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Cross-Desensitization of CCR1, but Not CCR2, following Activation of the Formyl Peptide Receptor FPR1

Filip Bednar,*1,2 Changcheng Song,†,1 Giuseppe Bardi,†,3 William Cornwell,† and Thomas J. Rogers†

The cross-regulation of G protein–coupled receptors (GPCRs) plays an important role in the immune response. Studies from several laboratories have suggested that a hierarchy of sensitivities to cross-desensitization exists for the chemoattractant GPCRs. We carried out experiments to study the capacity of the formyl peptide receptor-1 (FPR1) to desensitize chemokine receptors CCR1 and CCR2. Our results show that activation of FPR1 resulted in the desensitization and partial internalization of CCR1, but not CCR2, in both primary human monocytes and HEK293 cells coexpressing CCR1, CCR2, and FPR1 (HR1R2F cells). The desensitization of CCR1 by FPR1 stimulation was not due to the simple depletion of the Ca2+ stores, but was dependent on activation of protein kinase C. Furthermore, we found that the cross-desensitization of CCR1 by FPR1 was associated with CCR1 phosphorylation and moderate reduction of CCR1 cell-surface expression. In contrast, CCR2 was not phosphorylated or internalized after FPR1 activation. Additional studies showed that optimal cross talk between FPR1 and CCR1 was dependent on the functional activity of protein kinase CB. These results provide a mechanistic basis for the capacity of certain GPCR ligands to exert rapid and selective cross-inactivation of other chemoattractant receptors, and suggest that FPR1 is able to exert “traffic control” in the migration of inflammatory cells by rapidly inhibiting the cell responses to potentially “low-priority” chemoattractants such as CCR1 agonists without inhibiting the response to “higher priority” CCR2 chemoattractants. The Journal of Immunology, 2014, 192: 5305–5313.

Several of the chemokine receptors are expressed by leukocytes, and these must collectively coordinate their migration to sites of inflammation and microbial infection, in response to various locally produced chemotactic ligands. The classical chemoattractant receptor (formyl peptide receptor-1 [FPR1]) and the receptors for chemokines are key participants in the innate and acquired defense systems, and guide leukocytes to sites of inflammation. CCR1 is a chemokine receptor that may play a role in early immune responses and is expressed by T and B cells (1), monocytes and dendritic cells (2), eosinophils (3), and bone marrow progenitor cells (4). CCR1 can be activated by several chemokine ligands including CCL3 and CCL5 (5). Although CCR1 is well established to contribute to the accumulation of T cells and monocytes in chronic inflammatory disease states, the role of CCR1 in acute inflammation, or in early acquired immune responses, is not entirely clear. A second chemokine receptor, CCR2, is expressed by monocytes, T cells, NK cells, basophils, mast cells, dendritic cells, and B cells (6–8), and is activated primarily by the ligand CCL2. In host defense against bacterial infections, inflammatory monocytes respond rapidly to microbial stimulation by expression of CCR2, and traffic in response to elevated CCL2 secretion. In murine models of infection with bacterial, protozoal, and fungal pathogens, CCR2-mediated recruitment of monocytes is required to suppress pathogen growth. In addition, the high-affinity receptor for bacterial and mitochondrial N-formyl peptides (FPR1) is highly expressed by monocytes and neutrophils (9–11), and the locally produced formyl peptides are potent stimuli to attract monocyte/macrophages and neutrophils to the site of pathogen infection and tissue damage (10, 11).

The proper guidance of leukocytes to the site of inflammation requires that inflammatory cells recognize appropriate chemoattractant signals because agonists for chemoattractant receptors can be produced by multiple sources, including bacteria and host cells within and surrounding the inflammatory stimulus site, and proper guidance of inflammatory cells is required. Our laboratory and others have shown that G protein–coupled receptors (GPCRs) exert mutual functional regulation through the process of heterologous desensitization. In this study, we evaluated the capacity of FPR1 to cross talk with the chemokine receptors CCR1 and CCR2, which chemoattract monocytes. We show that CCR1, CCR2, and FPR1 are coexpressed in primary human monocytes, and FPR1 activation rapidly desensitizes CCR1, but not CCR2, in a protein kinase CB (PKCB)–dependent signaling pathway.

Materials and Methods

Isolation of PBMCs

Human PBMCs were isolated from blood by using Ficoll-Paque plus (Amersham Biosciences) density gradient centrifugation. The CD14+...
monocytes were isolated using the Midi-MACS magnetic separation system and CD14+ isolation kit (Miltenyi Biotec, Auburn, CA) from PBMCs according to the manufacturer’s directions. In brief, cells were incubated with 80 μl MACS buffer (PBS containing 2 mM EDTA and 0.5% BSA) and 20 μl anti-CD14 beads per 107 cells. After incubation, the cells were washed with buffer, resuspended, and loaded onto the LS magnetic column. The columns were then washed three times with MACS buffer, and the cells were eluted from the column using RPMI 1640 containing 10% FBS, 25 mM HEPES, 2 mM l-glutamine, or HBSS.

Flow cytometry

Agonist or inhibitor-treated cells were incubated with undiluted goat serum for 30 min at 4°C, then washed in FACS buffer (1% BSA in PBS). Cells were resuspended with FACS buffer containing anti–CCR1-PE, anti–CD3-allophycocyanin-Cy7, or anti–CD14-Pacific Blue (BD Biosciences Pharmingen, Palo Alto, CA), or anti–CCR2-PE Ab (R&D Systems, Minneapolis, MN), and incubated at 4°C for 30 min. After washing twice with FACS buffer, the cells were resuspended in FACS buffer and analyzed using FACSCalibur, FACSaria, or LSRII flow cytometers (BD Biosciences). A minimum of 10,000 gated cells were analyzed per sample for the relevant cell-surface receptor expression. For quantification analysis of CCR1 and CCR2, the mean fluorescent intensities of CCR1-PE or CCR2-PE–labeled cells were converted to the number of Ab-bound receptors with Quantibrite PE calibration beads (Becton Dickinson, San Jose, CA) according to the manufacturer’s instructions.

Cell lines and cell culture

HR1R2F, human embryonic kidney cells stably expressing pcDNA3.0-FPR1, CCR1, and CCR2, were cultured in D10 medium (DMEM medium containing 10% FBS, 25 mM HEPES, 2 mM l-glutamine) supplemented with 500 μg/ml neomycin. Constructs of human CCR1 and CCR2 were obtained from Missouri S&T cDNA Resource Center (Rolla, MO). Human fMLF receptor FPR1 construct was a gift from Dr. J.M. Wang (National Cancer Institute, Frederick, MD). HEK293 cells were transfected with the constructs of CCR1, CCR2, and FPR1 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells were cultured in D10 medium for 2 wk with 500 μg/ml neomycin. Then the CCR1+, CCR2+, and FPR1+ cells were sorted using flow cytometry. Cells with high or low levels of receptor expression were fractioned as 75–100% and 25–50% of the mean fluorescent intensity of CCR1-PE. For certain experiments, HR1RF cells were further transfected with PKCδ-GFP (OriGene Technologies, Rockville, MD) using Lipofectamine 2000 (Invitrogen). Cells were cultured in D10 medium supplemented with 1 mg/ml neomycin for 1 wk; then cells were sorted using Influx cell sorter (Becton Dickinson).

Intracellular calcium flux

Cells were loaded with 5 μM fura 2-AM and 2.5 mM probenecid (Molecular Probes, Invitrogen, Carlsbad, CA) in either RPMI 1640 medium or HBSS with calcium/Mg2+ for 30 min at room temperature in the dark. After washing the samples, the cells were transfected into constantly stirred cuvettes containing HBSS with Ca2+/Mg2+ and maintained at 37°C in a fluorescence spectrophotometer (Aminco Bowman AB2 spectrofluorometer [SLM Aminco, Rochester, NY] or Fluoromax-3 [Jobin Yvon, Edison, NJ]). Stimulants at different concentrations were added to each cuvette at the indicated time points, and intracellular calcium was monitored by measuring fura 2 fluorescence at 510 nm, using 340/380-nm dual-wavelength excitation. Calibration was performed at the end of analysis by the addition of 0.05% Triton X-100 for maximal fura 2 ratio (Rmax) and then 5 mM EGTA for minimal fura 2 ratio (Rmin). The intracellular calcium concentration was then calculated from the 340/380 nm fluorescence ratio according to the method of Grynkiewicz et al. (12).

Chemotaxis

Chemotaxis analysis was carried out in response to recombinant CCL3, CCL2, or fMLF (PeproTech, Rocky Hill, NJ) diluted in chemotaxis medium (RPMI 1640 containing 1% BSA and 25 mM L-HEPES, pH 7.0). Chemotacticants were placed in the upper chambers, and cells were loaded in the upper, of 48-well chemotaxis chambers (NeuroProbe, Gaithersburg, MD), and the chambers were separated by a polycarbonate membrane (10-μm pore size) precoated with 50 μg/ml mouse collagen I. After 2 h of incubation at 37°C in a humidified CO2 chamber, the membranes were removed from the chemotaxis chambers and nonadherent cells were gently mechanically removed from the upper side of the membrane. The cells on the lower side of the membrane were stained using the HEMA3 staining system (Fisher Diagnostics, Middletown, VA). The number of migrated cells in three high-powered fields (×40) was counted by light microscopy. All experiments were repeated at least three times. Results are expressed as the chemotaxis index, which is calculated as the ratio of cells per high-power (40×) field in the presence of chemotacticant divided by the number of cells per field in the absence of chemotacticant.

Analysis of receptor phosphorylation

HR1RF cells were serum starved in DMEM medium without phosphate and pyruvate, containing 1% BSA for 24 h. After treatment with 1 μM fMLF, the cells were lysed in lysis buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 2 mM EDTA), complete mini-protease inhibitors, phosphatase inhibitor mixture II [Calbiochem, La Jolla, CA] and 1% Triton X-100). After centrifugation at 13,000 rpm for 15 min, rabbit polyclonal anti-CCR1 or -CCR2 Abs (Santa Cruz Biotechnology) and washed protein G-Sepharose (GE, Amersham, Carlsbad, CA) were added to the supernatants for immunoprecipitation. The immunoprecipitated complexes were subjected to SDS-PAGE and Western blot analysis with the PhosphoDetect Phosphospecific Detection Kit (Millipore). The level of phosphorylation was analyzed by measuring absorbance and using Image 1.43a image analysis software. After subtraction of background absorbance, the data were normalized with GAPDH as a loading control. The normalized absorbance of the control preparation was arbitrarily set to 100.

Fluorescence microscopy

HR1RF cells cultured in 96-well plates were starved in serum-free DMEM for 16 h, followed by treatment with 0.5 μM fMLF for 20 min. Cells were fixed and the immunofluorescence was assessed after staining with Becton Dickinson Cytofix/Cytoperm Fixation/Permeabilization Solution Kit following the manufacturer’s protocol. Phospho-PKD/PKu (Ser147/148), phospho-PKAc/a/b II (Thr364/365), phospho-PKc/b (Thr505), phospho-PKc0 (Thr589), and phospho-PKCa/A (Thr404/407) Cell Signaling Technology were prepared in the BD washing buffer and added to the cells at 4°C, then stained with Alexa 488–labeled secondary Abs for 2 h at 4°C, and imaged with a Nikon inverted fluorescence microscope using a 20× objective lens.

Statistical analysis

Statistical analyses were performed using unpaired Student t test or ANOVA.

Results

Activation of FPR1 cross-desensitizes CCR1, but not CCR2, in human PBMCs and monocytes

We have previously demonstrated that activation of receptors for bacterial formyl peptide fMLF in human monocytes led to the functional cross-desensitization of chemokine receptor CCR5 (9). To determine whether FPR1, CCR1, and CCR2 are cross-regulated, we first analyzed the pattern of receptor expression in CD14+ monocytes. We found that 99.5% of CCR1+ cells, and 99.7% of CCR2+ cells, coexpress FPR1. Analysis of receptor-induced calcium mobilization responses showed that after pretreatment with fMLF, CCL3 failed to induce a normal calcium flux response, whereas CCL2 retained a normal response (Fig. 1A), suggesting that the activation of FPR1 cross-desensitizes CCR1, but not CCR2. In reversing the order of agonist administration, neither CCL3 (Fig. 1B) nor CCL2 (Fig. 1C) were able to desensitize the cellular response to fMLF, showing that the cross talk between FPR1 and CCR1 is not bidirectional. More extensive analysis (Fig. 2A) showed that CCL3 induced a potent CCR1 calcium mobilization response in monocytes (EC50 of 1.75 nM). However, after FPR1 activation, the CCR1 response was substantially reduced (EC50 increased to >100 nM), which is consistent with FPR1-induced cross-desensitization of CCR1. In contrast, the functional activity of CCR2 remained essentially normal after FPR1 activation (Fig. 2B). Similar results were obtained from nonfractionated PBMCs. Additional analysis (Fig. 2C) shows that the fMLF-induced inhibition of the response to CCL3 is fMLF dose dependent, with greater inhibition achieved with increasing fMLF concentrations.

Because cross-desensitization of GPCRs may be associated with internalization of the target receptor (9, 13–15), we determined
whether the desensitized CCR1 undergoes internalization. The results (Fig. 2D) showed that treatment with 1 μM fMLF induced internalization of ~35% of CCR1 at 5 min, and 40–50% of the receptor was internalized by 30 min (Fig. 2D). In contrast, the cell-surface expression level of CCR2 was reduced by ~20% at 30 min (Fig. 2E). These data show that the FPR1-induced cross-desensitization of CCR1 was associated with partial internalization of the receptor, whereas less internalization (or desensitization) of CCR2 was detected.

Both CCR1 and CCR2 use phospholipase C and the endoplasmic reticulum calcium store in monocytes

The major signaling pathway driving calcium mobilization during Gα12-linked GPCR activation is the Gβγ-mediated activation of phospholipase Cβ (PLCβ) isoforms, with subsequent generation of diacylglycerol and inositol 1,4,5-trisphosphate (IP3). IP3 then releases the endoplasmic reticulum (ER) calcium stores through the activation of ER membrane resident IP3 receptors (16, 17). However, multiple PLC isoforms have been shown to serve as signaling mediators for chemokine receptor–induced calcium mobilization (18, 19). We determined whether the lack of CCR2 desensitization after fMLF pretreatment might be due to the use of distinct calcium signaling mechanisms for CCR2 and CCR1. We pretreated fresh monocytes with 1-[6-((17b)-3-Methoxyestra-1,3,5(10)-trien-17-yl)[amino]hexyl]-1H-pyrole-2,5-dione (U73122), a highly selective PLC antagonist (20), and found (Fig. 3A and 3B) that the treatment completely inhibited both CCL2- and CCL3-induced calcium mobilization, indicating that PLC is critical for the calcium flux responses mediated by CCR1 and CCR2.

The magnitude of the calcium mobilization response can be potentially dependent on both intracellular and extracellular stores of calcium. We assessed the response of monocytes in the presence of EGTA, a calcium chelating agent, to eliminate the contribution of extracellular calcium stores to the chemokine response. The results show (Fig. 3C and 3D) that the initial absolute magnitude of the overall CCL3 and CCL2 responses was essentially unchanged in the presence of EGTA. Thus, dissimilar usage of extracellular and intracellular calcium cannot explain the difference in susceptibility to FPR1-mediated cross-desensitization of CCR1 and CCR2.

We also characterized the role of ER calcium stores in the response through CCR1 and CCR2. We pretreated human monocytes with 2,5-di-tert-butylhydroquinone (DBHQ), a compound that inhibits the sarcoplasmic/endooplasmic reticulum calcium-ATPase pump responsible for calcium reuptake into the ER from the cytosol (21, 22). The results (Fig. 3E and 3F) show that depletion of this store led to nearly complete abrogation of the calcium mobilization response to CCL3 and CCL2. Therefore, CCR1 and CCR2 both use the PLC/IP3 pathway to induce ER calcium store mobilization in human monocytes, and the difference in the susceptibility of CCR1 and CCR2 to heterologous desensitization by FPR1 does not appear to be due to the existence of divergent signaling mechanisms for these two receptors.

We then examined the possibility that the rapid CCR1 desensitization by FPR1 might be due to cytosolic calcium store depletion. We pretreated monocytes with fMLF for 5–10 min in the absence of EGTA and assessed the release of ER calcium stores with DBHQ. The results (data not shown) show that there was no significant difference in the release of stored calcium. In fact, these experiments showed a consistent trend toward a more rapid, and more robust, calcium release, when compared with the release in the untreated (baseline) cells. This suggests that the calcium efflux pathways from the ER calcium stores were modestly potentiated by fMLF pretreatment. These results suggest that calcium store depletion is not responsible for the observed heterologous desensitization of CCR1 by FPR1.

Susceptibility of CCR1 and CCR2 to cross-desensitization is not related to the level of cell-surface expression

We examined the relation between receptor expression levels and the calcium mobilization response after activation of CCR1 and CCR2. We found that when using the same agonist concentration, CCL2 induced an ~2-fold higher calcium flux response than CCL3 (Fig. 4A), and the expression level of CCL2 was ~2-fold greater than CCR1 (Fig. 4B). To investigate whether the receptor expression level correlates directly with receptor desensitization susceptibility, we generated a cell line designated HR1R2F, which coexpresses CCR1, CCR2, and FPR1. This cell line eliminates the contribution of other potential receptors that might be activated by either fMLF, CCL3, or CCL2. The results show (Fig. 4C and 4D)
that in HR1R2 cells, fMLF desensitized the calcium response of CCR1, but not CCR2. Quantitative flow cytometry showed that the expression levels of CCR1 and CCR2 are similar (data not shown). These results suggest that the relative receptor expression levels of CCR1 and CCR2 are not a major factor in determining the sensitivity to cross talk with FPR1.

We further sorted the HR1R2F cells into two populations with high (HR1R2F-H) or low CCR1 (HR1R2F-L) expression (Fig. 5A and 5B). Flow cytometry shows that these cell populations express essentially identical levels of FPR1 (average mean fluorescence intensity for HR1R2F-H was 381 and for HR1R2F-L was 405). The calcium mobilization response to CCL3 is significantly reduced in both HR1R2F-L (Fig. 5C) and HR1R2F-H (Fig. 5D) cell populations after fMLF pretreatment. We also found that pretreatment with fMLF resulted in reduced efficacy of the CCL3 chemotactic response using either of the HR1R2F cell populations

**FIGURE 2.** Suppression of calcium flux and induction of receptor internalization of CCR1 by fMLF pretreatment in monocytes. Fura 2-AM loaded monocytes were treated with 1 μM fMLF for 5 min and subsequently treated with the designated concentrations of CCL3 (A) or CCL2 (B). Alternatively, cells were pretreated with the designated concentrations of fMLF (C), and subsequently treated with 12.5 nM CCL3. Change in intracellular calcium of untreated cells (●) and fMLF-pretreated cells (▲) is computed as peak [Ca²⁺]i - baseline [Ca²⁺]i, and normalized to the change observed at the highest concentration of chemokine without any fMLF pretreatment. Data are displayed as mean ± SD of three to four independent donor responses. *p < 0.01, untreated cells versus fMLF-pretreated cells at each concentration. CCR1 and CCR2 internalization after fMLF treatment in monocytes was measured using flow cytometry analysis (D and E). Monocytes were untreated (●), treated with 1 μM fMLF (▲), or treated with 12.5 nM CCL3 (D, ▲) or 11.1 nM CCL2 (E, ▲). Aliquots of treated monocytes were collected into cold FACS buffer at the indicated times, and CCR1 (D) or CCR2 (E) cell-surface expression was assessed by flow cytometry. Data are displayed as mean ± SD of three independent donors. *p < 0.05, for comparison with baseline of CCR1 or CCR2 cell-surface expression.

**FIGURE 3.** CCR1 and CCR2 calcium signaling in primary human monocytes. (A and B) Role of PLC in the initiation of CCR1 and CCR2 calcium signaling in human monocytes. Fura 2-AM–loaded monocytes were left untreated or pretreated with 10 μM U73122 for 5 min. Calcium responses were elicited by 12.5 nM CCL3 (A, C, and E) or 11.1 nM CCL2 (B, D, and F). (C and D) Role of extracellular and intracellular calcium stores was determined by addition of 5 mM EGTA to the cells 30 s before agonist addition. Responses in the presence and absence of 5 mM EGTA were measured. (E and F) Role of the ER calcium stores was determined by 5 min. Pretreatment with 50 μM DBHQ before agonist addition. Cells with and without DBHQ were then stimulated by the chemokines. Representative data from one of three independent donors are presented.
CCL2 or PBS (control). (A) Fura 2-AM-loaded monocytes stimulated with the indicated concentrations of CCL3 (▲) or CCL2 (●). The change in intracellular calcium was calculated and normalized as described earlier. Data are displayed as mean ± SD of two independent donor responses. *p < 0.05 for CCL2 response versus CCL3 response at each chemokine concentration. (B) The expression levels of CCR1 and CCR2 in monocytes were measured by flow cytometry. Monocytes were labeled with PE-conjugated anti-CCR1 or anti-CCR2 mAbs, and receptor density was determined based on fluorescence intensity calibration with Quantibrite PE Beads (Becton Dickinson). Data are presented as the expression of receptor as a percentage of CCR2, and are the mean ± SD of three independent donors. *p < 0.001. (C) The calcium flux response with or without 5 μM fMLF pretreatment for 5 min was measured with 5 nM CCL3 or buffer (control). (D) The calcium flux response was measured with or without pretreatment for 5 min with or without 5 μM fMLF, and induced with 5 nM CCL2 or PBS (control).

Phosphorylation of CCR1 after FPR1 activation

To better understand the molecular mechanism of the CCR1 desensitization, we examined the receptor phosphorylation of CCR1 and CCR2 after the cells were treated with fMLF, after labeling HR1R2F cells with [32P]orthophosphate. CCR1 and CCR2 were immunoprecipitated from the cell lysates and subjected to SDS-PAGE. The results (Fig. 6) show that CCR1, but not CCR2, was phosphorylated after the fMLF treatment. These results suggest that a signaling process is initiated by FPR1 activation that results in the phosphorylation of CCR1, whereas CCR2 remains unaffected.

Biochemical basis for cross-desensitization of CCR1

We examined the capacity of fMLF pretreatment to inhibit the capacity of cells to manifest a chemotactic response to CCL3. The results show (Fig. 7A) that HR1R2F cells pretreated with fMLF exhibited a significant reduction in the chemotactic response over a broad dose range of CCL3 (Fig. 7A). Moreover, we examined the capacity of the PKC inhibitor staurosporine to block the cross-desensitization of CCR1, and the results show (Fig. 7B) that staurosporine significantly blocked the fMLF-induced inhibition of the chemotactic response to CCL3 at doses as low as 10 nM. These results confirm that staurosporine-sensitive kinases play an important role in the signaling processes that are responsible for FPR1-induced cross-desensitization of CCR1.

In an effort to further characterize the requirement for PKC activity in the cross-desensitization of CCR1, we evaluated the response of the major PKC isoforms after fMLF activation. HR1R2F cells were treated with 0.5 μM fMLF for 20 min, and expression of phosphorylated PKD, PKCa/βII, PKCδ, PKCζ, or PKCζ/λ was assessed. The results show (Fig. 8) that the fMLF treatment resulted in the activation of both PKCa/βII and PKCζ. Under the conditions of this experiment, we detected little activation of either PKCβ or PKCζ/λ. Based on these results, we examined the kinetics of PKCβ, PKCβII, and PKCζ activation after fMLF treatment using Western blot analysis. Our results show (Fig. 9A) that PKCβI phosphorylation is apparent by 10 min, and the induction of PKCβII and PKCζ phosphorylation is detectable with 0.5 and 1 μM fMLF, respectively (Fig. 9B and 9C). The results from the Western blot analysis also show that these cells constitutively exhibit a detectable level of both PKCβI and PKCβII.

In an effort to assess the role of PKCβ in the cross-talk between FPR1 and CCR1, we examined the role of PKCβ in the FPR1-induced cross-phosphorylation of CCR1. HR1R2F cells were first pretreated with a PKCβ pseudosubstrate inhibitor (PPβi), and these cells were then induced with either fMLF or CCL3. The results of Western blot analysis show (Fig. 9D) that PPβi treatment substantially blocked the fMLF-induced phosphorylation of CCR1. Next, we wished to determine the role of PKCβ in the FPR1-induced cross-desensitization of CCR1 activity. Cells were pretreated with either staurosporine or PPβi; then the cells were treated with either fMLF or buffer, and the CCL3-induced calcium flux response was determined. The results show (Fig. 10A) that fMLF-treated cells exhibit a weak calcium response to CCL3, and both staurosporine and PPβi partially returned the response to a control level. Additional experiments were carried out with cells transfected to overexpress PKCβ-GFP, and the results show (Fig. 10B) that these cells exhibit a reduced calcium mobilization response to CCL3. Moreover,
pretreatment of cells overexpressing PKCβ with fMLF resulted in a response to CCL3, which was somewhat less than the response of normal cells pretreated with fMLF (Fig. 10B).

Finally, we wished to more directly examine the role of PKCβ in the cross talk between FPR1 and CCR1. Cells were first treated with the PKCβ-selective inhibitor PPβi, followed by fMLF pretreatment, and the functional activity of CCR1 was measured by a calcium mobilization response. The results show (Fig. 10C) that the PPβi treatment partially reduced the inhibitory effect of the fMLF treatment, suggesting that PKCβ is required for optimal cross talk between FPR1 and CCR1. In contrast, the data also demonstrate that the fMLF-induced cross-desensitization of CCR1 remained normal in cells initially treated with the PKCδ-selective inhibitor PPδi, suggesting that PKCδ does not play an apparent role in this cross-talk interaction.

Discussion

A protective inflammatory response is dependent on the appropriate migration of leukocytes from one anatomic site to another. The traffic of these cells is dependent on their ability to decipher a complex mixture of chemotactic signals that accumulate at a given anatomical location and to "prioritize" among the chemoattractants so that the cells migrate to the most appropriate target site. A variety of processes have been proposed to participate in this "prioritization" including the regulation of adhesion molecules on the leukocyte and/or endothelial cell surface (23, 24).

FIGURE 5. Cross-desensitization of CCR1 by fMLF in HR1R2F-L and HR1R2F-H cell lines. (A and B) The HR1R2F parental cell line was sorted on the basis of CCR1 expression into high- and low-receptor–bearing cell lines. HR1R2F cell lines were immunostained with PE anti-CCR1 or PE anti-FPR1, and the relative expression levels were determined by flow cytometry. (C and D) The calcium flux response induced by 6.25 nM CCL3 in 1 μM fMLF pretreated or nonpretreated HR1R2F-L (C) or HR1R2F-H (D) cell lines was measured. (E) Chemotaxis induced by CCL3 in untreated (open bar) or fMLF pretreated (gray bar) HR1R2F cells with high or low expression level of CCR1. Chemotaxis is reported as the chemotaxis index. Data are displayed as mean ± SD of three independent experiments, **p < 0.001.

FIGURE 6. Phosphorylation of CCR1, but not CCR2, after FPR1 activation. The HR1R2F cells were radiolabeled with [32P]orthophosphate and left untreated or pretreated with 1 μM fMLF. The cell lysates were immunoprecipitated with anti-CCR1 or -CCR2 Abs. The protein samples were dissolved in SDS loading buffer and subjected to SDS-PAGE. Phosphorylation of the CCR1 and CCR2 was assessed by autoradiography. The levels of GAPDH were determined by Western blot analysis. The normalized absorbance of each of the phosphorylated bands is presented below the GAPDH panel (results are representative of three independent experiments).

FIGURE 7. Stauroporine inhibits the fMLF-induced cross-desensitization of CCR1-driven chemotactic activity of HR1R2F cells. (A) The chemotaxis response of HR1R2F cells to CCL3 at the indicated agonist concentrations was determined. Cells were pretreated with (▼) or without (●) 1 μM fMLF for 5 min before analysis of the chemotactic response to CCL3. (B) HR1R2F cells were pretreated with stauroporine at the designated concentrations for 5 min before treatment with 1 μM fMLF for 5 min, and the chemotactic response of these cells to 5 nM CCL3 was determined. The results are expressed as the chemotactic response where the cells that were not staurosporine or fMLF treated were set arbitrarily to 100%. *p < 0.05 compared with the chemotaxis response in the absence of staurosporine.
chemokine receptors because PAF or LTB4 receptor signaling. The present studies show that the C5a and CXCL8 receptors, and is not significantly altered by
in the biochemical consequences for the target receptor. Although many cross-desensitized GPCRs fail to undergo internalization, some GPCRs are at least partially internalized as a consequence of the heterologous desensitization process. For example, the k-opioid receptor is partially internalized after cross-desensitization induced by CXCR4 (14), and cross-desensitization of CCR5 after activation of FPR1 in monocytes and dendritic cells is associated with virtually complete staurosporine-sensitive internalization (9, 32). The biochemical basis for the internalization of CCR5 in these studies is uncertain because desensitization of G_{i}-linked GPCRs is typically dependent on PKCs, and not on GPCR kinases, as would normally be the case for homologous desensitization (37). Moreover, PKC-mediated phosphorylation is not typically linked to β-arrestin association with the receptor complex (38).

Previous reports have shown that cross-desensitization between G_{i}-linked GPCRs is dependent on the activation of one or more members of the PKC family of protein kinases (reviewed in Ref. 15). For example, the μ-opioid receptor is a strong inducer of PKCζ activation, and this GPCR induces cross-desensitization of both CCR1 and CCR5, and previous reports show that opioid-induced cross talk with CCR1 and CCR5 is dependent on the activation of this atypical PKC (39, 40). In contrast, the activation of CXCR1 induces activation and rapid outer-membrane translocation of PKCe,

The heterologous desensitization phenomenon has now been studied for a number of GPCRs, and there is a great deal of diversity

FIGURE 8. FPR1-mediated activation of PKCβ and PKCθ. HR12F cells were treated with 0 (A, B, E, F, J, M, N, Q, R, U, and V) or 0.5 μM (C, D, G, H, K, L, O, P, S, T, W, and X) fMLF for 20 min, followed by staining with the designated anti–phospho-PKC Abs as described in Materials and Methods. Nuclei were stained with DAPI. A higher magnification of a selected region of (G) and (H) is presented in (U)–(X). Scale bars, 100 μm (Q–T); 25 μm (U–X). (A–P) Original magnification ×20.

24), the production of cytokines such as TNF-α that can implement a “stop” signal (25–27), and homologous desensitization of chemoattractant receptors once the concentration of a given chemoattractant in a tissue microenvironment reaches a sufficient concentration (28). We believe an important additional part of this prioritization process is mediated by the mechanism of heterologous desensitization and selective cross talk between chemoattractant receptors (reviewed in Ref. 15). Gi protein–coupled chemoattractant receptors exhibit a hierarchy in initiating cross-desensitization, and this is inversely correlated with their susceptibility to regulation by cross-desensitization (9, 29, 32, 33). Moreover, FPR1 induces a desensitizer and is relatively resistant as a target for cross-desensitization.

The heterologous desensitization phenomenon has now been studied for a number of GPCRs, and there is a great deal of diversity

FIGURE 9. FPR1-mediated PKC activation and the role of PKCθ in the cross-phosphorylation of CCR1. HR12F cells were treated with 0.5 μM fMLF for the designated times (A) or doses of fMLF for 20 min (B and C), followed by Western blot analysis of phospho-PKC using Abs for phosho-PKCβI (Thr673) (A), anti–phospho-PKCβII (Thr638/641) (B), and anti–phospho-PKCθ (Thr538) (C) Abs. GAPDH was also probed as loading controls. (D) Cells were also pretreated with 1 μM PP2i 20 min, followed by treatment with vehicle or 0.5 μM fMLF or 10 nM CCL3 for 20 min. The assessment of phosphorylation of CCR1 was then carried out as described in Materials and Methods. CCR1 and GAPDH were also analyzed to control for loading. The normalized absorbance of each of the phosphorylated bands is presented below the GAPDH panel (A and B). The densitometry results for three replicate experiments are shown for (D). *p < 0.05.
In this regard, because FPR1 cross-desensitizes both CCR5 and CCR1, these receptors may both possess PKCβ target sequences, whereas CCR2b does not. CCR2b is most likely the splice variant of CCR2 that is expressed on the outer membrane of these cells, because CCR2a is poorly expressed, in part, because of the presence of a retention signal motif in the C-terminal region of this CCR2a (44). Previous work has shown that the PKC target site in the C terminus of CCR5 is at S337 (45), and this corresponds to S341 in CCR1 and S345 in CCR2b. We have not determined the PKC phosphorylation site in CCR1, but it would be expected to be S341 because the adjacent sequence of S341 is homologous to S337 in CCR5, and these sequences are consistent with common PKCβ phosphorylation sites (including arginine at position -3). In contrast, the sequence of CCR2b adjacent to S345 has little homology to either CCR1 or CCR5, and does not contain common sequence elements for a PKC phosphorylation site (46).

In this study, we considered the possibility that CCR1 and CCR2 might use distinct calcium stores, and that this might provide an explanation for the difference in susceptibility to cross-desensitization. We found that both CCR1 and CCR2 use a PLC signaling pathway to activate the same ER calcium store, and fMLF-treated monocytes recover their ER calcium stores quickly. These data demonstrate that calcium store depletion is not responsible for the observed heterologous desensitization of CCR1 by FPRs.

The relative resistance of both FPR1 and CCR2 to cross-desensitization may reflect the strength of these receptors in performing leukocyte recruitment during inflammation. We suggest that the production of ligands for these receptors, in the context of many other (potentially competing) chemoattractants, will direct the successful migration of receptor-bearing cells. In the case of FPR1, a number of microbial and endogenous agonists are likely to be present at the sites of inflammation, including mitochondrial components, cathepsin G, and annexin 1, and collectively the localized concentration of FPR1 agonists may approach or surpass micromolar concentrations (reviewed in Ref. 47). The cross talk contributes to the traffic control of leukocytes, which must emigrate from a blood vessel, navigate through numerous chemoattractants and regulatory factors, and finally arrive at the correct anatomical location. The capacity of cells to traffic in response to CCR2 or CCR1 ligands is likely to be influenced by the collection of other chemoattractants, and the combination of these diverse ligands is likely to differ depending on the nature of the inflammatory response, the presence of microbial agents, and the tissue type. Indeed, in certain situations, CCR1 may be more important than CCR2 for the mobilization of inflammatory cells. For example, the migration of monocytes to the synovium appears to be directed most strongly by CCR1, and not CCR2 (48). In addition, the resistance to cross-desensitization of more than a single receptor allows the migration of receptor-bearing cells. In the case of FPR1, many other (potentially competing) chemoattractants, will direct the successful migration of receptor-bearing cells. In the case of FPR1, many other (potentially competing) chemoattractants, will direct the successful migration of receptor-bearing cells. In the case of FPR1, many other (potentially competing) chemoattractants, will direct the successful migration of receptor-bearing cells. In the case of FPR1, many other (potentially competing) chemoattractants, will direct the successful migration of receptor-bearing cells. In the case of FPR1, many other (potentially competing) chemoattractants, will direct the successful migration of receptor-bearing cells.
recent immigrant cell populations produces a new collection of their own chemokine receptors.

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Disclosures
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