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Abstract

To define the molecular mechanisms of cross-regulation among chemoattractant receptors, we stably coexpressed, in a rat basophilic leukemia (RBL-2H3) cell line, epitope-tagged receptors for the chemoattractants formylmethionylleucylphenylalanine (fMLP), a peptide of the fifth component of the complement system (C5a), and interleukin-8 (IL-8). All the expressed receptors underwent homologous phosphorylation and desensitization upon agonist stimulation. When co-expressed, epitope-tagged C5a receptor (ET-C5aR) and epitope-tagged IL-8 receptor (ET-IL-8RA) were cross-phosphorylated by activation of the other. Activation of epitope-tagged fMLP receptor (ET-FR) also cross-phosphorylated ET-C5aR and ET-IL-8RA, but ET-FR was totally resistant to cross-phosphorylation. Similarly, C5a and IL-8 stimulation of [³⁵S]guanosine 5'-3-P-(thio) triphosphate (GTPγS) binding and Ca²⁺ mobilization were cross-desensitized by each other and by fMLP. Stimulation of [³⁵S]GTPγS binding by fMLP was also not cross-desensitized by C5a or IL-8, however, Ca²⁺ mobilization was, suggesting a site of inhibition distal to G protein activation. Consistent with this desensitization of Ca²⁺ mobilization, inositol 1,4,5-trisphosphate release in RBL-2H3 cells expressing both ET-C5aR and ET-FR revealed that fMLP and C5a cross-desensitized each other's ability to stimulate phosphoinositide hydrolysis. Taken together, these results indicate that receptor cross-phosphorylation correlates directly with desensitization at the level of G protein activation. The ET-FR was resistant to this process. Of note, cross-desensitization of ET-FR at the level of phosphoinositide hydrolysis and Ca²⁺ mobilization was demonstrated in the absence of receptor phosphorylation. This suggests a new form of chemoattractant cross-regulation at a site distal to receptor/G protein coupling, involving the activity of phospholipase C.

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Cross-desensitization of Chemoattractant Receptors Occurs at Multiple Levels

EVIDENCE FOR A ROLE FOR INHIBITION OF PHOSPHOLIPASE C ACTIVITY*

(Received for publication, August 25, 1995)

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To define the molecular mechanisms of cross-regulation among chemoattractant receptors, we stably co-expressed, in a rat basophilic leukemia (RBL-2H3) cell line, epitope-tagged receptors for the chemoattractants formylmethionylleucylphenylalanine (fMLP), a peptide of the fifth component of the complement system (C5a), and interleukin-8 (IL-8). All the expressed receptors underwent homologous phosphorylation and desensitization upon agonist stimulation. When co-expressed, epitope-tagged C5a receptor (ET-C5aR) and epitope-tagged IL-8 receptor (ET-IL-8RA) were cross-phosphorylated by activation of the other. Activation of epitope-tagged fMLP receptor (ET-FR) also cross-phosphorylated ET-C5aR and ET-IL-8RA, but ET-FR was totally resistant to cross-phosphorylation. Similarly, C5a and IL-8 stimulation of [³⁵S]guanosine 5'-3-O-(thio)triphosphate (GTP γ S) binding and Ca²⁺ mobilization were cross-desensitized by each other and by fMLP. Stimulation of [³⁵S]GTP γ S binding by fMLP was also not cross-desensitized by C5a or IL-8, however, Ca²⁺ mobilization was, suggesting a site of inhibition distal to G protein activation. Consistent with this desensitization of Ca²⁺ mobilization, inositol 1,4,5-trisphosphate release in RBL-2H3 cells expressing both ET-C5aR and ET-FR revealed that fMLP and C5a cross-desensitized each other's ability to stimulate phosphoinositide hydrolysis. Taken together, these results indicate that receptor cross-phosphorylation correlates directly with desensitization at the level of G protein activation. The ET-FR was resistant to this process. Of note, cross-desensitization of ET-FR at the level of phosphoinositide hydrolysis and Ca²⁺ mobilization was demonstrated in the absence of receptor phosphorylation. This suggests a new form of chemoattractant cross-regulation at a site distal to receptor/G protein coupling, involving the activity of phospholipase C.

Leukocytes migrate to sites of inflammation where they participate in host-defensive and/or tissue-destructive activities via activation of chemoattractant receptors. Upon stimulation by proinflammatory agents such as a peptide component of the fifth complement system (C5a),¹ formylpeptides (fMLP), inter-

leukin-8 (IL-8), platelet-activating factor, or leukotriene B₄ chemoattractant receptors couple to guanine nucleotide binding regulatory proteins (G proteins) to induce cellular responses (1). Prolonged stimulation of these receptors results in desensitization. Originally, two types of desensitization were described: homologous and heterologous (2). Homologous desensitization is specific for a receptor and its agonist. Heterologous desensitization refers to a process whereby activation of one type of receptor results in the desensitization of different receptors. Homologous desensitization occurs as a result of phosphorylation of the active form of a receptor by a receptor kinase, whereas heterologous desensitization affects active and inactive receptor forms by kinases activated by second messengers (2, 3).

Cross-desensitization studies of chemoattractant receptors using Ca²⁺ mobilization as a measurement of receptor activation have led us to the description of a novel type of desensitization whose specificity falls between heterologous and homologous desensitization (4, 21). This type of desensitization was defined as cross-inhibition of Ca²⁺ mobilization among a particular class of chemoattractant receptors, *i.e.* those for peptide but not for lipid chemotactic factors (4, 21). Other studies have shown that phosphorylation of the cytoplasmic domains of G protein-coupled receptors, followed by their uncoupling from G proteins, can be responsible for desensitization (2, 5). However, experiments in human neutrophils indicated that component(s) distal from receptor/G-protein may also be involved in chemoattractant receptor cross-desensitization (4). To better define the multiple types of receptor desensitization, we developed a model system, a rat basophilic leukemia cell line (RBL-2H3), in which chemoattractant receptors can be expressed and induced to elicit cellular responses similar to those in neutrophils. Using this model, we recently showed that agonist-stimulation of the chemoattractant receptors for fMLP, C5a, IL-8, and platelet-activating factor expressed in RBL-2H3 cells resulted in phosphorylation and desensitization of these receptors (6, 7).² In the present work, we sought to better define the mechanism(s) of cross-desensitization of chemoattractant receptors. For that purpose, chemoattractant receptors were co-expressed in RBL-2H3 cells and studied for their ability to undergo and/or mediate cross-phosphorylation and correlate this with consequent GTP γ S binding, generation of inositol trisphosphates, and mobilization of intracellular calcium. The results presented here demonstrate that receptor phosphoryl-

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¹ The abbreviations used are: C5a, peptide of the fifth component of the complement system; fMLP, formylmethionylleucylphenylalanine;

ET-FR, epitope-tagged fMLP receptor; ET-C5aR, epitope-tagged C5a receptor; IL-8, interleukin-8; ET-IL-8R, epitope-tagged IL-8 receptor; IP₃, inositol 1,4,5-trisphosphate; GTP γ S, guanosine 5'-3'-O-(thio)triphosphate; G protein, GTP-regulatory protein; PKC, protein kinase C; PLC, phospholipase C.

² Richardson, M. R., DuBose, R. A., Ali, H., Tomhave, E., Haribabu, B., and Snyderman, R. (1995) *Biochemistry*, in press.

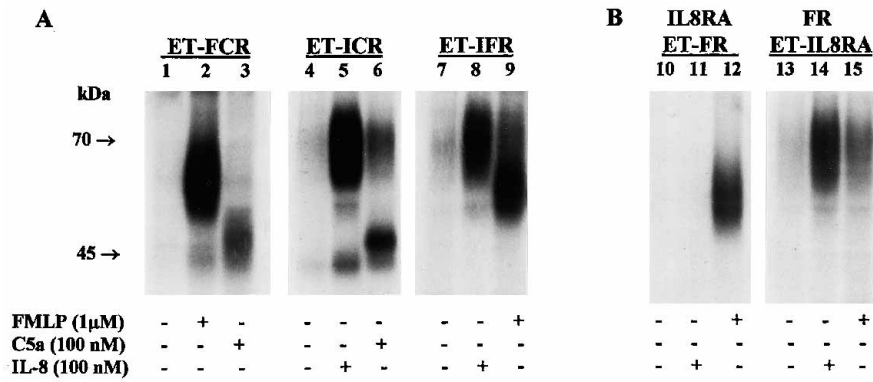


FIG. 1. Immunoprecipitation of epitope-tagged chemoattractant receptors expressed in RBL-2H3 cells. A, ^{32}P -labeled double-transfected RBL-2H3 cells ($2.5 \times 10^6/60\text{-mm}$ plate) expressing epitope-tagged receptors for FR and C5aR (ET-FCR), IL-8RA and C5aR (ET-ICR), or IL-8RA and FR (ET-IFR) 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, and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. This experiment was repeated five times with similar results. B, RBL-2H3 cells expressing either ET-FR and IL-8RA (lanes 10–12) or ET-IL-8RA and FR (lanes 13–15) were stimulated in the presence or absence of either fMLP or IL-8, and receptor phosphorylation was assessed as described above.

ation and modification of a downstream component(s) of the chemoattractants signaling cascade participate in different forms of chemoattractant receptor cross-regulation.

EXPERIMENTAL PROCEDURES

Materials— ^{32}P Orthophosphate (8,500–9,120 Ci/mmol), myo-[2- ^3H]inositol (24.4 Ci/mmol), and ^{35}S GTP γS (1300 Ci/mmol) were purchased from DuPont NEN. ^{125}I -IL-8 and ^3H Inositol 1,4,5-trisphosphate (IP_3) assay kits were from Amersham. IL-8 (monocyte derived) was purchased from Genzyme. Monoclonal 12CA5 antibody was from BabCo. PSV2neo was from American Type Culture Collection. Geneticin (G418) and all tissue culture reagents were purchased from Life Technologies, Inc. Protein G-agarose and protease inhibitors were from Boehringer Mannheim. fMLP, indo-1 acetyloxymethyl ester, and pluronic acid were from Molecular Probes. C5a, GDP, GTP, GTP γS , and ATP were purchased from Sigma. Thapsigargin and ionomycin were from Calbiochem. All other reagents were from commercial sources.

Construction of Epitope-tagged Receptors—Nucleotides encoding a nine-amino acid epitope sequence (YPYDVPDYA) was inserted between the N-terminal initiator methionine and the second amino acid of each cDNA by polymerase chain reaction as described previously (6, 7).²

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 $\mu\text{g}/\text{ml}$) (6). These cells (1×10^7 cells) were transfected by electroporation with either pRK5 or pCDNA3 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), and cell surface expression of the receptors was monitored by fluorescence-activated cell sorter analysis as described previously (6, 7).² For double transfectants, RBL-2H3 cells expressing one receptor were electroporated in the presence of pRK5 vectors (20 μg) containing the second receptor cDNA. Double transfectants were isolated by fluorescence-activated cell sorter using fluorescein isothiocyanate ligand for the second receptor. The receptors co-expressed in RBL-2H3 cells displayed pharmacological and functional properties similar to those obtained with single expression of the receptors (K_d values were ~ 80 nM, ~ 8 pM, ~ 2 nM for ET-FR, ET-C5aR, and ET-IL-8RA, respectively) (6, 7).²

Phosphorylation of the Epitope-tagged Receptors—Phosphorylation of ET receptors was performed as described previously (6, 7).² Briefly, RBL-2H3 cells (2.5×10^6) expressing each 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 $\mu\text{Ci}/\text{dish}$) for 90 min to metabolically label the intracellular ATP pool. Then, labeled cells were 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 with the 12CA5 antibody, analyzed by SDS-electrophoresis, and visualized by autoradiography (6, 7).²

GTP γS Binding—Cells were treated with appropriate concentrations of stimulants, and membranes were prepared as already described (7).² ^{35}S GTP γS binding, using 10–20 μg of membrane preparations,

was carried out as described previously.²

Calcium Measurement—Cells (3×10^6) were removed, washed with HEPES-buffered Hank's balanced salt solution, and loaded with 1 μM indo 1-acetoxymethyl ester in the presence of 1 μM pluronic acid for 30 min at room temperature. Then, 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 (fMLP, 100 nM; C5a, 10 nM; IL-8, 10 nM) was measured as described (6).

Inositol Phosphate Extraction and Measurement—RBL-2H3 cells expressing both ET-FR and ET-C5aR were plated in 60-mm dishes (2.5×10^6 cells/dish) and equilibrated in serum-free medium for 1 h. Cells were then treated with or without agonists in the same medium at 37 °C. The reactions were terminated by addition of an equal volume of ice-cold 15% (w/v) trichloroacetic acid, and the samples were kept on ice for 30 min. Inositol phosphates were extracted three times with 10 volumes of water-saturated diethyl ether and neutralized to pH 7.5 with 1 M NaHCO_3 (9). 50 μl of each extract was used to determine the IP_3 mass using the Amersham radioreceptor binding assay kit.

RESULTS

Phosphorylation and Immunoprecipitation of ET Receptors in RBL-2H3 Cells—RBL-2H3 cells expressing epitope-tagged receptors for FR and C5aR, IL-8RA and C5aR, or FR and IL-8RA were ^{32}P labeled and treated with different ligands (1 μM fMLP, 0.1 μM C5a, or 0.1 μM IL-8) and immunoprecipitated with 12CA5 antibody. As shown in Fig. 1, ET-C5aR (~ 45 kDa) was phosphorylated by C5a (lanes 3 and 6) and cross-phosphorylated by fMLP (lane 2) and IL-8 (lane 5). ET-IL-8RA (~ 70 kDa) was phosphorylated by IL-8 (lanes 5 and 8) and cross-phosphorylated by C5a (lane 6) and fMLP (lane 9). In contrast, ET-FR (~ 65 kDa) was phosphorylated by fMLP (lanes 2 and 9) but resistant to cross-phosphorylation by either C5a (lane 3) or IL-8 (lane 8) stimulation. The identity of these phosphorylated bands as the respective receptors has been previously demonstrated by immunoprecipitation of iodinated and phosphorylated receptors in the presence and absence of the epitope tag peptide (6).² Since ET-IL-8RA and ET-FR migrate as broad overlapping bands in these SDS gels, double-transfected RBL-2H3 cell lines with one wild type and the other epitope-tagged receptors were prepared to more clearly resolve cross-phosphorylation of these two receptors. As shown in Fig. 1B, RBL-2H3 cells expressing ET-IL-8RA and wild type FR and *vice versa* showed similar results to ET-IFR cells when stimulated with either IL-8 (0.1 μM) (Fig. 1B, lanes 11 and 14) or fMLP (1 μM) (Fig. 1B, lanes 12 and 15).

We also determined whether ligand cross-reactivity could result in an apparent receptor cross-phosphorylation. Single transfected RBL-2H3 cells expressing either ET-C5aR or ET-IL-8RA were ^{32}P labeled, treated with C5a (0.1 μM), IL-8

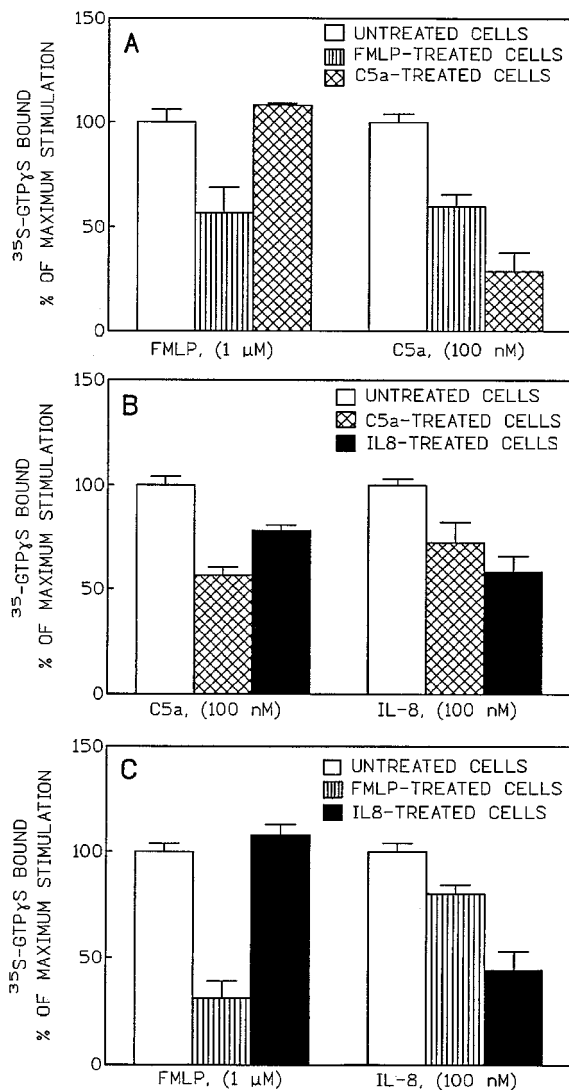


FIG. 2. Homologous and cross-desensitization of peptide chemoattractant receptors stimulated [35 S]GTP γ S binding. Double-transfected RBL-2H3 cells were treated with fMLP (1 μ M), C5a (100 nM), or IL-8 (100 nM) for 5 min. Membranes were prepared and assayed for agonist-stimulated [35 S]GTP γ S binding. 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 of [35 S]GTP γ S bound to control membranes (untreated cells) after 10 min of reaction. Basal activities were \sim 0.2–0.3 pmol of [35 S]GTP γ S bound/mg of protein. Maximum stimulation was 0.23 ± 0.008 (fMLP) and 0.19 ± 0.010 (C5a) pmol of [35 S]GTP γ S bound/mg of protein for untreated ET-FCR cells (panel A), 0.2 ± 0.011 (C5a) and 0.21 ± 0.02 (IL-8) pmol of [35 S]GTP γ S bound/mg of protein for untreated ET-ICR cells (panel B), and 0.19 ± 0.008 (fMLP) and 0.22 ± 0.0132 (IL-8) pmol of [35 S]GTP γ S bound/mg of protein for untreated ET-IFR cells (panel C). Specific activity was \sim 380–450 cpm/fmol of GTP γ S.

(0.1 μ M), or fMLP (1 μ M), and immunoprecipitated. Only homologous phosphorylation was observed for each receptor (*i.e.* ET-C5aR only by C5a and ET-IL-8RA only by IL-8) (data not shown), indicating that ligand cross-reactivity does not occur in these receptors.

GTP γ S Binding in Cross-desensitized Membranes—To determine the effect of cross-phosphorylation in receptor cross-desensitization, agonist-stimulated [35 S]GTP γ S binding was measured in membranes prepared from double transfectant RBL-2H3 cells pretreated with fMLP (1 μ M), C5a (100 nM), or IL-8 (100 nM). As shown in Fig. 2, pretreatment of cells with fMLP (A and C), C5a (A and B), or IL-8 (B and C) resulted in

homologous desensitization (50–70%) of agonist-induced [35 S]GTP γ S binding to membranes. Membranes from cells pretreated with fMLP (ET-FCR (Fig. 2A) and ET-IFR (Fig. 2C)) showed a \sim 40 and \sim 22% decrease in C5a and IL-8-stimulated [35 S]GTP γ S binding, respectively, whereas no decrease was observed for fMLP stimulation in cells pretreated with either C5a or IL-8 (Fig. 2, A and C). Both, ET-C5aR and ET-IL-8RA cross-desensitized (\sim 28 and \sim 25%, respectively) each other's ability to stimulate [35 S]GTP γ S binding in ET-ICR cells pretreated with either C5a or IL-8 (Fig. 2B).

Cross-desensitization of Receptor-induced Ca $^{2+}$ Mobilization—Ca $^{2+}$ mobilization was also measured to determine the relationship between cross-phosphorylation and cross-desensitization of receptor-mediated cellular responses. As shown in Fig. 3, all three receptors induced Ca $^{2+}$ mobilization in response to agonist stimulation. Ca $^{2+}$ mobilization in response to an EC $_{100}$ dose of fMLP (100 nM), C5a (10 nM), or IL-8 (10 nM) was homologically desensitized by a first dose of the same ligand (data not shown). C5a-induced Ca $^{2+}$ mobilization was cross-desensitized by pretreatment of the cells with a first dose of either fMLP (Fig. 3A) or IL-8 (Fig. 3B). IL-8-induced Ca $^{2+}$ mobilization was similarly cross-desensitized by pretreatment of the cells with a first dose of either C5a (Fig. 3B) or fMLP (Fig. 3C). However, in contrast to fMLP-stimulated [35 S]GTP γ S binding, fMLP-induced Ca $^{2+}$ mobilization was also cross-desensitized by pretreatment of the cells with a first dose of either C5a (Fig. 3A) or IL-8 (Fig. 3C).

It was determined whether a depletion of the intracellular calcium pool caused by the first ligand could account for the attenuation of Ca $^{2+}$ mobilization in response to a second stimuli. Treatment of ET-FCR cells with 2 μ M thapsigargin before stimulation (301 ± 21 nM), 3 min after the first ligand (362 ± 13 nM) and after the second ligand (388 ± 17 nM), followed by 10 μ M ionomycin (621 ± 76 nM) showed no significant change in the intracellular Ca $^{2+}$ pool. These results indicate that the cross-desensitization of receptor-mediated Ca $^{2+}$ mobilization was not due to an impairment of the intracellular Ca $^{2+}$ storage.

Cross-desensitization of Receptor-mediated IP $_3$ Generation—The ability of fMLP and C5a to stimulate PIP $_2$ hydrolysis in control *versus* desensitized cells was determined by measuring the intracellular concentration of IP $_3$. As shown in Fig. 4, pretreatment of RBL-2H3 cells expressing both fMLPR and C5aR with an EC $_{100}$ dose of either fMLP (1 μ M) or C5a (100 nM) decreased by 85–95% the ability of the receptors to mediate intracellular increase of IP $_3$ levels.

DISCUSSION

Despite a large body of evidence indicating that chemoattractant-mediated inflammatory responses are regulated by desensitization, little is known about the molecular events governing this process. Wilde *et al.* (10) reported that C5a-stimulated GTPase activity was desensitized in membranes from neutrophils pretreated with fMLP. These results were confirmed by our previous work, which further indicated that exposure of neutrophils to fMLP cross-desensitized C5a, IL-8, platelet-activating factor, and leukotriene B mediated-GTP γ S binding in membranes. In contrast, receptors for formylpeptide were resistant to this type of cross-desensitization due presumably to the absence of the necessary phosphorylation site (see below) (4). Heterologous phosphorylation of chemoattractant receptors by second messenger-activated kinases (such as PKC) followed by their uncoupling from G protein has been thought to be the molecular mechanism responsible for chemoattractant cross-desensitization (4, 6). However, Tardif *et al.* (11) reported that fMLP stimulation of HL-60 cells did not induce C5aR phosphorylation. The data presented in the work reported here clearly

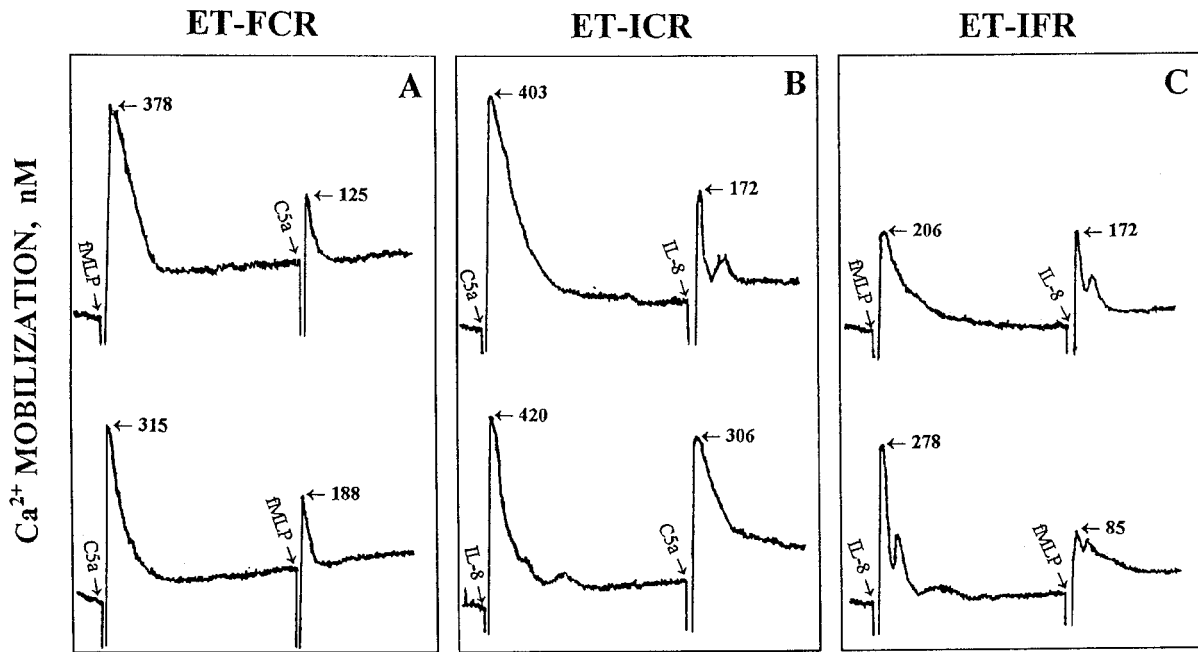


FIG. 3. Cross-desensitization of chemoattractant receptors mediated Ca^{2+} mobilization. Double-transfected RBL-2H3 cells (3×10^6 cells/assay) were loaded with indo-1 and stimulated with fMLP (100 nM), C5a (10 nM), or IL-8 (10 nM). Cells were rechallenged 3 min later with the same concentration of ligand. Traces are representative of three experiments.

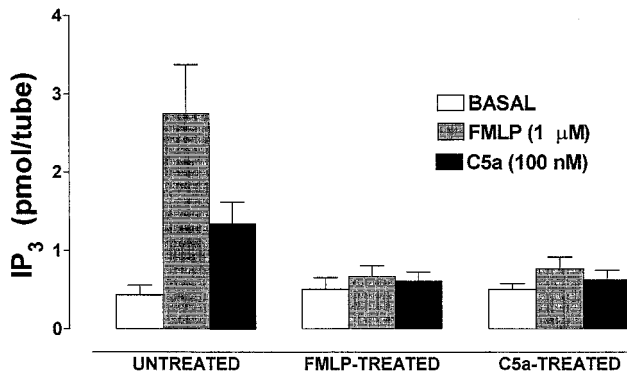


FIG. 4. Measurement of IP_3 concentration in control and desensitized RBL-2H3 cells. RBL-2H3 cells (2.5×10^6 cells) expressing epitope-tagged FR and C5aR were treated with $1 \mu\text{M}$ fMLP (fMLP-treated), 100 nM C5a (C5a-treated), or in the absence of stimulants (untreated cells) for 10 min at 37°C in serum-free medium. Cells were then rechallenged for 10 s with fMLP, C5a, or buffer, and IP_3 was extracted as described under "Experimental Procedures." The IP_3 concentration in each extract was determined using the IP_3 ^3H assay system from Amersham. Data are means \pm S.E. of four separate determinations performed in duplicate.

indicate that fMLP stimulation resulted in phosphorylation of both C5aR and IL-8RA in double-transfected RBL-2H3 cells (Fig. 1), and both C5a and IL-8-mediated $\text{GTP}\gamma\text{S}$ binding were desensitized under such conditions. The failure of Tardif *et al.* (11) to find such cross-phosphorylation in HL-60 cells is not understood.

The extent of fMLP-mediated phosphorylation of ET-C5aR and ET-IL-8RA mirrored the ones previously obtained upon exposure of these receptors to the protein kinase C activator, phorbol 12-myristate 13-acetate (6, 7).² These results suggest that fMLP cross-desensitization of ET-C5aR and ET-IL8RA, as well as C5a of ET-IL8RA and IL-8 of ET-C5aR, may be mediated by receptor phosphorylation by PKC. Indeed, the PKC inhibitor staurosporine inhibited fMLP-mediated phosphorylation of ET-C5aR in the ET-FCR cell line (data not shown). fMLP has been shown to increase PKC activity in neutrophils

and several other cell lines (12–14). Molecular cloning has revealed that the receptor for fMLP lacks sequence motif for PKC phosphorylation (RXXSXXR). This likely explains its resistance to PKC-mediated phosphorylation (15). Neither C5a nor IL-8 pretreatment resulted in cross-desensitization of fMLP-mediated $\text{GTP}\gamma\text{S}$ binding, which correlated with its resistance to cross-phosphorylation. Taken together, these results are in agreement with the current concept that receptor phosphorylation leads to desensitization and indicate that PKC-mediated phosphorylation results in one form of chemoattractant receptor cross-desensitization at the level of receptor/G protein activation.

Interestingly, receptor cross-phosphorylation cannot explain the cross-desensitization of formylpeptide receptor-mediated Ca^{2+} mobilization by other chemoattractants since the formylpeptide receptors are totally resistant to the heterologous phosphorylation. Thus, the formylpeptide receptors provide an important tool to determine the downstream site(s) for chemoattractant receptor cross-desensitization. The chemoattractant receptors studied here are coupled to phospholipase C and mediate intracellular signals via stimulation of phosphatidylinositol hydrolysis and production of IP_3 and diacylglycerol (1). It has been shown that IP_3 plays a pivotal role in stimulating intracellular Ca^{2+} mobilization (16). Thus, cross-desensitization of fMLP-mediated Ca^{2+} mobilization could be at the level of PIP_2 hydrolysis or IP_3 activity. Indeed, cAMP-mediated phosphorylation of the receptor for intracellular generated IP_3 markedly decreases its ability to stimulate Ca^{2+} release (17). Therefore, the possibility existed that cross-desensitization of fMLP-induced intracellular Ca^{2+} mobilization reflected either a decrease in the level of intracellular IP_3 produced or desensitization of the receptor for IP_3 . As shown in fig. 4, fMLP stimulated IP_3 production was decreased by $\sim 90\%$ in cells pretreated with C5a. These results indicate that the cross-desensitization of Ca^{2+} mobilization in response to fMLP by the other chemoattractants is likely due to a decrease in the level of IP_3 production. There are several possible explanations for diminished IP_3 production. A depletion of the pool of PIP_2 prior hydrolysis or stimulation of phosphatidylinositol-3 kinase ac-

tivity is one explanation. Against this hypothesis is that pretreatment of neutrophils with C5a decreased fMLP-induced IP₃ production with no significant change in the level of PIP₂ (18). Moreover, in neutrophils that have been cross-desensitized by fMLP, purinergic receptor ability to stimulate PLC and Ca²⁺ release is normal, indicating adequate IP₃ receptor and PIP₂ level (4). A second explanation for diminished IP₃ production is a decrease in the catalytic activity of the phospholipase C either by modification of the enzyme or its activating components. Both α and $\beta\gamma$ subunits of G protein have been shown to activate PLC in reconstituted systems (19). Chemoattractant receptors couple to G_i and mediate PLC β_2 activation via G $\beta\gamma$ subunits (1, 19). γ subunits are known to be isoprenylated and methylated (20). It has recently been shown that demethylation of the $\beta\gamma$ subunit, which does not affect receptor-mediated GTP γ S binding to G protein, caused a 10-fold decrease in $\beta\gamma$ -mediated activation of PLC and, thus, production of IP₃ (8). Therefore, it is possible that C5a and IL-8-mediated cross-desensitization of FR-induced Ca²⁺ mobilization may be due to either a demethylation or other modification of $\beta\gamma$ subunits, rendering them less effective in activating PLC. Modification of PLC itself could also result in its diminished activity. In any case, the cross-desensitization of formylpeptide receptor as presented is likely due to a modification in its ability to activate PLC. C5a- and IL-8-induced Ca²⁺ mobilization and IP₃ production are also inhibited in cells pretreated with fMLP. Since all three chemoattractant receptors studied here apparently utilize the same signal transduction pathways, the downstream effect observed with the fMLP receptor likely plays a role in the attenuation of C5a- and IL-8-induced responses in addition to the impairment of receptor/G protein coupling due to receptor phosphorylation.

In summary, we have developed a system to stably co-express two G protein-coupled receptors and study their cross-regulation. The results presented herein indicate that chemoattractant receptor-mediated inflammatory response are regulated at multiple sites. One is at the level of receptor phosphorylation affecting receptor/G protein coupling. The sec-

ond is at a site distal to that, presumably involving the activity of phospholipase C. Cross-desensitization at different levels of the signaling cascades may be used by the receptors to control each other's activity at sites of inflammation where multiple chemoattractants are present. It will be important to determine if receptor cross-desensitization at the level of PLC occurs more generally than the subgroup of chemoattractant receptors studied here.

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