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Deficient Homologous Desensitization of Formyl Peptide Receptors Stably Expressed in Undifferentiated HL-60 Cells

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ABSTRACT. The ability of formyl peptide receptors (FPRs) stably expressed in undifferentiated HL-60 cells to undergo ligand-induced desensitization was compared with their ability in normal and vector-transfected HL-60 cells following granulocyte differentiation with DMSO. fMet-Leu-Phe failed to induce uncoupling of FPRs from G-proteins in FPR-transfected cells, whereas uncoupling was induced in differentiated HL-60 cells and differentiated vector-transfected HL-60 cells, as determined by ligand-stimulated guanosine 5′-(γ-thio)triphosphate (GTPγS) binding and GTPγS inhibition of fMet-Leu-Phe binding to isolated membranes. Immunoprecipitation of $G\alpha_{i2}$ from solubilized, azidoanalide (AA-γGTP) photolabeled membranes showed that receptors in desensitized FPR-transfected HL-60 cells remained coupled to $G\alpha_{i2}$, whereas desensitized receptors in differentiated HL-60 cell membranes were uncoupled from $G\alpha_{i2}$. As determined by immunoblotting, $G\alpha_{i2}$ expression was similar in undifferentiated and differentiated HL-60 cells and FPR-transfected cells. Ligand-stimulated receptor internalization and desensitization of calcium redistribution were similar in all three groups of cells. Immunoblotting also indicated that G-protein-coupled receptor kinases (GRKs) 2 and 4 were present in undifferentiated FPR-transfected HL-60 cells at 50% of the level seen in differentiated HL-60 cells. However, differentiation did not increase GRK2 or GRK4 expression, indicating that differences in GRK expression do not explain deficient desensitization. The data indicated that undifferentiated HL-60 cells are unable to induce homologous desensitization of FPRs. BIOCHEM PHARMACOL **60**;2:179–187, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. desensitization; receptors; HL-60 cells; formyl peptide; G-proteins

PMNs¶ play an essential role in the elimination of microbes through a highly regulated series of events, including migration from the vascular space to a site of inflammation and generation of reactive oxygen metabolites and microbicidal proteins. These actions are stimulated by a diverse group of extracellular ligands termed chemoattractants, which include formylated peptides, C5a, platelet-activating factor, leukotriene B_4, and CXC chemokines [1, 2]. Desensitization of chemoattractant receptors limits PMN damage to normal tissue and is associated with receptor uncoupling from G-proteins and reduced plasma membrane expression [3, 4].

All chemoattractant receptors are predicted to possess 7 transmembrane spanning regions connected by 3 intracellular loops and 3 extracellular loops, and an intracellular C-terminal tail typical of the large superfamily of G-protein-coupled receptors [1, 5–7]. Substantial evidence indicates that phosphorylation of multiple serines and threonines in the C-terminal tail of chemoattractant receptors by GRKs is required for desensitization [8–13]. FPRs are phosphorylated by GRK2 and GRK3, whereas GRK5 and GRK6 have no activity [13]. Additional mechanisms of desensitization also have been proposed. For example, activation of protein kinase C uncouples some chemoattractant receptors from phospholipase C activation, possibly by phosphorylation of phospholipase Cβ3 [9, 14–19] or phosphorylation of chemoattractant receptors themselves [9, 20–22].

The stable expression of functionally competent FPRs in U937 cells and undifferentiated HL-60 cells has been accomplished [23, 24]. FPRs expressed in undifferentiated U937 cells demonstrate normal G-protein-mediated signaling and undergo ligand-initiated desensitization [23]. FPRs expressed in undifferentiated HL-60 cells also demonstrate G-protein-mediated signaling [24]; however, desensitization in this model has not been documented previously.

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¶ *Abbreviations*: PMN, polymorphonuclear leukocyte; GTPγS, guanosine 5′-(γ-thio)triphosphate; AA-GTP, [γ-^{32}P]GTP azidoanalide; FPR, formyl peptide receptor; PMA, 4β-phorbol-12β-myristate-13γ-acetate; and GRK, G-protein-coupled receptor kinase.

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The present studies were designed to examine the ability of FPRs stably expressed in undifferentiated HL-60 cells to undergo homologous desensitization.

MATERIALS AND METHODS

Reagents

fMet-Leu-Phe was obtained from the Sigma Chemical Co. GTPγS was obtained from Boehringer Mannheim. Fura-2 AM was obtained from Calbiochem. [^{35}S]GTPγS and fMet-Leu-[^{3}H]Phe were purchased from DuPont-New England Nuclear. Antisera to α_{i2} were produced in rabbits by immunization with a synthetic peptide representing the terminal decapeptide of α_t (KENLKDCGLF). Antisera to this peptide recognize α_{i1} and α_{i2} [25], but human PMNs do not express α_{i1} [26].

Transfection of FPRs into HL-60 Cells

HL-60 cells were transfected with FPRs using the vector pSSFV.neo by electroporation, and transfected cells were selected by cultivation in G418, as previously described [24].

Cell Culture

HL-60 cells, obtained from the American Type Culture Collection, were grown in suspension culture in RPMI 1640 medium supplemented with 10% (v/v) horse serum, 1% (v/v) non-essential amino acids, 1 mM l-glutamine, 50 U/mL of penicillin, and 50 μg/mL of streptomycin in a humidified atmosphere with 8% CO_2 at 37°. To induce myeloid differentiation, cells were seeded at a density of 10^6 cells/mL and cultivated for 5 days in medium containing 1.25% (v/v) DMSO [3, 27]. HL-60 cell membranes were prepared by nitrogen cavitation and stored at −70° until used, as previously described [27].

GTPγS Binding Assay

GTPγS binding was performed as described previously [27]. Specific binding was calculated by subtracting the amount of [^{35}S]GTPγS bound in the presence of 10 μM GTPγS from total [^{35}S]GTPγS bound and expressed as femtomoles of GTPγS bound per milligram of membrane protein.

Receptor Binding Assay

fMet-Leu-Phe binding assays were performed in a reaction mixture (100 μL) containing 50 mM Tris (pH 7.5), 1 mM EDTA, 5 mM $MgCl_2$, as previously described [27]. Binding parameters were estimated using a nonlinear least squares curve fitting procedure (SCTFIT), as previously described [27].

Flow Cytometric Assay for FPRs

Binding of N-formyl-Nleu-Leu-Phe-Nleu-Tyr-Lys-fluorescein to FPRs was performed as previously described [24]. Specificity of binding sites was determined by the addition of 10 μM fMet-Leu-Phe. The cells were analyzed by flow cytometry (Epics Profile II, Coulter). Quantum 24 fluorescein microbeads (Flow Cytometry Standards Corp.) containing 4×10^3 to 6.6×10^4 molecules of equivalent soluble fluorochromes were used as standards to determine the average number of FPRs per cell. Data were stored and analyzed using WinList 3.0 (Verity Software House, Inc.).

Photoaffinity Labeling of Plasma Membrane G Proteins

Photoaffinity labeling of G proteins from plasma membranes (200 μg/condition) with AA-GTP was performed as previously described [28].

Intracellular Calcium

Intracellular calcium was measured using the fluorescent indicator Fura-2 as previously described [14]. The 350/380 nm fluorescence ratio was determined on suspensions of Fura-2-loaded cells with an emission frequency of 510 nm using an Aminco-Bowman ratio spectrofluorimeter. At the end of the experiment, the cells were treated with Triton X-100 to determine the maximum 350/380 nm fluorescence ratio, followed by the addition of the calcium chelator EGTA to determine the minimum ratio. Intracellular calcium was calculated from these values according to the equation of Grynkiewicz *et al.* [29] using a K_d of 224 nM.

Immunoblotting for GRKs

Immunoblotting was performed as previously described [30], using antisera toward $G\alpha_{i2}$, GRK2, GRK3, GRK4, GRK5, and GRK6 (Santa Cruz Biotechnology, Inc.). Proteins were separated by 10% SDS–PAGE, transferred onto nitrocellulose, and blotted with $G\alpha_{i2}$ or GRK antisera. Immunoreactivity was localized by enhanced chemiluminescence (Renaissance, Dupont-NEN).

RESULTS

We reported previously that a 10-min exposure of differentiated HL-60 cells to 10^{-7} M fMet-Leu-Phe resulted in desensitization of FPRs, including uncoupling of the receptor–G-protein interaction, reduction in membrane expression of receptors, and loss of functional responses to fMet-Leu-Phe [3]. Therefore, this concentration and time of exposure to fMet-Leu-Phe were used throughout these studies.

The expression of FPRs in transfected HL-60 cells before and after desensitization with 10^{-7} M fMet-Leu-Phe was determined by two separate methods. First, receptor expression was determined in intact, differentiated HL-60 cells or

undifferentiated FPR-transfected HL-60 cells by a flow cytometric assay using N-formyl-Nleu-Leu-Phe-Nleu-Tyr-Lys-fluorescein. FPRs were present on at least 70% of cells from both groups. Desensitization reduced FPR expression on differentiated HL-60 cells from 7700 ± 3100 receptors per cell to 2340 ± 420 receptors per cell, a 59% reduction (N = 4, mean ± SEM). Desensitization reduced FPR expression on undifferentiated FPR-transfected HL-60 cells from 2230 ± 410 receptors per cell to 550 ± 230 receptors per cell, a 69% reduction (N = 7, mean ± SEM). Second, FPR density was determined by radioligand saturation binding in membranes isolated from normal or desensitized undifferentiated FPR-transfected HL-60 cells. The B_{max} of fMet-Leu-Phe binding sites was 2.86 pmol/mg with a K_d of 3.5 nM in normal membranes (mean, N = 2), while the B_{max} and K_d for desensitized membranes were 1.01 pmol/mg and 4.4 nM, respectively (mean, N = 2), a 65% reduction in B_{max} in desensitized membranes. Thus, exposure to fMet-Leu-Phe reduced FPR density in both differentiated HL-60 cells and undifferentiated FPR-transfected HL-60 cells, whereas receptor affinity was not reduced in FPR-transfected HL-60 cells.

Previous studies indicate that desensitization uncouples FPRs from G-proteins, resulting in loss of ligand-stimulated GTPγS binding and GTPase activity and the failure of guanine nucleotides to reduce ligand binding [3, 4]. Therefore, receptor–G-protein coupling was examined in normal and desensitized membranes from undifferentiated FPR-transfected HL-60 cells and from differentiated HL-60 cells stably transfected with the pSSFV.neo vector. The vector-transfected cells were used as a control to eliminate the possibility of the vector interfering with the desensitization process. Figure 1 shows that fMet-Leu-Phe stimulated a concentration-dependent increase in GTPγS binding in normal membranes from both types of cells. Whereas fMet-Leu-Phe-stimulated GTPγS binding to desensitized membranes from differentiated vector-transfected HL-60 cells was impaired markedly (Fig. 1B), desensitized membranes from FPR-transfected HL-60 cells showed an fMet-Leu-Phe-stimulated increase in GTPγS binding similar to that in normal membranes (Fig. 1A). Figure 2 shows that GTPγS inhibited fMet-Leu-Phe binding to normal membranes from both FPR-transfected HL-60 and differentiated vector-transfected HL-60 cells. GTPγS inhibition of fMet-Leu-Phe binding to desensitized membranes from differentiated vector-transfected HL-60 cells was attenuated markedly (Fig. 2B), whereas GTPγS inhibition of fMet-Leu-Phe binding to desensitized membranes from FPR-transfected cells remained intact (Fig. 2A). Thus, exposure to fMet-Leu-Phe failed to uncouple FPRs from G-proteins in undifferentiated FPR-transfected HL-60 cells, whereas FPRs in differentiated vector-transfected cells underwent typical desensitization. $G\alpha_{i2}$ protein expression in membranes from undifferentiated HL-60 cells, differentiated HL-60 cells, and FPR-transfected HL-60 cells was determined by immunoblotting. $G\alpha_{i2}$ protein expression levels were similar in all three groups, indicating that differences in receptor–G-

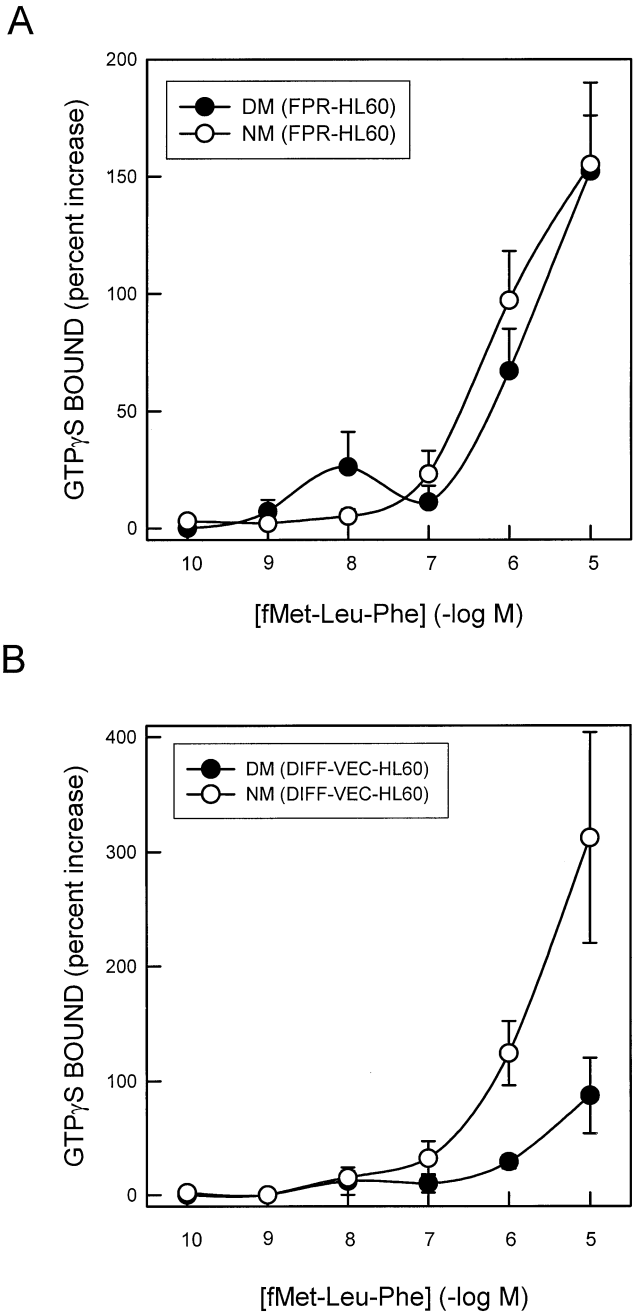

FIG. 1. Effect of desensitization of G-protein activation in FPR-transfected HL-60 cells and differentiated vector-transfected cells. Panel A shows the ability of fMet-Leu-Phe to stimulate GTPγS binding to normal membranes (NM) and desensitized membranes (DM) from FPR-transfected HL-60 cells (FPR-HL-60). Results are the means ± SEM of six separate experiments in three different membrane preparations, expressed as the percent increase in GTPγS binding over basal levels. Basal values for NM and for DM were 186 ± 28 (mean ± SEM) and 187 ± 36 fmol/mg GTPγS bound, respectively. Panel B shows fMet-Leu-Phe stimulation of GTPγS binding to NM and DM from differentiated HL-60 cells that were transfected with pSSFV.neo vector alone (Diff-Vec-HL-60). Results are the means ± SEM of three separate experiments in two different membrane preparations, expressed as percent increase in GTPγS binding over basal levels. Basal values for NM and for DM were 205 ± 58 and 196 ± 56 fmol/mg GTPγS bound, respectively.

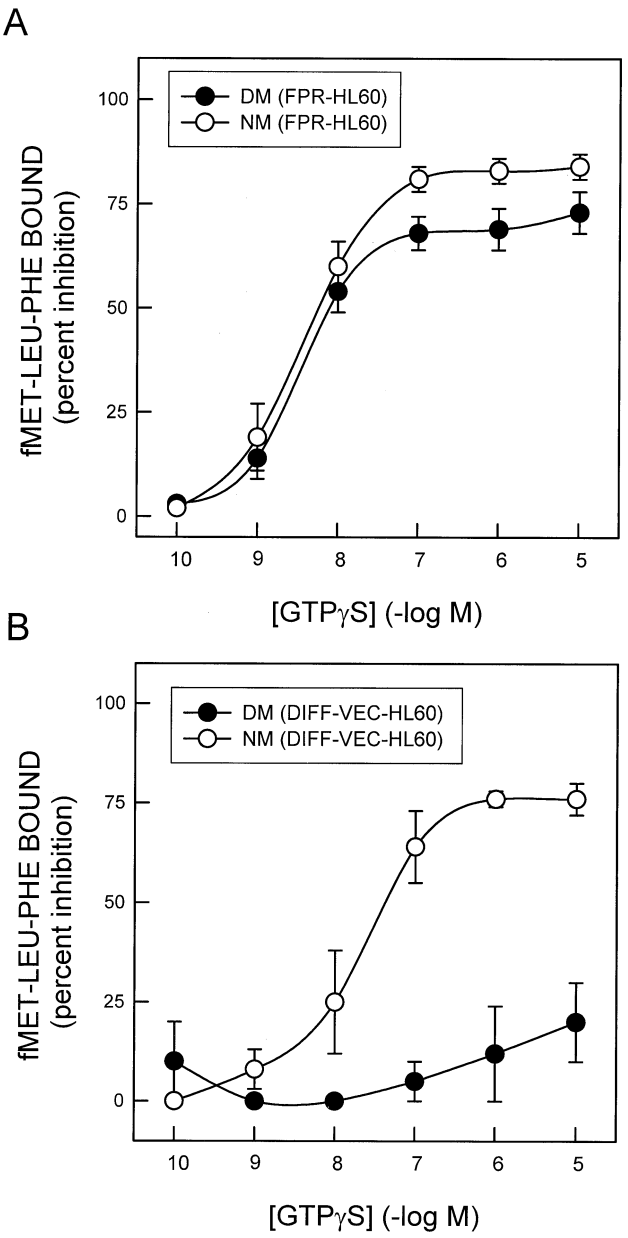

FIG. 2. Effect of desensitization on GTPγS inhibition of fMet-Leu-Phe binding in FPR-transfected HL-60 cells and differentiated vector-transfected HL-60 cells. Panel A shows the response of fMet-Leu-Phe binding to increasing concentrations of GTPγS in normal membranes (NM) and desensitized membranes (DM) from FPR-transfected HL-60 cells (FPR-HL-60). Results represent the means ± SEM of seven separate experiments in three different membrane preparations, expressed as percent inhibition of fMet-Leu-Phe bound compared with that in the absence of GTPγS. Basal values for NM and for DM were 534 ± 176 (mean ± SEM) and 454 ± 134 fmol/mg of fMet-Leu-Phe bound, respectively. Panel B shows the response of fMet-Leu-Phe binding to increasing concentrations of GTPγS in NM and DM from differentiated HL-60 cells that were transfected with pSSFV.neo vector alone (Diff-Vec-HL-60). Results represent the means ± SEM of three separate experiments in two different membrane preparations expressed as percent inhibition of fMet-Leu-Phe bound compared with that in the absence of GTPγS. Basal values for NM and for DM were 244 ± 38 and 131 ± 17 fmol/mg of fMet-Leu-Phe bound, respectively.

protein uncoupling were not due to differences in G-protein expression (data not shown).

Photoaffinity labeling with AA-GTP was performed to determine if FPRs expressed by undifferentiated HL-60 cells couple to pertussis toxin-sensitive G_i proteins, as has been shown for differentiated HL-60 cells. Membranes from FPR-transfected HL-60 cells were treated with or without 10^{-5} M fMet-Leu-Phe prior to photolabeling. As shown in Fig. 3A, fMet-Leu-Phe stimulated increased labeling of a protein at 42 kDa in intact membranes. Immunoprecipitation of $G\alpha_{i2}$ from solubilized membranes showed that fMet-Leu-Phe stimulated increased photolabeling of $G\alpha_{i2}$ in FPR-transfected cells. Immunoprecipitation of $G\alpha_{q/11}$, $G\alpha_{12}$, and $G\alpha_{13}$ failed to show any labeling in the presence or absence of fMet-Leu-Phe (data not shown).

To determine if fMet-Leu-Phe desensitization uncouples FPRs from $G\alpha_{i2}$ in FPR-transfected HL-60 and differentiated HL-60 cells, photoaffinity labeling of normal and desensitized membranes from these cells was performed. Desensitized membranes from differentiated HL-60 cells demonstrated no increase in photolabeling of $G\alpha_{i2}$ in the presence of fMet-Leu-Phe. On the other hand, fMet-Leu-Phe stimulated increased photolabeling in desensitized membranes from FPR-transfected cells (Fig. 3B). These results confirm that exposure to fMet-Leu-Phe fails to uncouple the FPR-$G\alpha_{i2}$ interaction in FPR-transfected HL-60 cells.

Possible explanations for the failure of FPR–G-protein uncoupling in undifferentiated FPR-transfected HL-60 cells include an inability of transfected receptors to undergo desensitization or the absence of receptor-specific kinases. To determine if differentiation of FPR-transfected cells permitted normal desensitization of transfected FPRs, normal and desensitized membranes were prepared after DMSO differentiation for 5 days. As shown in Fig. 4, desensitized membranes from differentiated, FPR-transfected HL-60 cells demonstrated a marked attenuation of fMet-Leu-Phe-stimulated GTPγS binding, a reduction in basal fMet-Leu-Phe binding, and a loss of GTPγS inhibition of fMet-Leu-Phe binding. Although these cells possess both FPRs induced by differentiation and FPRs expressed following transfection, the marked attenuation of responses suggests that desensitization of both types of receptors occurred.

The possibility that GRKs necessary to induce desensitization are not present was examined by immunoblotting for GRK2, GRK3, GRK4, GRK5, and GRK6. Figure 5 shows immunoblots of GRK2 and GRK4 in lysates from undifferentiated FPR-transfected HL-60 cells, differentiated FPR-transfected HL-60 cells, and differentiated HL-60 cells. GRK2 and GRK4 expression was detected in all three cell types; however, expression was reduced by 50% in differentiated and undifferentiated FPR-transfected HL-60 cells. GRK3, GRK5, and GRK6 were not detected.

Previous reports suggest that another mechanism of desensitization of chemoattractant receptors results from protein kinase C-dependent uncoupling of receptors from

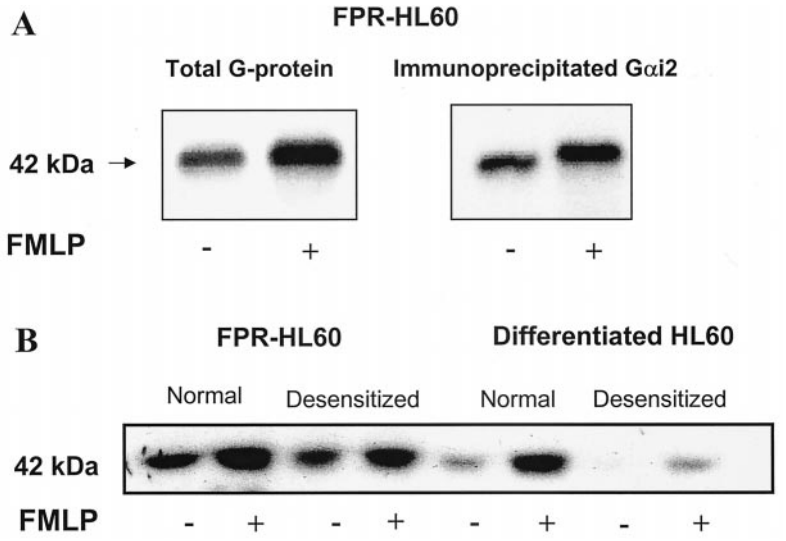

FIG. 3. AA-GTP photolabeling of G-proteins in membranes of FPR-transfected HL-60 cells and differentiated HL-60 cells. Panel A shows autoradiograms of AA-GTP photolabeled G-proteins in solubilized membranes from receptor-transfected HL-60 cells. Photolabeling of intact membranes was performed in the presence or absence of 1×10^{-5} M fMet-Leu-Phe prior to solubilization. Proteins in solubilized membranes were separated by 10% SDS–PAGE (total G-protein), or $G\alpha_{i2}$ was immunoprecipitated from solubilized membranes as described in Materials and Methods and 10% SDS–PAGE was performed. Densitometric analysis of these autoradiograms showed a 72% increase in photolabeling of total G-proteins following fMet-Leu-Phe stimulation and a 54% increase in photolabeling of $G\alpha_{i2}$ following fMet-Leu-Phe stimulation. Panel B shows an autoradiogram of AA-GTP-photolabeled G-proteins in solubilized normal and desensitized membranes from receptor-transfected and differentiated HL-60 cells. Plasma membranes were incubated in the presence or absence of 1×10^{5} M fMet-Leu-Phe prior to solubilization. $G\alpha_{i2}$ was immunoprecipitated from solubilized membranes as described in Materials and Methods, and 10% SDS–PAGE was performed. Results are representative of two separate experiments.

phospholipase C activation and calcium mobilization [14–17]. Therefore, we examined desensitization of the calcium transient response to fMet-Leu-Phe in FPR-transfected HL-60 cells and differentiated HL-60 cells. Figure 6 shows that fMet-Leu-Phe and ATP stimulated similar calcium transients in both groups of cells. Pretreatment with 10^{-7} M fMet-Leu-Phe for 10 min at 37° resulted in loss of the fMet-Leu-Phe- but not the ATP-stimulated calcium transient in both FPR-transfected cells and differentiated HL-60 cells. We have established previously that protein kinase C activation by PMA desensitizes the fMet-Leu-Phe-stimulated calcium transient in differentiated HL-60 cells without uncoupling FPRs from G proteins [14]. To determine if protein kinase C desensitized FPR-transfected HL-60 cells, FPR-transfected cells were incubated with 10 nM PMA for 10 min prior to stimulation with fMet-Leu-Phe or ATP. Pretreatment with PMA inhibited the calcium response to both fMet-Leu-Phe and ATP (Fig. 7). We also compared ligand-stimulated GTPγS binding in PMA-desensitized, fMet-Leu-Phe-desensitized, and normal FPR-transfected HL-60 membranes. fMet-Leu-Phe stimulated a concentration-dependent increase in GTPγS binding in all three membranes (Fig. 8). Thus, the ability of FPRs to stimulate calcium mobilization was desensitized following PMA treatment in the absence of receptor–G-protein uncoupling.

DISCUSSION

Chemoattractant receptors expressed by differentiated HL-60 granulocytes stimulate G-protein-coupled responses similar to those seen in human neutrophils, including homologous desensitization [3, 8, 14, 15, 27, 28]. Our results and a previous report showed that FPRs overexpressed in undifferentiated HL-60 cells stimulate many of the same responses, including fMet-Leu-Phe-stimulated GTPγS binding, guanine nucleotide inhibition of fMet-Leu-Phe binding, fMet-Leu-Phe-stimulated GTP binding to $G\alpha_{i2}$, pertussis toxin-inhibited calcium mobilization, and actin polymerization [24]. The data presented herein demonstrated that undifferentiated HL-60 cells that express FPRs do not reproduce the full range of FPR responses. Some aspects of homologous desensitization were impaired, as fMet-Leu-Phe-stimulated guanine nucleotide exchange, GTPγS inhibition of fMet-Leu-Phe binding, FPR affinity for ligand, and fMet-Leu-Phe-stimulated GTP photoaffinity labeling of $G\alpha_{i2}$ were similar in normal and desensitized membranes from FPR-transfected HL-60 cells. On the other hand, exposure to fMet-Leu-Phe resulted in reduced receptor expression, and exposure to fMet-Leu-Phe or PMA resulted in loss of ligand-stimulated calcium mobilization.

Desensitization of chemoattractant receptors normally results in a number of alterations that produce loss of receptor function. Ligand-stimulated phosphorylation of serines and threonines within the C-terminal tail by GRK2 and GRK3 is associated with uncoupling of receptors from their G-proteins and reduced plasma membrane expression during homologous desensitization [3, 4, 8–13, 19]. Class-specific heterologous desensitization of some chemoattractant receptors has been described [20, 31]. This heterologous desensitization may result from protein kinase C-

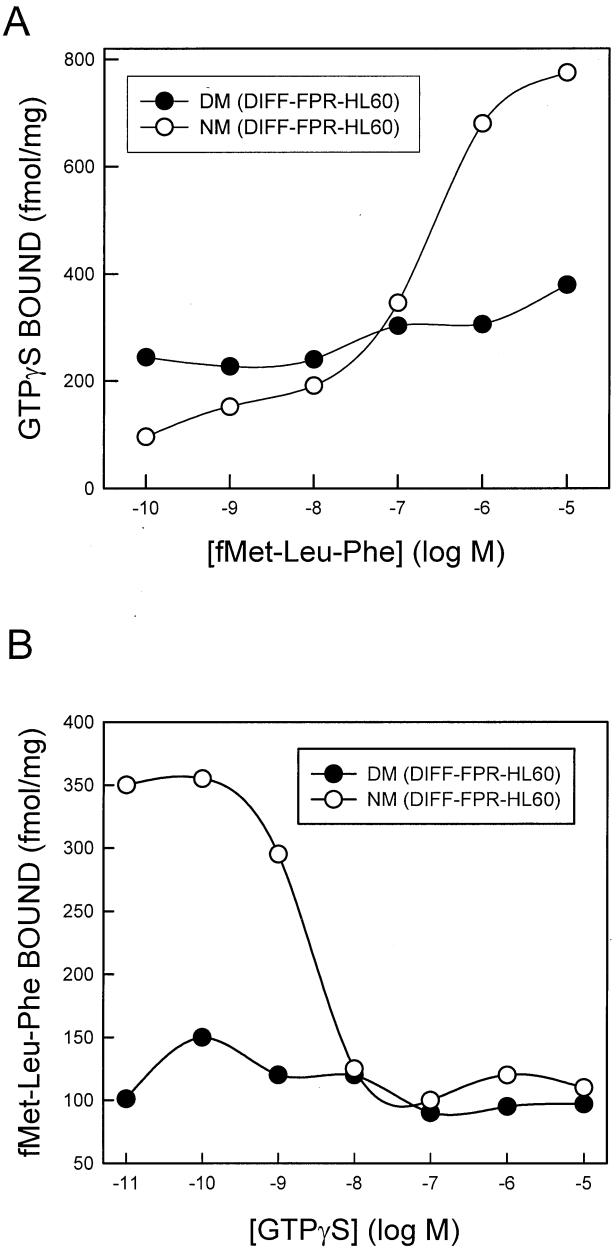

FIG. 4. Desensitization of differentiated FPR-transfected HL-60 cells. Panel A shows the ability of fMet-Leu-Phe to stimulate GTPγS binding to normal membranes (NM) and desensitized membranes (DM) from FPR-transfected HL-60 cells differentiated by a 5-day cultivation in 1.25% DMSO (Diff-FPR-HL-60). Results represent the means of triplicate samples from one experiment that is representative of four experiments in two different membrane preparations, expressed as femtomoles of [^{35}S]GTPγS/milligram of membrane protein. Panel B shows the response of fMet-Leu-Phe binding to increasing concentrations of GTPγS in NM and DM from Diff-FPR-HL-60 cells. Results represent the means of triplicate samples from one experiment representative of three experiments in two different membrane preparations, expressed as femtomoles of [^{3}H]fMet-Leu-Phe bound/milligram of membrane protein.

induced phosphorylation of these receptors [9, 20–22]. Evidence also exists that protein kinase C desensitizes calcium mobilization by interrupting receptor activation of phospholipase C [14–18].

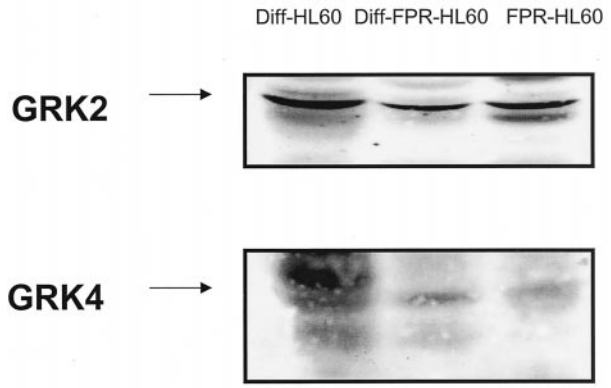

FIG. 5. Immunoblot analysis of GRK expression. Plasma membrane proteins (25 μg/lane) from FPR-transfected HL-60 cells (FPR-HL-60), differentiated FPR-transfected HL-60 cells (Diff-FPR-HL-60), and differentiated HL-60 cells (Diff-HL-60) were separated by 10% SDS–PAGE, transblotted, and incubated with antisera to GRK2 and GRK4 followed by goat anti-rabbit IgG-horseradish peroxidase. The results are representative of three separate experiments. Immunoblots with antisera to GRK3, GRK5, and GRK6 performed simultaneously with those shown revealed no staining.

Several possible explanations for the failure of receptor–G-protein uncoupling to occur following ligand binding were examined. The possibility that the vector used to transfect HL-60 cells interfered with desensitization is unlikely, as differentiated, vector-transfected HL-60 cells demonstrated normal FPR–G-protein uncoupling following exposure to fMet-Leu-Phe. The ability of differentiated FPR-transfected HL-60 cells to undergo ligand-induced FPR–G-protein uncoupling suggests that the receptors expressed by transfected cells maintain the ability to be desensitized. Interpretation of these data is complicated by the expression of both transfected and natively expressed receptors on these cells. However, the marked reduction of G-protein activation in membranes from differentiated FPR-transfected HL-60 cells following desensitization suggests that both native and transfected receptors were uncoupled from G-proteins.

The acquisition of homologous desensitization during differentiation of FPR-transfected HL-60 cells suggested that undifferentiated cells lack a necessary component of the signal transduction pathway leading to uncoupling of FPRs from G-proteins. Therefore, the presence of GRKs in undifferentiated and differentiated FPR-transfected HL-60 cells was compared with differentiated HL-60 cells by immunoblot analysis. GRK2 and GRK3, but not GRK5 or GRK6, phosphorylate serines and threonines on the C-terminal tail of FPRs, leading to uncoupling of receptors from their G-proteins [10]. The ability of GRK4 to phosphorylate FPRs has not been reported. GRK2 and GRK4, but not GRK3, GRK5, and GRK6, were expressed in undifferentiated and differentiated FPR-transfected HL-60 cells and differentiated HL-60 cells. Expression of GRK2 and GRK4 was reduced by 50% in undifferentiated FPR-transfected HL-60 cells, compared with differentiated HL-60 cells. However, differentiation of FPR-transfected

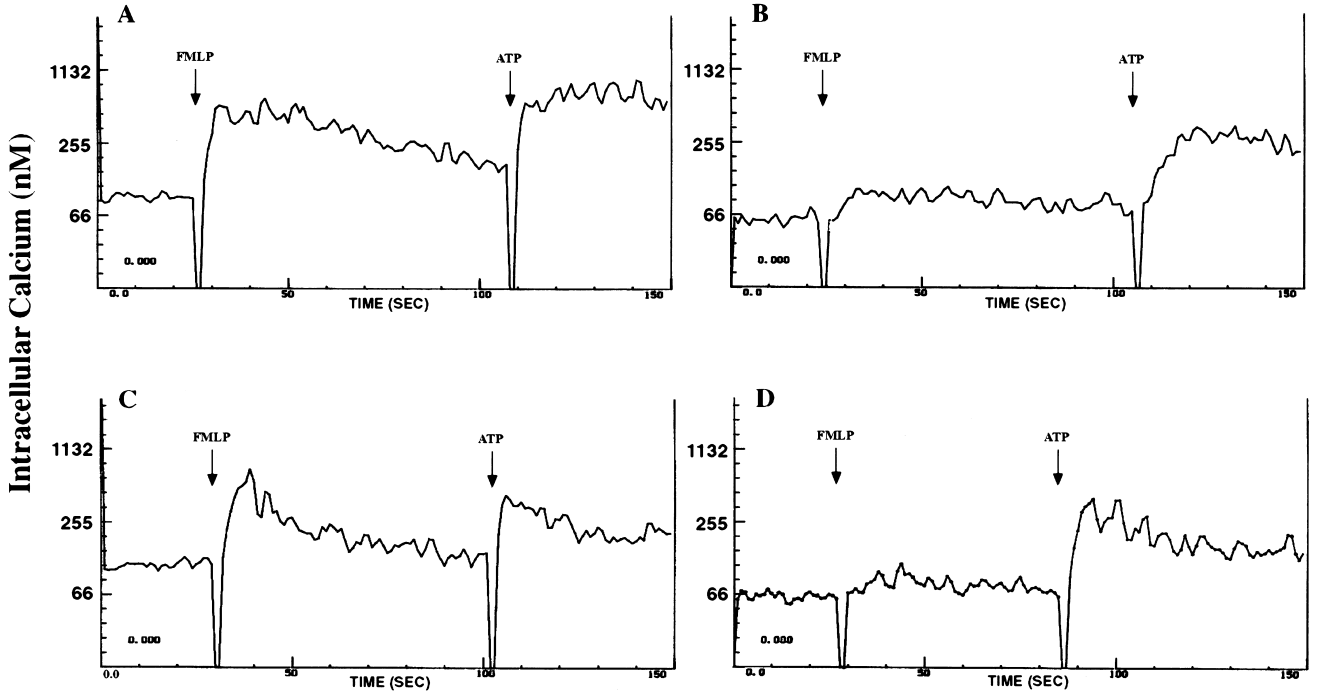

FIG. 6. Calcium transients stimulated by fMet-Leu-Phe in normal and desensitized FPR-transfected and differentiated HL-60 cells. Cells were loaded with Fura-2 prior to determination of intracellular calcium as the 350/380 nm fluorescence ratio, determined with an emission frequency of 510 nm using an Aminco–Bowman ratio spectrofluorimeter. Results are depicted as the plot of the emission intensity at 510 nm of a single experiment representative of four separate experiments. Panel A shows the increase in intracellular calcium in differentiated HL-60 cells following stimulation with 10^{-7} M fMet-Leu-Phe followed by the addition of 1 μM ATP. Both fMet-Leu-Phe and ATP stimulated an increase in intracellular calcium concentration. Panel B shows the intracellular calcium in differentiated HL-60 cells desensitized by a 10-min exposure to 10^{-7} M fMet-Leu-Phe, washed, and then stimulated with 10^{-7} M fMet-Leu-Phe followed by 1 μM ATP. Desensitization eliminated the calcium transient stimulated by fMet-Leu-Phe, whereas that stimulated by ATP remained. Panel C shows the intracellular calcium of undifferentiated FPR-HL-60 cells, in which both 10^{-7} M fMet-Leu-Phe and 1 μM ATP stimulated a calcium transient similar to that seen in differentiated HL-60 cells. Panel D shows the effect of desensitization with 10^{-7} M fMet-Leu-Phe on the calcium transient in undifferentiated FPR-HL-60 cells. The ability of 10^{-7} M fMet-Leu-Phe to stimulate a calcium transient was eliminated by prior exposure to fMet-Leu-Phe, whereas ATP stimulated a normal calcium transient.

HL-60 cells did not increase GRK2 or GRK4 expression. Therefore, it is unlikely that the component missing in undifferentiated cells is a GRK. The identity of this component remains to be determined.

Receptor down-regulation is another process that contributes to the loss of cellular response to ligand [32]. The basis for receptor down-regulation remains to be elucidated fully. It is independent of G-protein coupling [33], but receptor phosphorylation has been suggested to be a necessary component [8]. Our results indicate that down-regulation of FPR expression occurs during desensitization of undifferentiated FPR-transfected HL-60 cells, despite the failure of these receptors to undergo uncoupling from their G-proteins. These data support previous reports suggesting that the processes of G-protein uncoupling and receptor internalization are independent [34–39].

Desensitization of fMet-Leu-Phe-stimulated calcium mobilization occurred in the absence of FPR–G-protein uncoupling. Previous reports suggested that desensitization of chemoattractant-stimulated responses occurs at two levels of the signal transduction pathway, at receptor–G-protein interaction and distal to G-protein activation. Several studies have reported that treatment of neutrophils or differentiated HL-60 cells with phorbol diesters results in the inability of chemoattractants to stimulate phospholipase C activation and calcium mobilization [14–17]. The basis for this effect may be protein kinase C-mediated phosphorylation of phospholipase Cβ3 [18]. On the other hand, protein kinase C activation leads to phosphorylation of C5a, platelet-activating factor, and interleukin-8 receptors, but not FPRs, in the absence of agonist, and this phosphorylation results in receptor desensitization [9, 12, 20–22]. It has been suggested that this mechanism of desensitization contributes to class-specific cross-desensitization of chemoattractant receptors [40]. To determine if desensitization of calcium mobilization observed in the present study was due to protein kinase C activation, the pattern of calcium responses to fMet-Leu-Phe and ATP was examined in formyl peptide and PMA-desensitized cells. Desensitization by formyl peptide resulted in loss of calcium mobilization by fMet-Leu-Phe. Desensitization by PMA produced loss of calcium mobilization stimulated by both fMet-Leu-Phe and ATP. These results may be explained by different effects of short-term protein kinase C activation

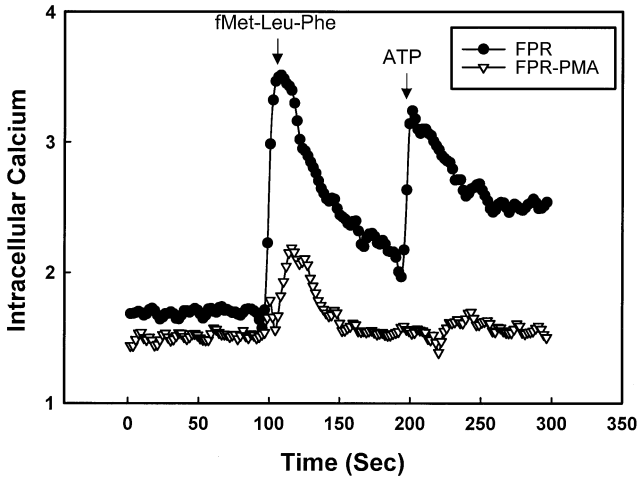

FIG. 7. Effect of PMA desensitization on calcium transients stimulated by 10^{-7} M fMet-Leu-Phe and 1 μM ATP in FPR-transfected HL-60 cells. Cells were loaded with Fura-2 prior to determination of intracellular calcium as the 350/380 nm fluorescence ratio, determined with an emission frequency of 510 nm using an Aminco–Bowman ratio spectrofluorimeter. Results are depicted as a plot of the emission intensity at 510 nm of a single experiment. This figure shows the effect of desensitization with 10 nM PMA for 10 min on the calcium transient in undifferentiated FPR-transfected HL-60 cells (FPR-HL-60).

by fMet-Leu-Phe and prolonged activation by PMA. On the other hand, desensitization of calcium mobilization by fMet-Leu-Phe may be related to reduced FPR expression and appears to be independent of protein kinase C activation.

Our finding that desensitization was impaired in undifferentiated HL-60 cells differs from results obtained with desensitization of FPRs expressed in undifferentiated U937 cells [10, 23]. Chemotaxis and G-protein activation stimulated by fMet-Leu-Phe were lost upon desensitization of U937 cells in which FPRs were expressed, and this desensitization was prevented when C-terminal tail serines and threonines were replaced by alanine or glycine [10, 23]. Desensitization of calcium mobilization and loss of G-protein coupling did not occur in the absence of receptor phosphorylation in U937 cells [10]. Our results indicated that desensitization of calcium mobilization and receptor down-regulation occur independently of FPR–G-protein uncoupling in undifferentiated FPR-transfected HL-60 cells. FPRs expressed on undifferentiated HL-60 cells failed to undergo ligand-induced uncoupling from G-proteins, whereas differentiation resulted in acquisition of this process. The mechanism for abnormal desensitization in undifferentiated HL-60 cells was not identified by our studies and remains to be determined. FPR expression by undifferentiated HL-60 cells may provide a useful model in which to study signal components necessary for receptor–G-protein uncoupling and to examine mechanisms of receptor internalization in the absence of uncoupling from G proteins.

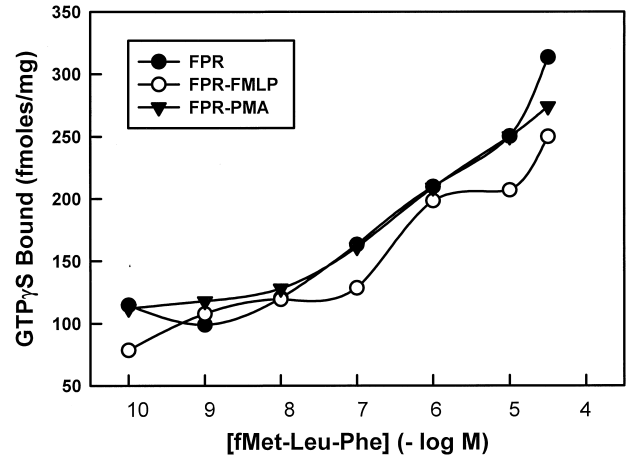

FIG. 8. Effect of fMet-Leu-Phe and PMA desensitization on receptor–G protein coupling in FPR-transfected HL-60 cells. Plasma membranes from FPR-transfected HL-60 cells (solid circles), fMet-Leu-Phe desensitized FPR-transfected HL-60 cells (open circles) or PMA-desensitized FPR-transfected cells (solid inverted triangles) were examined for fMet-Leu-Phe-stimulated GTPγS binding. Results are expressed as femtomoles of GTPγS bound/milligram of membrane protein. The results are representative of two experiments performed.

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References

1. Murphy PM, The molecular biology of leukocyte chemoattractant receptors. *Annu Rev Immunol* **12:** 593–633, 1994.
2. McLeish KR and Jakobs KH, G-proteins in the signal transduction of the neutrophil. In: *GTPases in Biology II* (Eds. Dickey BF and Birnbaumer L), pp. 363–373. Springer, Berlin, 1993.
3. McLeish KR, Gierschik P and Jakobs KH, Desensitization uncouples the formyl peptide receptor-guanine nucleotide-binding protein interaction in HL60 cells. *Mol Pharmacol* **36:** 384–390, 1989.
4. Wilde MW, Carlson KE, Manning DR and Zigmond SH, Chemoattractant-stimulated GTPase activity is decreased on membranes from polymorphonuclear leukocytes incubated in chemoattractant. *J Biol Chem* **264:** 190–196, 1989.
5. Gerard C and Gerard NP, The pro-inflammatory seven-transmembrane segment receptors of the leukocyte. *Curr Opin Immunol* **6:** 140–145, 1994.
6. Kunz D, Gerard NP and Gerard C, The human leukocyte platelet-activating factor receptor. cDNA cloning, cell surface expression, and construction of a novel epitope-bearing analog. *J Biol Chem* **267:** 9101–9106, 1992.
7. Yokomizo T, Izumi T, Chang K, Takuwa Y and Shimizu T, A G-protein-coupled receptor for leukotriene B_4 that mediates chemotaxis. *Nature* **387:** 620–624, 1997.
8. Giannini E and Boulay F, Phosphorylation, dephosphorylation, and recycling of the C5a receptor in differentiated HL60 cells. *J Immunol* **154:** 4055–4064, 1995.
9. Ali H, Richardson RM, Tomhave ED, Didsbury JR and Snyderman R, Differences in phosphorylation of formylpep-

tide and C5a chemoattractant receptors correlate with differences in desensitization. *J Biol Chem* **268:** 24247–24254, 1993.
10. Prossnitz ER, Desensitization of *N*-formylpeptide receptor-mediated activation is dependent upon receptor phosphorylation. *J Biol Chem* **272:** 15213–15219, 1997.
11. Mueller SG, White JR, Schraw WP, Lam V and Richmond A, Ligand-induced desensitization of the human CXC chemokine receptor-2 is modulated by multiple serine residues in the carboxyl-terminal domain of the receptor. *J Biol Chem* **272:** 8207–8214, 1997.
12. Tardif M, Mery L, Brouchon L and Boulay F, Agonist-dependent phosphorylation of *N*-formylpeptide and activation peptide from the fifth component C (C5a) chemoattractant receptors in differentiated HL60 cells. *J Immunol* **150:** 3534–3545, 1993.
13. Prossnitz ER, Kim CM, Benovic JL and Ye RD, Phosphorylation of the *N*-formyl peptide receptor carboxyl terminus by the G protein-coupled receptor kinase, GRK2. *J Biol Chem* **270:** 1130–1137, 1995.
14. Lederer ED, Jacobs AA and McLeish KR, Desensitization by protein kinase C activation differentially uncouples formyl peptide receptors from effector enzymes in HL-60 granulocytes. *Cell Signal* **5:** 735–745, 1993.
15. Kikuchi A, Kozawa O, Hamamori Y, Kaibuchi K and Takai Y, Inhibition of chemotactic peptide-induced phosphoinositide hydrolysis by phorbol esters through the activation of protein kinase C in differentiated human leukemia (HL-60) cells. *Cancer Res* **46:** 3401–3406, 1986.
16. Smith CD, Uhing RJ and Snyderman R, Nucleotide regulatory protein-mediated activation of phospholipase C in human polymorphonuclear leukocytes is disrupted by phorbol esters. *J Biol Chem* **262:** 6121–6127, 1987.
17. Naccache PH, Molski TFP, Borgeat P, White JR and Sha'afi RI, Phorbol esters inhibit the fMet-Leu-Phe- and leukotriene B_4-stimulated calcium mobilization and enzyme secretion in rabbit neutrophils. *J Biol Chem* **260:** 2125–2131, 1985.
18. Ali H, Fisher I, Haribabu B, Richardson RM and Snyderman R, Role of phospholipase Cβ3 phosphorylation in the desensitization of cellular responses to platelet-activating factor. *J Biol Chem* **272:** 11706–11709, 1997.
19. O'Flaherty JT, Jacobson DP and Redman JF, Regulation of platelet-activating-factor receptors and the desensitization response in polymorphonuclear neutrophils. *Biochem J* **288:** 241–248, 1992.
20. Richardson RM, Haribabu B, Ali H and Snyderman R, Cross-desensitization among receptors for platelet activating factor and peptide chemoattractants. Evidence for independent regulatory pathways. *J Biol Chem* **271:** 28717–28724, 1996.
21. Richardson RM, Dubose RA, Ali H, Tomhave ED, Haribabu B and Snyderman R, Regulation of human interleukin-8 receptor A: Identification of a phosphorylation site involved in modulating receptor functions. *Biochemistry* **34:** 14193–14201, 1995.
22. Mueller SG, Schraw WP and Richmond A, Activation of protein kinase C enhances the phosphorylation of the type B interleukin-8 receptor and stimulates its degradation in nonhematopoietic cells. *J Biol Chem* **270:** 10439–10448, 1995.
23. Kew RR, Peng T, DiMartino SJ, Madhavan D, Weinman SJ, Cheng D and Prossnitz ER, Undifferentiated U937 cells transfected with chemoattractant receptors: A model system to investigate chemotactic mechanisms and receptor structure/function relationships. *J Leukoc Biol* **61:** 329–337, 1997.
24. Prossnitz ER, Quehenberger O, Cochrane CG and Ye RD, Signal transducing properties of the N-formyl peptide receptor expressed in undifferentiated HL60 cells. *J Immunol* **151:** 5704–5715, 1993.
25. Goldsmith P, Gierschik P, Milligan G, Unson CG, Vinitsky R, Malech HL and Spiegel AM, Antibodies directed against synthetic peptides distinguish between GTP-binding proteins in neutrophil and brain. *J Biol Chem* **262:** 14683–14688, 1987.
26. Gierschik P, Falloon J, Milligan G, Pines M, Gallin JI and Spiegel A, Immunochemical evidence for a novel pertussis toxin substrate in human neutrophils. *J Biol Chem* **261:** 8058–8062, 1986.
27. Schepers TM, Brier ME and McLeish KR, Quantitative and qualitative differences in guanine nucleotide binding protein activation by formyl peptide and leukotriene B_4 receptors. *J Biol Chem* **267:** 159–165, 1992.
28. Rane MJ, Arthur JM, Prossnitz ER and McLeish KR, Activation of mitogen-activated protein kinases by formyl peptide receptors is regulated by the cytoplasmic tail. *J Biol Chem* **273:** 20916–20923, 1998.
29. Grynkiewicz G, Poenie M and Tsien RY, A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* **260:** 3440–3450, 1985.
30. Rane MJ, Carrithers SL, Arthur JM, Klein JB and McLeish KR, Formyl peptide receptors are coupled to multiple mitogen-activated protein kinase cascades by distinct signal transduction pathways. Role in activation of reduced nicotinamide adenine dinucleotide oxidase. *J Immunol* **159:** 5070–5078, 1997.
31. Tomhave ED, Richardson RM, Didsbury JR, Menard L, Snyderman R and Ali H, Cross-desensitization of receptors for peptide chemoattractants. Characterization of a new form of leukocyte regulation. *J Immunol* **153:** 3267–3275, 1994.
32. Hausdorff WP, Caron MG and Lefkowitz RJ, Turning the signal off: Desensitization of β-adrenergic receptor function. *FASEB J* **4:** 2881–2889, 1990.
33. Clark RB, Friedman J, Prashad N and Ruoho AE, Epinephrine-induced sequestration of the β-adrenergic receptor in cultured S49 WT and cyc^- lymphoma cells. *J Cyclic Nucleotide Protein Phosphor Res* **10:** 97–119, 1985.
34. Hausdorff WP, Campbell PT, Ostrowski J, Yu SS, Caron MG and Lefkowitz R, A small region of the β-adrenergic receptor is selectively involved in its rapid regulation. *Proc Natl Acad Sci USA* **88:** 2979–2983, 1991.
35. Hausdorff WP, Bouvier M, O'Dowd BF, Irons GP, Caron MG and Lefkowitz RJ, Phosphorylation sites on two domains of the β_2-adrenergic receptor are involved in distinct pathways of receptor desensitization. *J Biol Chem* **264:** 12657–12665, 1989.
36. Cheung AH, Dixon RAF, Hill WS, Sigal IS and Strader CD, Separation of the structural requirements for agonist-promoted activation and sequestration of the β-adrenergic receptor. *Mol Pharmacol* **37:** 775–779, 1990.
37. Strader CD, Sigal IS, Blake AD, Cheung AH, Register RB, Rands E, Zemcik BA, Candelore MR and Dixon RAF, The carboxyl terminus of the hamster β-adrenergic receptor expressed in mouse L cells is not required for receptor sequestration. *Cell* **49**: 855–863, 1987.
38. Campbell PT, Hnatowich M, O'Dowd BF, Caron MG, Lefkowitz RJ and Hausdorff WP, Mutations of the human β_2-adrenergic receptor that impair coupling to G_s interfere with receptor down-regulation but not sequestration. *Mol Pharmacol* **39:** 192–198, 1991.
39. Bouvier M, Hausdorff WP, De Blasi A, O'Dowd BF, Kobilka BK, Caron MG and Lefkowitz RJ, Removal of phosphorylation sites from the β_2-adrenergic receptor delays onset of agonist-promoted desensitization. *Nature* **333:** 370–373, 1988.
40. Didsbury JR, Uhing RJ, Tomhave ED, Gerard C, Gerard N and Snyderman R, Receptor class desensitization of leukocyte chemoattractant receptors. *Proc Natl Acad Sci USA* **88:** 11564–11568, 1997.