studies in intact neutrophils indicated that whereas peptide chemoattractants desensitized the responses to lipid chemoat-

tractants, the reverse was not true (8). The studies presented here provide a molecular basis for this observation. RBL-2H3 cells expressing PAFR and FR, C5aR, or IL-8RA showed that PAFR was readily phosphorylated and desensitized by FR, C5aR, and IL-8RA but was incapable of phosphorylating or desensitizing the peptide chemoattractant receptors. This is of interest since the PAFR clearly activated PLC and undergo phosphorylation by both PKC-dependent and PKC-independent processes (13). Several explanations were considered. First, the level of PLC activation might be insufficient to generate enough PKC activity for cross-phosphorylation. Second, cross-phosphorylation may be a consequence of PLD activation and PAFR is a poor PLD activator (1). Finally the type of PKC or other second messenger-dependent kinase activated by PAFR may be unable to phosphorylate peptide chemoattractant receptors. The development of the mPAFR allowed these questions to be addressed. mPAFR displayed striking differences when compared to the PAFR in stimulating far greater activation of PLC as well as sustained Ca^{2+} influx and activation of PLD (Figs. 4 and 5). The mechanism for this enhanced receptor activity appears to be a consequence of greater turnover of G proteins apparently due to resistance of the receptor to undergo phosphorylation and, thus, desensitization. The mPAFR is instructive in that it shows that the cytoplasmic tail of the PAFR, presumably through phosphorylation, regulates the extent of G protein activation mediated by a single dose of ligand. It moreover shows that greater activation of G proteins by the receptor allows for the opening of a channel allowing Ca^{2+} influx (30). When this was blocked by EGTA, PLD activation did not occur, demonstrating the requirement of Ca^{2+} influx for the activation of this enzyme. Stimulation of the mPAFR (Fig. 6), unlike PAFR (Fig. 1), led to cross-phosphorylation of IL-8RA. This cross-phosphorylation was dependent on PLC but not on PLD activation, since EGTA which blocked the latter, but had no effect on IL-8RA phosphorylation (Fig. 9). This suggests that the level of PLC activation by PAFR determines its ability to cross-phosphorylate and cross-desensitize peptide chemoattractant receptors. Under normal conditions, given the level of receptors expressed in neutrophils or in the studies here, the PAFR apparently does not activate enough PLC to mediate cross-phosphorylation.

The extent of mPAFR-mediated cross-phosphorylation of IL-8RA was minor as compared to the extent of cross-phosphorylation of wild type PAFR by IL-8RA (Figs. 6 and 9 *versus* Figs. 1 and 2). Nonetheless this phosphorylation of the IL-8RA was significant in that it resulted in a ~50 and 61% cross-desensitization of IL-8RA-induced GTPγS binding and intracellular Ca^{2+} mobilization, respectively. Furthermore, staurosporine blocked both the cross-phosphorylation and cross-desensitization of IL-8RA indicating a role for PKC in this process. It has been shown that homologous phosphorylation of rhodopsin, β-adrenergic receptors, and muscarinic receptors by receptor specific kinases (rhodopsin kinase and β-adrenergic receptor kinases) do not cause receptor desensitization unless specific protein such as arrestin or β-arrestin are present (35–38). However, heterologous phosphorylation of these receptors by PKC and PKA (although to lower extents than homologous) caused 35–50% inhibition of receptor/G protein coupling (4, 5, 37). This suggest that receptor desensitization at the level of receptor/G protein coupling for a number of receptors, including those for IL-8, may be due to the phosphorylation of a small number of specific residue(s) involved in such interaction.

Another finding of note is that neither the PAFR nor the mPAFR at the dose tested (EC_{100} *versus* EC_{100}) generated the signal which results in downstream inhibition of PLC activation by peptide chemoattractant receptors, since neither PAFR nor mPAFR inhibited Ca^{2+} mobilization by FR (Tables I and III). Thus, not only are PAFR not subject to regulation at the level of PLC activation by peptide chemoattractant receptors, they do not produce the signal(s) sufficient to mediate this activity. Since mPAFR mediates substantial activation of PLC, Ca^{2+} influx, and PLD, it can be presumed that second messengers produced by these pathways are not sufficient to produce class-desensitization of peptide chemoattractants. More likely, a component(s) unique to the peptide chemoattractant receptors pathways is involved in its susceptibility to regulation of PLC activation. The nature of this signal(s) or second messenger(s) remains to be determined. A clue may be found in the differences in G proteins and PLCs used by PAFR *versus* peptide chemoattractant receptors. Whereas the latter interact preferentially with G_i (pertussis toxin (Ptx)-sensitive) to stimulate PLCβ_2 via G$\beta\gamma$ (10, 11, 19), PAFR is thought to couple to members of the Gq family (Ptx-insensitive) of G proteins to activate PLCγ via Gα (13, 19, 31–34). Thus the G$\beta\gamma$ of G_i may be a site for cross-regulation of chemoattractant receptors for peptide.

In all, these studies point out the utility of cellular and genetic models to elucidate the complexities of receptor regulation and cross-regulation. While all the chemoattractant receptors studied are effective mediators of directed cellular migration, they are likely to play different physiological roles in other aspects of phagocyte regulation. The PAFR clearly is subject to different regulatory processes than the peptide chemoattractant receptors as reflected in its resistance to inhibition at the level of PLC activation and its inability to affect PLC activation by the peptide chemoattractant receptors. These data are likely to reflect the distinct usage of G proteins and PLC isozymes by the different groups of receptors and indicates that receptor cross-regulation involves mechanisms beyond receptor phosphorylation.

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