Allosteric Transinhibition by Specific Antagonists in CCR2/CXCR4 Heterodimers*\textsuperscript{1,2}

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Chemokine receptors are presently used as targets for candidate drugs in the frame of inflammatory diseases and human immunodeficiency virus infection. They were shown to dimerize, but the functional relevance of dimerization in terms of drug action remains poorly understood. We reported previously the existence of negative binding cooperativity between the subunits of CCR2/CCR5 heterodimers. In the present study, we extend these observations to heterodimers formed by CCR2 and CXCR4, which are more distantly related. We also show that specific antagonists of one receptor inhibit the binding of chemokines to the other receptor as a consequence of their heterodimerization, both in recombinant cell lines and primary leukocytes. This resulted in a significant functional cross-inhibition in terms of calcium mobilization and chemotaxis. These data demonstrate that chemokine receptor antagonists regulate allosterically the functional properties of receptors on which they do not bind directly, with important implications on the effects of these potential therapeutic agents.

CCR2 is a member of the CC chemokine receptor family that plays an important role in the recruitment of monocytes to atherosclerotic lesions and in the formation of intimal hyperplasia after arterial injury (3). Two isoforms of CCR2 have been described that are generated by alternative splicing and differ in their C-terminal tail, but only the longer variant (CCR2b) is expressed at high level in leukocytes. CCR2 interacts with MCP-1 (CCL2) and other CC chemokines such as MCP-2 (CCL7), MCP-3 (CCL8), and MCP-4 (CCL13) (4). CCR2 was shown to form homodimers, as well as heterodimers with CCR5, its closest structural relative within the chemokine receptor family, resulting in a negative binding cooperativity of allosteric nature (5–8). CCR2 was also reported recently to form heterodimers with CXCR4, a more distantly related chemokine receptor (9). However, no pharmacological characterization of this heterodimer was performed so far. CXCR4 binds specifically SDF-1α, an evolutionary conserved chemokine known to play a role in a number of physiological processes, such as the homing of T cell populations to sites of inflammation and the maintenance of the cellular microenvironment in bone marrow (10). SDF-1α and CXCR4 have also been shown to control the migration of neuronal cell populations during brain development (11), to regulate the growth and/or survival of tumoral cells (12, 13), and to play a role in the metastatic spread of various tumors (14, 15). In addition, CXCR4 is the main coreceptor for the T cell line tropic human immunodeficiency virus, type 1 strains (16). CXCR4 and CCR2 share only 34% of amino acid identity over their entire length. The ligand binding specificity of CCR2 and CXCR4 has been mapped to the extracellular domains of the receptors, particularly the second extracellular loop (17). Both CXCR4 and CCR2 are expressed by memory T lymphocytes and the monocyte-macrophage lineage (18, 19). As other chemokine receptors, they are coupled to the G\textsubscript{αi} class of heterotrimeric G proteins, inhibit adenyl cyclase, promote intracellular calcium mobilization, stimulate the mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathways, and promote chemotaxis. With the aim of understanding better the functional consequences of chemokine receptor oligomerization, we have generated cells coexpressing CCR2 and CXCR4 and characterized them in terms of binding and signaling properties. We confirmed that CCR2 and CXCR4 form constitutive heterodimers, which appear unaffected by chemokine stimulation. As previously described for the CCR2/CCR5 heterodimer, negative binding cooperativity was observed between CCR2 and CXCR4 when coexpressed, a receptor heterodimer binding only a single chemokine with high affinity. In addition, it was...
shown that specific antagonists of one receptor can inhibit the binding of chemokines to receptors on which these antagonists do not bind, resulting in a cross-inhibition of the functional response, and these observations were attributed as well to an allosteric interaction between the two binding sites of a dimer. Finally, similar observations were made on primary T cells ex vivo, demonstrating the functional relevance of CCR2/CXCR4 heterodimers in native cells.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Phycoerythrin-labeled anti-CXCR4 (MAB173), anti-CCR2 (FAB151P) monoclonal antibodies, and recombinant chemokines were obtained from R & D Systems. The DOC-1 anti-CCR2 antibody was kindly provided by Matthias Mack (University of Regensburg, Regensburg, Germany). TAK-779 was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program, Division of AIDS, NIAID and AMD3100 from Sigma-Aldrich.

**Cell Lines and Leukocyte Populations**—CHO-K1 cells were cultured in Ham’s F12 medium supplemented with 10% fetal bovine serum (Invitrogen), 100 units/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). The CCR2 coding sequence was cloned between the BamHI and XbaI sites of the bicstronic expression vector pEFIB3, as described (20), and the construct was transfected by FuGENE (Roche Applied Science) into a CHO-K1 cell line expressing apoaequorin, Get16 and wild-type CXCR4. Cells expressing CCR2 were selected by 10 µg/ml blasticidin (Invitrogen). Human peripheral blood lymphocytes were prepared from buffy coats of healthy blood donors as described previously (8). CD4⁺ blasts were generated by incubating the lymphocytes with anti-CD3 (1:100) and anti-CD28 (1:1000) antibodies for 3 days. The cells were maintained in a medium supplemented with human interleukin 2 (2 ng/ml; R & D Systems) for an additional 7 days.

**Bioluminescence Resonance Energy Transfer Assays**—The cDNAs encoding EYFP³ and a humanized form of *Renilla* luciferase were fused in frame to the 3’ end of CXCR4 and CCR2 cDNAs in the pcDNA3.1 vector. A BRET protocol adapted to cell monolayers was used (21). Briefly, human embryonic kidney cells (HEK-293T) were transfected by the calcium phosphate precipitation method, using a constant amount of plasmid DNA but various ratios of plasmids encoding the fusion protein partners (22). A control corresponding to mock transfected cells was included to subtract raw basalm fluorescence and fluorescence from the data. Expression of EYFP fusion proteins was estimated by measuring fluorescence at 535 nm following excitation at 485 nm, using a Mithras LB940 multilabel reader (Berthold). Expression of Rluc fusion proteins was estimated by measuring the luminescence of the cells after incubation with 2.5 µM coelenterazine H (Promega Corporation) and then diluted 5-fold before use. Variable concentrations of chemokines were added to cell suspension (25,000 cells/well), and luminescence was measured for 30 s in an EG & G Berthold luminometer (PerkinElmer Life Sciences). Half-maximal effective concentrations (EC₅₀) were determined with the PRISM software (Graphpad Softwares) using nonlinear regression applied to a sigmoidal dose-response model.

**Intracellular Calcium Mobilization Assay**—The functional responses were analyzed with an aequorin-based assay as described (23). The cells were incubated for 4 h in the dark in the presence of 5 µM coelenterazine H (Promega Corporation) and then diluted 5-fold before use. Variable concentrations of chemokines were added to cell suspension (25,000 cells/well), and luminescence was measured for 30 s in an EG & G Berthold luminometer (PerkinElmer Life Sciences). Half-maximal effective concentrations (EC₅₀) were determined with the PRISM software (Graphpad Softwares) using nonlinear regression applied to a sigmoidal dose-response model.
response to the chemoattractant over cells migrating toward medium.

RESULTS

Heterodimerization of CCR2 and CXCR4 in Recombinant Cell Lines—In previous studies, we showed that CCR2 forms constitutive homodimers, as well as heterodimers with its close structural relative CCR5 (6, 7). The functional consequence of this heterodimerization was a negative binding cooperativity between the CCR5 and CCR2 ligands, as a result of an allosteric interaction between the two binding sites of the heterodimer (8). In this work, we investigated further the ability of CCR2 to interact with more distant members of the chemokine receptor family and whether a negative binding cooperativity could apply as well for other such heterodimers. In a first step, we investigated by a BRET technique the extent of heterodimerization between CXCR4-hRluc and CCR2-EYFP. The BRET50 and BRETmax values were obtained from three independent experiments carried out with triplicate data points. The values represent the means ± S.E.

|          | SDF-1α | MCP-1 |
|----------|--------|-------|
| **No stimulation** |        |       |
| CCR2 hRluc/CXCR4 EYFP | 0.13 ± 0.02 | 0.14 ± 0.00 |
| CXCR4 hRluc/CCR2 EYFP | 0.13 ± 0.03 | 0.18 ± 0.01 |

The estimated KD values for CXCR4 and CCR2 were coexpressed. In the 125I-SDF-1α competition binding assay, SDF-1α competed exclusively CCR2 (Fig. 1B). MCP-1 was a stronger competitor than SDF-1α (IC50 = 0.01 nM) (Fig. 1C). Similarly, MCP-1 was a stronger competitor than SDF-1α (IC50 = 0.02 ± 0.01 nM) (Fig. 1D). The IC50 values were representative of two independent experiments.

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Construction and Characterization of Cell Lines Coexpressing CXCR4 and CCR2—To study the functional consequences of the heterodimerization, CHO-K1 cell lines stably coexpressing CXCR4 and CCR2 were isolated and analyzed for the level of expression over time. The parental CHO-K1 cell line expressing CXCR4 (clone 40) and CCR2 (clone 3) were selected. The expression of the two receptors by FACS and saturation binding assays (data not shown). The parental CHO-K1 cell line expressing CXCR4 (clone 40) and CCR2 (clone 3) was used as the recipient for CCR2 coexpression, and the C2X4–40 clone expressing both receptors was selected (7). In saturation binding assays, the Bmax was estimated to 3.2 ± 0.07 pmol/mg proteins for CXCR4 and to 2.5 ± 0.04 pmol/mg proteins for CCR2. FACS analysis demonstrated that the selected clones were homogeneous in terms of receptor expression and regular testing confirmed the stability of this expression over time.

Binding Properties of Cells Expressing CXCR4 and CCR2—We next examined the ability of the ligands of each receptor to compete for 125I-SDF-1α and 125I-MCP-1 binding to membranes of cells expressing CXCR4 and/or CCR2. As expected, SDF-1α inhibited with high affinity the binding of 125I-SDF-1α on membranes containing CXCR4 only, whereas MCP-1 had no effect (Fig. 1A). Similarly, MCP-1 but not SDF-1α competed efficiently the binding of 125I-MCP-1 on membranes containing exclusively CCR2 (Fig. 1B). A drastic modification of the competition patterns was observed when CXCR4 and CCR2 were coexpressed. In the 125I-SDF-1α binding assay, SDF-1α behaved similarly, but MCP-1 now competed partially (IC50 = 0.02 ± 0.01 nM) (Fig. 1C). Conversely, in the 125I-MCP-1 binding assay, competition by MCP-1 was unaffected, whereas a significant competition by SDF-1α was observed (IC50 = 0.78 ± 0.09 nM) (Fig. 1D). The IC50 values were calculated for heter-
TABLE 2

Binding parameters of CHO-K1 cells expressing CCR2 and/or CXCR4

The binding parameters were measured on CHO-K1 cells expressing CCR2 and/or CXCR4. The IC50 and percentage of inhibition were obtained from competition binding experiments as displayed in Figs. 1 and 2. The values represent the means ± S.E. of at least three independent experiments.

| Cells | Tracer | Competitor | IC50 (nM) | Inhibition (%) |
|-------|--------|------------|-----------|----------------|
| CCR2  | 125I-MCP-1 | MCP-1       | 0.18 ± 0.09 | 100            |
|       |        | TAK-779    | 0.25 ± 0.07 | 91.3 ± 3.2    |
|       |        | AMD-3100   | 0.81 ± 0.05 | 100            |
| CXCR4 | 125I-SDF-1α | SDF-1α     | 0.79 ± 0.06 | 100            |
|       |        | AMD-3100   | 0.09 ± 0.01 | 36.2 ± 6.0    |
|       |        | TAK-779    | 0.03 ± 0.00 | 90.7 ± 8.3    |
| CCR2 + CXCR4 | 125I-MCP-1 | MCP-1       | 0.14 ± 0.02 | 100            |
|       |        | SDF-1α     | 0.78 ± 0.09 | 39.7 ± 5.5    |
|       |        | AMD-3100   | 0.09 ± 0.01 | 36.2 ± 6.0    |
|       |        | TAK-779    | 0.03 ± 0.00 | 90.7 ± 8.3    |
| CCR2 + CXCR4 | 125I-SDF-1α | SDF-1α     | 0.19 ± 0.03 | 100            |
|       |        | MCP-1      | 0.02 ± 0.01 | 33.9 ± 6.7    |
|       |        | AMD-3100   | 0.04 ± 0.00 | 87.1 ± 3.1    |
|       |        | TAK-779    | 0.08 ± 0.01 | 38.1 ± 3.0    |

To characterize further the negative binding cooperativity between CXCR4 and CCR2, the rate of radioligand dissociation from heterodimers, in the presence or absence of chemokines or antagonists, was assayed in “infinite” tracer dilution conditions (8). We showed that, on membranes prepared from cells expressing CCR2 only, dissociation of prebound 125I-MCP-1 was slow in the absence of unlabeled ligands (t1/2 > 200 min; Fig. 3 E and Table 3), but complete dissociation was observed after 24 h (not shown). The dissociation rate of 125I-MCP-1 was increased when CCR2 and CXCR4 were coexpressed (t1/2 = 27.6 min; Fig. 3F). Conversely, the presence of CCR2 increased significantly the dissociation rate of prebound 125I-SDF-1α from CXCR4 (t1/2 = 35.6 min versus t1/2 > 200 min; Fig. 3, A and B). As expected, we showed that the specific CXCR4 ligands SDF-1α and AM3100 had no effect on the dissociation of bound 125I-MCP-1 from membranes containing CCR2 only. However, both molecules accelerated the dissociation of 125I-SDF-1α from CXCR4, but also of 125I-MCP-1 when CXCR4 and CCR2 were coexpressed (t1/2 = 14.7 and 23.0 min, respectively; Fig. 3, F and H). Similarly, MCP-1 and TAK779 promoted a rapid dissociation of 125I-MCP-1 from CCR2 and of 125I-SDF-1α when CXCR4 and CCR2 were coexpressed (t1/2 = 15.4 and 13.8 min, respectively; Fig. 3, B and D). These data suggest that the stability of the interaction of CXCR4 and CCR2 with their respective chemokine ligands is lower in heterodimers than in homodimers. In addition, the binding of a chemokine agonist or a small molecule antagonist to one of the partners in a heterodimer modifies further the conformation in the other partner, resulting in a faster dissociation rate of the tracers.

Functional Properties of Cells Coexpressing CXCR4 and CCR2—We first compared the functional response of cells coexpressing CXCR4 and CCR2 to cell lines expressing either CCR2 or CXCR4. The concentration-action curve of calcium mobilization following stimulation by MCP-1 was similar for cells coexpressing both receptors or expressing CCR2 only.
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Similarly, the functional response to SDF-1α was identical in CXCR4-expressing cells whether CCR2 was coexpressed or not (supplemental Fig. S3). We also showed that the costimulation by MCP-1 and SDF-1α at equimolar concentrations resulted in cells coexpressing the two receptors, in a functional response essentially similar to that of MCP-1 alone, which is the ligand displaying the highest potency on its receptor. These data indicate the absence of cooperative signaling between CCR2 and CXCR4 in this calcium mobilization assay (supplemental Table S1). We next investigated the effect of specific antagonists in cells coexpressing both receptors. In line with the binding data, AMD3100 antagonized CXCR4, but also CCR2 signaling, whereas TAK-779 inhibited both CCR2 and CXCR4 signaling, demonstrating the ability of antagonists to inhibit the signaling of receptors on which they do not bind directly (Fig. 4).

![Figure 3. Dissociation kinetics in nonequilibrium binding assays.](image)

**TABLE 3**

Dissociation rates from CHO-K1 cells expressing CCR2 and/or CXCR4

| Cells          | Tracer   | Ligands | Half-life (min) |
|----------------|----------|---------|-----------------|
| CCR2           | 125I-MCP-1 | Buffer  | >200            |
|                |          | SDF-1α  | >200            |
|                |          | AMD-3100| 7.0 ± 2.8       |
|                |          | TAK-779 | 11.4 ± 5.9      |
| CXCR4          | 125I-SDF-1α| Buffer  | >200            |
|                |          | SDF-1α  | >200            |
|                |          | MCP-1   | 8.2 ± 1.9       |
|                |          | AMD-3100| 12.1 ± 3.9      |
|                |          | TAK-779 | >200            |
| CCR2 + CXCR4   | 125I-MCP-1| Buffer  | 3.9 ± 2.9       |
|                |          | SDF-1α  | 13.3 ± 3.9      |
|                |          | AMD-3100| 15.4 ± 7.7      |
|                |          | TAK-779 | 8.3 ± 2.9       |
|                |          | MCP-1   | 10.3 ± 2.9      |

![Figure 4. Aequorin-based functional assay, antagonists.](image)

**Functional CCR2-CXCR4 Heterodimerization in Native Cells**—Finally, we investigated whether negative binding cooperativity could be demonstrated in cells expressing CCR2 and CXCR4 endogenously. Human CD4+ T lymphocytes were isolated and activated with anti-CD3 antibodies (OKT3) and interleukin 2. Specific 125I-MCP-1 and 125I-SDF-1α binding could be
Competition binding assays were performed on CD4 blasts by using 125I-MCP-1 partially by the CXCR4-specific antagonist AMD3100. Similarly, migration toward MCP-1 was inhibited by TAK-779 but also suggesting the absence of cooperative signaling in this setting. Cell equimolar concentrations was similar (data not shown), suggesting CCR2 and CXCR4, 125I-MCP-1 binding was inhibited by AMD3100 (Fig. 5, C and D). Altogether, these data indicate that CCR2-CXCR4 heterodimers do exist in native cells and that negative binding cooperativity occurs between the two binding pockets of these receptor heterodimers. Importantly, these data also demonstrate that chemokine receptor heterodimerization results in a cross-inhibitory effect of small molecule antagonists on the functional responses of receptors on which these antagonists do not bind.

**DISCUSSION**

During the past few years, the number of reports demonstrating GPCR dimerization has increased tremendously, and it is presently well accepted that most GPCRs are able to form homodimers (24). Chemokine receptors make no exception to this rule, and dimerization was reported so far for four chemokine receptors: CCR2, CCR5, CXCR2, and CXCR4 (8). Coimmunoprecipitation, BRET, and fluorescence resonance energy transfer experiments have shown that CCR2 and CCR5 form both homo- and heterodimers constitutively (7). Although ligand-promoted dimerization was suggested at some point (25), it was later demonstrated that stimulation by chemokines did not affect the dimerization status of either homo- or heterodimers (26). In a previous study, we have demonstrated that heterodimerization between CCR2 and CCR5 resulted in a strong negative binding cooperativity of allosteric nature (7, 8). To extend the concept of allosteric interactions between dimer units, we showed by BRET that CCR2 and CXCR4 were able to form constitutive homo- and heterodimers and investigated the influence of this process on the pharmacological properties of both receptors. Following coexpression of CCR2 and CXCR4, we observed negative cooperativity interactions between the two receptor-binding pockets, very similar to what we reported previously for the CCR5/CCR2 heterodimer. The CXCR4-specific ligands inhibited MCP-1 binding when CXCR4 and CCR2 were coexpressed. Conversely, CCR2-specific ligands competed for SDF-1α binding only in cells coexpressing both receptors. The extent of this cross-competition (35%) is compatible with the expected proportion of receptors involved in the formation of heterodimers, considering similar expression levels for the two receptors, and no major differences in their relative tendency to form homo- or heterodimers. Dissociation kinetics after extensive tracer dilution showed that chemokines specific for one receptor can promote the dissociation of the tracer bound on the other receptor, demonstrating unambiguously the allosteric nature of the negative binding cooperativity. Interestingly, the coexpression of CCR2 and CXCR4 in CHO-K1 cells increased the spontaneous dissociation rate of both SDF-1α and MCP-1. Such changes in basal dissociation rates were not detected when CCR5 was coexpressed with CCR2 (8) and reflect probably the modification of the conformation of the receptors, according to the partners with which they interact. Additional studies will be required to determine whether such changes in kinetics properties can be extended to other chemokine receptor heterodimers.

Our study also reveals unexpected properties of chemokine receptor antagonists. AMD3100 is a bicyclam molecule described as a specific CXCR4 antagonist that inhibits the bind-
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Inhibiting the functional responses of heterodimers may involve both the binding and signaling of specific receptors. For example, in CHO-K1 cells, the inhibition of functional responses is greater when two (or more) chemokine receptors are coexpressed in a leukocyte population. It should be noted that both on recombinant systems and native cells, the inhibition of functional responses is stronger than binding cross-competition. This observation suggests that the functional interaction between different receptors may go beyond binding inhibition across heterodimers. It may involve functional cross-talk in large arrays of dimers, as proposed for the organization of rhodopsin in photoreceptor discs (34, 35). Such arrays might also involve receptors belonging to other classes. CXCR4 was proposed to interact with the T cell receptor (TCR-CD3), allowing SDF-1α-promoted CXCR4 signaling through a pre-existing TCR-ZAP70 complex and leading to more robust Ras and ERK (extracellular signal-regulated kinase) activation (36). Interestingly, TCR ligation was also reported to inhibit the CXCR4- and CXCR3-mediated signaling and chemotaxis in T cells, indicating a close and complex relationship between T cell activation and chemokine signaling (31, 37).

The heterodimerization of chemokine receptors has important implications in the field of drug development and the validation of specific receptors as drug targets. Antagonists characterized as selective for a chemokine receptor may indeed inhibit as well the functional response of other receptors coexpressed in leukocyte populations. This cross-inhibition can therefore lead to effects in vivo more diverse than predicted on the basis of receptor selectivity determined in vitro. This can imply either an increased therapeutic benefit, as a result of the partial blockade of other receptors contributing to an inflammatory process or to the development of unexpected and detrimental side effects. These findings also imply that mouse invalidated for chemokine receptors may not represent ideal models for the pharmacological blockade of these receptors. Indeed, besides other established limitations (compensatory mechanisms during development, chronic versus acute inactivation, interspecies differences), the blockade of a receptor involved in heterodimers may have significantly different consequences than the absence of the receptor and therefore of all heterodimers that may form in cells. In addition, similar situations likely prevail in other receptor classes regulating other physiological functions. It was indeed reported recently that antagonists of the cannabinoid CB1 and the orexin-1 receptors regulate the cellular localization and the function of both receptors (33), although such demonstration was not extended to primary cells. Future evaluation of the therapeutic benefit of acting on chemokine receptors or other GPCRs classes will therefore have to consider the existence of heterodimers and the allosteric interactions that characterize them. We thus believe that these observations will have broad implications in the field of immunology and more generally in all aspects of biology involving GPCRs and their use as therapeutic targets.

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