Regulation of the Human Chemokine Receptor CCR1
CROSS-REGULATION BY CXCR1 AND CXCR2*

To investigate the regulation of the CCR1 chemokine receptor, a rat basophilic leukemia (RBL-2H3) cell line was modified to stably express epitope-tagged receptor. These cells responded to RANTES (regulated upon activation normal T expressed and secreted), macrophage inflammatory protein-1α, and monocyte chemotactic protein-2 to mediate phospholipase C activation, intracellular Ca\(^{2+}\) mobilization and exocytosis. Upon activation, CCR1 underwent phosphorylation and desensitization as measured by diminished GTPase stimulation and Ca\(^{2+}\) mobilization. Allosteric substitution of specific serine and threonine residues (S2 and S3) or truncation of the cytoplasmic tail (∆CCR1) of CCR1 abolished receptor phosphorylation and desensitization of G protein activation but did not abolish desensitization of Ca\(^{2+}\) mobilization. S2, S3, and ∆CCR1 were also resistant to internalization, mediated greater phosphatidylinositol hydrolysis and sustained Ca\(^{2+}\) mobilization, and were only partially desensitized by RANTES, relative to S1 and CCR1. To study CCR1 cross-regulation, RBL cells co-expressing CCR1 and receptors for interleukin-8 (CXCR1, CXCR2, or a phosphorylation-deficient mutant of CXCR2, 331T) were produced. Interleukin-8 stimulation of CXCR1 or CXCR2, cross-phosphorylated CCR1 and cross-desensitized its ability to stimulate GTPase activity and Ca\(^{2+}\) mobilization. Interestingly, CCR1 cross-phosphorylated and cross-desensitized CXCR2, but not CXCR1. Ca\(^{2+}\) mobilization by S3 and ∆CCR1 were also cross-desensitized by CXCR1 and CXCR2 despite lack of receptor phosphorylation. In contrast to wild type CCR1, S3 and ∆CCR1, which produced sustained signals, cross-phosphorylated and cross-desensitized responses to CXCR1 as well as CXCR2. Taken together, these results indicate that CCR1-mediated responses are regulated at several steps in the signaling pathway, by receptor phosphorylation at the level of receptor/G protein coupling and by an unknown mechanism at the level of phospholipase C activation. Moreover, selective cross-regulation among chemokine receptors is, in part, a consequence of the strength of signaling (i.e. greater phosphatidylinositol hydrolysis and sustained Ca\(^{2+}\) mobilization) which is inversely correlated with the receptor’s susceptibility to phosphorylation. Since many chemokines activate multiple chemokine receptors, selective cross-regulation among such receptors may play a role in their immunomodulation.

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RANTES\(^1\) is a member of the CC subfamily of chemokines, which participate in the recruitment and activation of leukocytes (1–3). RANTES interacts with specific cell surface receptors, which are coupled to pertussis toxin-sensitive guanine nucleotide regulatory proteins (G protein) to activate effectors such as phospholipase C (PLC), ion channels, phospholipase D, and protein kinase C (4–6). In addition to the CCR1 receptor, RANTES activates several members of the CC subfamily of chemokine receptors including CCR3, CCR4, and CCR5 (2, 3). CCR1 is also activated by MIP-1α, MCP-2, and MCP-3, although maximum responses are only obtained with RANTES and MIP-1α (2, 3, 7–9).

While much has been learned about the signaling pathways of chemokine receptors, little is known about their mechanism(s) of regulation or cross-regulation. Cellular responses to chemoattractants such as formyl peptides (fMLP), a complement cleavage product (CsA), interleukin-8 (IL-8), platelet-activating factor, monocyte chemoattractant protein-1 ( MCP-1) and leukotriene B\(_4\), are regulated via three forms of desensitization: 1) desensitization that involves receptor/G protein uncoupling via phosphorylation of the activated receptor by a receptor-specific kinase (GRK) (10–15), 2) desensitization that occurs via phosphorylation of receptors by a second messenger activated kinase (10–15), and 3) downstream inhibition of PLC activation among groups of chemoattractant receptors (16).

This present work studied the mechanisms of regulation and cross-regulation of CCR1. For that purpose, an epitope-tagged CCR1 was expressed in a rat basophilic leukemia (RBL-2H3) cell line and studied for its ability to undergo phosphorylation and desensitization upon agonist stimulation. In addition, CCR1 was co-expressed with the receptors for IL-8 (CXCR1 and CXCR2), and its ability to undergo or mediate cross-desensitization was investigated. The results show that CCR1 is regulated via receptor phosphorylation as well as a phosphorylation-independent mechanism. In addition, the data demonstrated unexpected differences in the ability of CCR1 to cross-regulate cellular responses to CXCR1 and CXCR2. These differences likely reflect the disparate susceptibility of CXCR1 and CXCR2 to time-dependent receptor signals from CCR1. More broadly, these data suggest that cross-regulation among classes of chemoattractant receptors is dependent on the strength of receptor’s signaling, which may be inversely correlated with the receptor’s susceptibility to phosphorylation by second messenger dependent kinases.

\(^1\) The abbreviations used are: RANTES, regulated upon activation normal T expressed and secreted; MIP-1α, macrophage inflammatory protein-1α; MCP-2, monocyte chemotactic protein-2; fMLP, formylmethionylleucylphenylalanine; CsA, complement cleavage product; CCR1, RANTES receptor; IL-8, interleukin-8; CXCR1, IL-8 receptor A; CXCR2, IL-8 receptor B; PMA, phorbol 12-myristate 13-acetate; G protein, GTP-regulatory protein; PLC, phospholipase C; HA, hemagglutinin; cpt-cAMP, 8-(4-chlorophenylthio)-adenosine 3’-cyclic monophosphate.
Experimental Procedures

Materials—[32P]Orthophosphate (8500–9120 Ci/mmol), nyc-2-[3H]inositol (24.4 Ci/mmol), and [γ-32P]GTP (6000 Ci/mmol) were purchased from NEN Life Science Products. 32P1-RANTES and 125I-IL-8 were obtained from Amersham Pharmacia Biotech. RANTES, IL-8 (monocyte-derived), MIP-1α, and MCP-2 were purchased from Genzyme. Geneticin (G418) and all tissue culture reagents were purchased from Life Technologies Inc. Monoclonal 1202 and anti-body, protein G-agarose and protease inhibitors were purchased from Roche Molecular Biochemicals. Anti-human IL-8Rb (CXC2) antibody was purchased from Pharmingen. Polyclonal antibody against PLCβ and anti-human III-5RA (CXC1) antibody were obtained from Santa Cruz Biotechnology. Indo-1 acetoxyethyl ester and pluronie acid were purchased from Molecular Probes. 8-4(Chlorophenyl)thio)adenosine 3′-cyclic monophosphate (cAMPS) phosphodiesterase type 1 (PDE1), phosphol 12-myristate 13-acetate (PMA), GDP, GTP, and ATP were purchased from Sigma. All other reagents are from commercial sources. The cDNAs encoding the hemagglutinin (HA) epitope-tagged CCR1 and the CXCR2 mutant 331T were kindly provided by Dr. Timothy N. C. Wells and Dr. Ann Richmond, respectively.

Construction of the Phosphorylation-deficient Mutants of CCR1—The polymerease chain reaction was used to generate phosphorylation-deficient mutants of CCR1 (S1, S2, and S3) as well as a carboxyl-terminal truncated CCR1 mutant (ΔCCR1). The 5′ oligonucleotide corresponding to the epoioptide-tagged CCR1 (YPDYDVPDYA) was used with a 3′ oligonucleotide complementary to the CCR1 tail replacing serine and threonine residues with alanine (S1, S2, and S3) or to the amino acids 325–331 in CCR1 following by a stop codon (ΔCCR1). The resulting polymerase chain reaction products were cloned into the eukaryotic expression vector pcDNA3, and the entire receptors were sequenced to confirm the intended mutations and lack of secondary mutations.

Cell Culture and Transfection—RBL-2H3 cells were maintained as monolayer cultures in Dulbecco’s modified Eagle’s medium supplemented with 15% fetal bovine serum, 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 mg/ml) (17). RBL-2H3 cells (1×107 cells) were transfected by electroporation with pcDNA3 containing the receptor of interest, cells were incubated on ice for 2 h in the same medium (200 μl) containing the radiolabeled ligand (0.1 nM). Reactions were stopped with 1 ml of ice-cold phosphate-buffered saline containing 10 mg/ml bovine serum albumin, and washed three times with the same buffer. Cells were then lysed with 200 μl of radioimmune precipitation buffer, and bound radioactivity was counted (18, 19). Nonspecific radioactivity bound was determined in the presence of 1 μM unlabeled ligands. For internalization, cells were subjected to two runs of sorting and then cloned into single cell. Cells expressing similar number of both receptors were used in this study.

Radioligand Binding Assays—RBL-2H3 cells were subcultured overnight in 24-well plates (0.5×105 cells/well) in growth medium. Cells were then rinsed with Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, and streptomycin (100 mg/ml) and incubated on ice for 2 h in the same medium (200 μl) containing the radiolabeled ligand (0.1 nM). Reactions were stopped with 1 ml of ice-cold phosphate-buffered saline containing 10 mg/ml bovine serum albumin, and washed three times with the same buffer. Cells were then lysed with 200 μl of radioimmune precipitation buffer, and bound radioactivity was counted (18, 19). Nonspecific radioactivity bound was determined in the presence of 1 μM unlabeled ligands. For internalization, cells were incubated with different ligands for 0–60 min. Then cells were washed with phosphate-buffered saline, and TCR (or PLCβ) was performed as described previously (17–19). RBL (5×105) expressing the receptors were incubated with [32P]orthophosphate (150 μCi/dish) for 90 min. Then labeled cells were stimulated with the indicated ligands for 5 min at 37 °C. Cell lysates were immunoprecipitated with specific antibodies against either the NH2 terminus of CXC1 or CXC2; the HA epitope tag of CCR1 or the PLCβ, analyzed by SDS electrophoresis and visualized by autoradiography.

GTPase Activity—Cells were treated with appropriate concentrations of stimuliants, and membranes were prepared as described previously (17). GTPase activity using 10–20 μg of membrane preparations was carried out as described previously (18, 19).

Results

Expression and Characterization of CCR1 in RBL-2H3 Cells—Competition binding assays using 125I-RANTES (Fig. 1A) and Scatchard analysis (data not shown) revealed that the CCR1 stably expressed in RBL-2H3 cells bound RANTES with a dissociation constant (Kd) of 12 ± 2 nM and Bmax of 7354 ± 462 receptors/cell. The Kd for RANTES binding in RBL-2H3 cells is similar to that of the CCR1 expressed in 293 cells 7.6 ± 1.5 nM (4). Activation of CCR1 by RANTES stimulated PI hydrolysis (Fig. 1B) and secretion (data not shown). CCR1-mediated comparable peak intracellular Ca2+ mobilization, respectively. CCR1 mediated comparable peak intracellular Ca2+ mobilization in response to both RANTES and MIP-1α (Fig. 1C). MIP-2-induced Ca2+ mobilization was ~50% less than RANTES and MIP-1α. No response was obtained with IL-8. Pretreatment of the cells with pertussis toxin completely inhibited the ability of all three ligands tested to stimulate PI hydrolysis, Ca2+ mobilization, and secretion (data not shown).

Desensitization of CCR1 in RBL-2H3 Cells—Ca2+ mobiliza-
tion was quantified to study desensitization of CCR1-mediated cellular responses in RBL. As shown in Fig. 2, prestimulation of Indo-1-loaded RBL cells expressing CCR1 with an EC100 dose (for Ca2+ mobilization) of RANTES (10 nM), MIP-1α (10 nM), or MCP-2 (10 nM) markedly inhibited response to a second dose of RANTES. Desensitization by RANTES and MIP-1α was >90%, whereas MCP-2 pretreatment caused ~50% desensitization. PMA (100 nM) and cpt-cAMP (1 mM) pretreatment of the cells completely inhibited RANTES-, MIP-1α-, and MCP-2-induced Ca2+ mobilization (Fig. 2, data not shown).

Pretreatment of CCR1-expressing RBL cells with either RANTES (100 nM) or PMA (100 nM) produced ~60% desensitization of RANTES-mediated GTPase activity in membranes compared with control or untreated cells (Fig. 3). In contrast to Ca2+ mobilization (Fig. 2), pretreatment with cpt-cAMP (1 mM) had no effect on RANTES-stimulated GTPase activity (Fig. 3).

Phosphorylation of CCR1—RANTES (Fig. 4A, lane 2), MIP-1α (lane 5), and MCP-2 (lane 6) induced homologous phosphorylation of CCR1 (~50 kDa). MCP-2-mediated phosphorylation was less than that of RANTES and MIP-1α. Heterologous phosphorylation by PMA (lane 3) was also lower (~50% less) than that of RANTES and MIP-1α. No phosphorylation of CCR1 was detected with cpt-cAMP (lane 4).

Homologous phosphorylation of CCR1 by RANTES (Fig. 4B, lanes 3 and 4) or MCP-2 (lanes 5 and 6) was partially inhibited by pretreatment of the cells with the protein kinase C inhibitor staurosporine (100 nM). PMA-induced heterologous phosphorylation of CCR1 was totally blocked by staurosporine (lanes 7 and 8). These results suggest that CCR1 is susceptible to phosphorylation by a GRK- and a protein kinase C-dependent mechanism.

IL-8-mediated Cross-desensitization of CCR1—It was determined whether CCR1-mediated cellular responses are regulated by cross-desensitization. Double transfectant RBL cells expressing CCR1 (7354 ± 462 receptors/cell) and the receptors for IL-8, CXCR1 (7009 ± 131 receptors/cell), CXCR1-CXR1, or CXCR2 (7452 ± 387 receptors/cell), CXCR2-CCR1, were constructed. The Kd for CXCR1 (1.19 ± 0.69 nM) and CXCR2 (3 ± 0.73 nM) in RBL co-expressing CCR1 were similar to that of RBL cells expressing single receptor (19). RANTES- and MIP-1α-mediated Ca2+ mobilization by CCR1 was cross-desensitized (~65%) by CXCR1 or CXCR2 upon pretreatment of the cells with IL-8 (Table I). Pretreatment of the cells with a first dose of either RANTES or MIP-1α diminished Ca2+ mobiliza-
Regulation and Cross-regulation of CCR1

TABLE I

| Cells/treatment | Ca2+ mobilization (%) | Cross-desensitization (%) |
|-----------------|-----------------------|---------------------------|
| CXCR1-CCR1      |                       |                           |
| IL-8 → RANTES   | 658 ± 63 → 254 ± 19   | 66                        |
| RANTES → IL-8   | 768 ± 62 → 271 ± 47   | -5                       |
| IL-8 → MIP1α    | 735 ± 34 → 260 ± 10   | 62                       |
| MIP1α → IL-8    | 697 ± 14 → 299 ± 23   | -8                       |
| CXCR2-CCR1      |                       |                           |
| IL-8 → RANTES   | 562 ± 18 → 175 ± 7    | 67                       |
| RANTES → IL-8   | 527 ± 34 → 362 ± 13   | 35                       |
| IL-8 → MIP1α    | 625 ± 26 → 178 ± 5    | 70                       |
| MIP1α → IL-8    | 590 ± 14 → 412 ± 21   | 34                       |
| 331T-CCR1       |                       |                           |
| IL-8 → RANTES   | 550 ± 27 → 128 ± 36   | 75                       |
| RANTES → IL-8   | 499 ± 32 → 386 ± 10   | 30                       |
| IL-8 → MIP1α    | 571 ± 25 → 174 ± 19   | 67                       |
| MIP1α → IL-8    | 530 ± 16 → 416 ± 9    | 27                       |

Notes: Pretreatment of CXCR2-CCR1 cells with RANTES (100 nM) inhibited GTPase activity mediated by both RANTES (~50%) and IL-8 (~40%) in membranes (Fig. 5). IL-8 pretreatment also inhibited GTPase activity in response to both IL-8 (~50%) and RANTES (~40%). Pretreatment with IL-8 for 5 min caused ~50% desensitization of IL-8-mediated GTPase activity in membranes, whereas ~90% of CXCR2 are internalized after 1–2 min of exposure of the cells to IL-8 (Ref. 19, Fig. 7). Two factors may account for this difference: loss of phosphates group by part of the receptors during membrane preparation or recovery of internalized (i.e., non-desensitized) receptors from membranes vesicles.

Cross-phosphorylation of CCR1, CXCR1, and CXCR2—CXCR1-CCR1 and CXCR2-CCR2 RBL cells were 32P-labeled and stimulated with either RANTES (100 nM) or IL-8 (100 nM). The cell lysates were immunoprecipitated first with the 12CA5 antibody specific for the HA epitope tag expressed at the NH2 terminus of CCR1 and then with specific antibodies directed against the NH2 terminus of CXCR1 or CXCR2 (20). CXCR1 was homologously phosphorylated by RANTES (Fig. 6, A and B, lanes 5) and cross-phosphorylated upon stimulation by IL-8 of either CXCR1 (Fig. 6A, lane 6) or CXCR2 (Fig. 6B, lane 6). CXCR1 and CXCR2 were homologously phosphorylated by IL-8 (Fig. 6, A and B, lanes 3). CXCR2, but not CXCR1, was cross-phosphorylated by RANTES stimulation of CXCR1 (Fig. 2; compare lane 2 in panel B with lane 2 in panel A).

Internalization of CCR1—CXCR1 undergo rapid receptor internalization (~7 min) upon exposure of either CXCR1-CCR1- or CXCR2-CCR2-expressing cells to 100 nM RANTES (Fig. 7, panels A and C). Maximum internalization was ~90% after 60 min. RANTES had no effect on CXCR1 or CXCR2 (Fig. 7, panels B and D). IL-8 caused ~65% and ~95% internalization of CXCR1 and CXCR2, respectively (Fig. 7, panels B and D) but showed no significant decrease in 125I-RANTES binding to CXCR1 on either CXCR1-CCR1 or CXCR2-CCR2 cells (Fig. 7, panels A and C). Despite inducing CXCR1 cross-phosphorylation, treatment of double transfected cells with IL-8 did not cause CCR1 internalization, indicating cross-phosphorylation does not stimulate this process.

Expression and Characterization of CCR1 Mutants in RBL-2H3 Cells—In order to assess the role of phosphorylation in the desensitization CCR1, four receptor mutants lacking specific serine and threonine residues were constructed (Table II) and stably expressed into RBL-2H3 cells. Competition binding using 125I-RANTES and Scatchard analysis indicated that the pharmacological properties of the mutants are similar to that of the wild type CCR1 (Table III). The differences in Kd observed between the mutants and the wild type CCR1 (Table III) are not statistically significant (p > 0.05), as determined by paired test. Clones expressing similar receptor numbers (Table III) were utilized to determine the functional properties of the mutants CCR1 versus the wild type receptor. S2, S3, and ΔCCR1 were more active than CCR1 and S1 in mediating RANTES-induced PI hydrolysis (Fig. 8A). Peaks of intracellular Ca2+ mobilization in response to RANTES (10 nM) were similar for wild type and mutants CCR1 (Fig. 8B). However, a more sustained response was obtained with S2, S3, and ΔCCR1 as compared with CCR1 and S1. S2, S3, and ΔCCR1 were resistant to RANTES-induced receptor internalization, relative to CCR1 and S1 (Fig. 8C).

S2, S3, and ΔCCR1 were also resistant to RANTES and PMA-mediated receptor phosphorylation (Fig. 8D) and desensitization of GTPase activity in membranes (Fig. 8A). The resistance of S2 to phosphorylation relative to S1 and CCR1...
and CXCR1; or cells expressing RANTES (Fig. 9B) desensitized by pretreatment of the cells to a first dose of RANTES (A and C) or IL-8 (B and D) binding. The values are presented as percentage of total, which is defined as the total amount of 125I-ligand bound to control (untreated) cells. The experiment was repeated four times with similar results.

**FIG. 7.** Internalization of CCR1 in RBL cells. CXCR1-CCR1 or CXCR2-CCR1 cells (0.5 x 10^6 cells/well) were incubated with either RANTES (100 nM) or IL-8 (100 nM) for 0–60 min. Cells were then washed and assayed for 125I-RANTES (A and C) or 125I-IL-8 (B and D) binding. The values are presented as percentage of total, which is defined as the total amount of 125I-ligand bound to control (untreated) cells. The experiment was repeated four times with similar results.

**TABLE II**

| Amino acid sequences of the carboxyl-terminal tail of the wild type CCR1 and the serine and threonine residues either replaced with alanine or truncated in each mutants |
|---|
| Bold serine and threonine residues are potential phosphorylation sites in the wild type CCR1. |
| C-tail CCR1 | 308 |
| S1 | FRKYLRGLFRRVAVHLVKWLPFL |
| S2 | FRKYLRGLFRRVAVHLVKWLPFL |
| S3 | FRKYLRGLFRRVAVHLVKWLPFL |
| ΔCCR1 | FRKYLRGLFRRVAVHLVKWLPFL |
| S3-CCR1 | 355 |
| S3-AAR | FRKSAPAGFHELKSSTS |
| S3-AP | FRKSAPAGFHELKSSTS |
| S3-P | FRKSAPAGFHELKSSTS |
| S3-CKS | FRKSAPAGFHELKSSTS |

Table shows 125I-RANTES binding site per cell and the apparent affinity binding (Kd) values for wild type and CCR1 mutants shown in Table II.

| Ligand binding affinity of wild type and mutant CCR1 expressed in RBL-2H3 cells |
|---|
| Table shows 125I-RANTES binding site per cell and the apparent affinity binding (Kd) values for wild type and CCR1 mutants shown in Table II. |

| Kd (nM) | Bmax (receptors/cell) |
|---|---|
| CCR1 | 12 ± 2 | 7354 ± 462 |
| S1 | 7.5 ± 0.9 | 6731 ± 537 |
| S2 | 10.2 ± 4 | 7011 ± 582 |
| S3 | 15.1 ± 3 | 8031 ± 603 |
| ΔCCR1 | 19 ± 5 | 7273 ± 312 |

indicate that the phosphorylation sites for CCR1 are located in the cluster of serine and threonine which comprises amino acids 340–346. Ca^{2+} mobilization in response to RANTES was desensitized by pretreatment of the cells to a first dose of RANTES (Fig. 9B). However, S2, S3, and ΔCCR1 (–67%, –59%, and 61%, respectively) were more resistant to desensitization than S1 and CCR1 (–82% and 86%, respectively).

Cross-phosphorylation and Cross-desensitization of S3 and ΔCCR1—RBL-2H3 cells co-expressing the CCR1 mutant S3 (8031 ± 603 receptors/cell) and CXCR1 (6585 ± 911 receptors/cell), (S3-CXCR1) or CXCR2 (6890 ± 511 receptors/cell), (S3-CXCR2); or cells expressing ΔCCR1 (7273 ± 312 receptors/cell) and CXCR1 (6990 ± 107 receptors/cell), ΔCCR1-CXCR1, or CXCR2 (7121 ± 539 receptors/cell), ΔCCR1-CXCR2, were generated to determine the role of receptor cross-phosphorylation on CCR1 cross-desensitization. S3 and ΔCCR1 were resistant to cross-phosphorylation by both CXCR1 and CXCR2 upon IL-8 activation (data not shown). In contrast to CCR1, RANTES stimulation of either S3 or ΔCCR1 resulted in cross-phosphorylation of both CXCR1 (Fig. 10, lanes 2 and 6) and CXCR2 (data not shown). CXCR1 was also homologously phosphorylated by IL-8 (Fig. 10, lanes 3 and 5). S3 and ΔCCR1 activation by RANTES caused no internalization of either CXCR1 or CXCR2 (data not shown).

S3- and ΔCCR1-mediated Ca^{2+} mobilization in response to RANTES were cross-desensitized by prior exposure of the double transfectant cells to IL-8 (Table IV), although to a lower extent than the wild type CCR1 (Table I). Both CXCR1- and CXCR2-mediated Ca^{2+} mobilization in whole cells and GTPase activity in membranes were cross-desensitized upon activation of S3 and ΔCCR1 by RANTES (Table IV, data not shown).

**CCR1 and ΔCCR1-mediated PLCβ mediation**

PLCβ has been shown to be the only PLCβ isozyme expressed in RBL cells (21, 22). Whether CCR1 activation resulted in PLCβ phosphorylation was studied. As shown in Fig. 11A, both CCR1 and ΔCCR1 mediated RANTES-induced phosphorylation of PLCβ to an extent similar (–2-fold over basal) (lanes 2 and 3) to that of CXCR1, CXCR2, and 331T (19). CCR1 induced phosphorylation of PLCβ to an extent similar to that of cpt-cAMP (–2-fold over basal) (Fig. 11B, lanes 2 and 3) but lower than that of PMA (–3-fold over basal) (lane 4).

**DISCUSSION**

Chemokines are inflammatory mediators of the chemotactic and cytotoxic functions of a large variety of cells including neutrophils, monocytes, eosinophils, basophils, and lymphocytes. Most chemokines activate more than one receptor on leukocytes. This redundancy in receptor activation has hampered the investigation of their mechanisms of regulation. In this study, the CCR1 receptor, which binds RANTES, MIP-1α, MCP-2, and MCP-3 with high affinity (1–3), was stably expressed in the leukocyte-like RBL-2H3 cell line. The data presented herein demonstrate that CCR1-mediated responses to RANTES, MIP-1α, and MCP-2 are regulated via receptor phos-
Prior exposure of cells expressing CCR1 to RANTES, MIP-1α, MCP-2, or PMA, which causes phosphorylation of the receptor (Fig. 2), inhibited both Ca²⁺ mobilization in whole cells and GTPase activity in membranes. Second, stimulation with IL-8 of either CXCR1 (CXCR1-CCR1 cells) or CXCR2 (CXCR2-CCR1 cells), which results in CCR1 cross-phosphorylation, desensitized RANTES- and MIP-1α-mediated GTPase activity and Ca²⁺ mobilization (Fig. 5 and Table I). Third, alanine substitution of specific serine and threonine residues as well as truncation of the cytoplasmic tail of CCR1 abolished receptor phosphorylation and desensitization of G protein activation but not desensitization of receptor-mediated Ca²⁺ mobilization (Figs. 8 and 9, Table II).

The cAMP analog, cpt-cAMP, which caused phosphorylation of PLCβ₃ but not CCR1, inhibited Ca²⁺ mobilization to RANTES, MIP-1α, and MCP-2 (Figs. 2 and 11). MIP-1α induced dose- and time-dependent increases in intracellular cAMP levels in the human megakaryocytic leukemia cell line M07e (23). In addition, RANTES- and MIP-1α-mediated lymphocyte uropod formation and adhesion receptor redistribution were inhibited by the cAMP-dependent protein kinase inhibitor H-89, suggesting a role for PKA as a downstream regulator of CCR1 (24).
Cross-desensitization of S3 and ΔCCR1-mediated Ca\(^{2+}\) mobilization by CXCR1 and CXCR2

RBL-2H3 cells (5 \times 10^5 cells/assay) expressing S3-CXCR1, S3-CXCR2, ΔCCR1-CXCR1, and ΔCCR1-CXCR2 were loaded with Indo-1 and stimulated with either RANTES (10 nM) or IL-8 (10 nM). Cells were rechallenged 3 min later with a second dose of the indicated ligand and peak intracellular Ca\(^{2+}\) mobilization was determined. Data are the means ± S.E. of three different experiments.

| Cells/treatment | Ca\(^{2+}\) mobilization | Desensitization |
|-----------------|--------------------------|-----------------|
| S3-CXCR1        |                          |                |
| IL-8 \(\rightarrow\) RANTES | 545 ± 18 \(\rightarrow\) 434 ± 23 | 39 |
| RANTES \(\rightarrow\) IL-8   | 712 ± 12 \(\rightarrow\) 272 ± 25 | 42 |
| S3-CXCR2        |                          |                |
| IL-8 \(\rightarrow\) RANTES | 561 ± 45 \(\rightarrow\) 461 ± 37 | 31 |
| RANTES \(\rightarrow\) IL-8   | 669 ± 21 \(\rightarrow\) 207 ± 39 | 63 |
| ΔCCR1-CXCR1     |                          |                |
| IL-8 \(\rightarrow\) RANTES | 592 ± 33 \(\rightarrow\) 302 ± 41 | 42 |
| RANTES \(\rightarrow\) IL-8   | 516 ± 12 \(\rightarrow\) 365 ± 22 | 38 |
| ΔCCR1-CXCR2     |                          |                |
| IL-8 \(\rightarrow\) RANTES | 672 ± 35 \(\rightarrow\) 514 ± 44 | 23 |
| RANTES \(\rightarrow\) IL-8   | 666 ± 32 \(\rightarrow\) 295 ± 10 | 56 |

Table IV

Cross-desensitization of S3 and ΔCCR1-mediated Ca\(^{2+}\) mobilization by CXCR1 and CXCR2

An unexpected finding is that CCR1 failed to cross-phosphorylate and cross-desensitize responses to CXCR1 (Fig. 6 and Table I). Previous studies in neutrophils and transfected RBL-2H3 cells have shown that responses to both CXCR1 and CXCR2, including GTPase activity and Ca\(^{2+}\) mobilization, are cross-desensitized by fMLP receptor and C5α receptor (19, 26–28). It was also shown that response CXCR1 was resistant to cross-regulation by CXCR2 (19). However, truncation of the cytoplasmic tail of CXCR2 (331T), which prolongs its signaling and increases its resistance to internalization led to cross-regulation of CXCR1 (19, 29, 30). Thus, the resistance of CXCR1 to cross-desensitization by CCR1 may also be due to the strength of the CCR1-mediated signal, which may not be sufficient to trigger the cross-desensitization mechanism required for CXCR1, although it is sufficient to cross-desensitize CXCR2. Indeed, S3 and ΔCCR1, which generated greater signals and were more resistant to internalization than CCR1 (3–5% (S3 and ΔCCR1) versus ~90% (CCR1) after 60 min), cross-phosphorylated and cross-desensitized CXCR1 (Fig. 10, Table IV).

Of interest is that CCR1 cross-desensitized Ca\(^{2+}\) responses to CXCR2 as well as the phosphorylation-resistant mutant of CXCR2, 331T, to the same extent (Table I). These results indicate that cross-desensitization of Ca\(^{2+}\) mobilization among CCR1 and CXCR2 is independent of receptor phosphorylation and further suggest the importance of downstream effector(s) in receptor-mediated cross-desensitization. The downstream effector(s) involved in cross-desensitization of Ca\(^{2+}\) mobilization is not known. However, several studies have indicated that phosphorylation of PLCβ upon receptor activation may result in a decrease of PLCβ-mediated inositol trisphosphate production and, thus, inhibition of intracellular Ca\(^{2+}\) mobilization (21, 22, 30, 31). Indeed, CCR1 as well as CXCR1 and CXCR2 induced PLCβ phosphorylation upon activation (Fig. 11). Nonetheless, phosphorylation of PLCβ cannot of itself explain downstream cross-desensitization since, despite mediating PLCβ phosphorylation to the same extent (~2-fold over basal), experiments were conducted. Several chemokine receptors, including CCR1, have been shown to couple to different G proteins to transduce signals, depending on the cell type in which they are being expressed (25).
both CCR1 and CXCR2 failed to cross-desensitize responses to CXCR1. Thus, an additional process must be involved. Since the only PLCβ isozyme expressed in RBL cells is PLCβ3 (21), this result may indicate that cross-desensitization requires modification of an additional signaling component needed to activate PLCβ3 and that CXCR1 versus CCR1 and CXCR2 use different pathways. Supporting that contention is the report to cross-desensitization by CCR1 but not by its phosphorylation and modification of downstream effector. CXCR1 was resistant to sensitize each other at two levels: receptor/G protein coupling and internalization of receptors with similar ligand specificity but different signal lengths based on cytoplasmic tail phosphorylation (32).

In summary, these data demonstrate that CCR1-mediated responses to RANTES, MIP-1α, and MCP-2 are regulated via multiple mechanisms of desensitization including homologous (presumably via a GRK-dependent mechanism), heterologous (via second messenger activated kinases), and class desensitization (inhibition of PLCβ activation) by CXC chemokines. In addition, they demonstrate that CCR1 and CXCR2 cross-desensitize each other at multiple levels: receptor/G protein coupling and modification of downstream effector. CXCR1 was resistant to cross-desensitization by CCR1 but not by its phosphorylation and modification of an additional signaling component needed to activate PLCβ3 and that CXCR1 versus CCR1 and CXCR2 use different pathways. Supporting that contention is the report that, in addition to phosphorylation of PLCβ, modification of either G proteins or G protein-related proteins such as RGS (regulators of G protein signaling) may also required for the regulation of PLCβ signaling (32).

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