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An Intracellular Allosteric Site for a Specific Class of Antagonists of the CC Chemokine G Protein-Coupled Receptors CCR4 and CCR5

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ABSTRACT

A novel mechanism for antagonism of the human chemokine receptors CCR4 and CCR5 has been discovered with a series of small-molecule compounds that seems to interact with an allosteric, intracellular site on the receptor. The existence of this site is supported by a series of observations: 1) intracellular access of these antagonists is required for their activity; 2) specific, saturable binding of a radiolabeled antagonist requires the presence of CCR4; and 3) through engineering receptor chimeras by reciprocal transfer of C-terminal domains between CCR4 and CCR5, compound binding and the selective structure-activity relationships for antagonism of these receptors seem to be associated with the integrity of that intracellular region. Published antagonists from other chemical series do not seem to bind to the novel site, and their interaction with either CCR4 or CCR5 is not affected by alteration of the C-terminal domain. The precise location of the proposed binding site remains to be determined, but the known close association of the C-terminal domain, including helix 8, as a proposed intracellular region that interacts with transduction proteins (e.g., G proteins and β-arrestin) suggests that this could be a generic allosteric site for chemokine receptors and perhaps more broadly for class A G protein-coupled receptors. The existence of such a site that can be targeted for drug discovery has implications for screening assays for receptor antagonists, which would need, therefore, to consider compound properties for access to this intracellular site.

The superfamily of G protein-coupled receptors (GPCRs) represents a productive area for drug discovery. Worldwide sales of drugs that act via GPCRs were estimated in 2004 to be in excess of $120 billion/year (Business Insights Research, 2005). In that same year, chemical programs to target GPCRs accounted for 40% of the portfolio of the pharmaceutical industry, with nearly 40% of these in clinical development (Business Insights Research, 2005).

Much of the progress in discovery of small-molecule ligands (i.e., <500 mol. wt.) for GPCRs comes from improvements in technologies to screen large numbers of compounds, where many campaigns will use functional assays with cell lines genetically engineered to express high amounts of the GPCR of interest, often coexpressed with a genetically modified G protein to improve signaling. This cell-based screening approach is supported by an understanding that the natural agonists for GPCRs interact with extracellular domains of the receptor that form a binding pocket, in analogy with the retinal-binding site of rhodopsin, and from mutagenesis studies of cloned GPCRs, which suggest that antagonists bind competitively at either the orthosteric or a syntopic site within the exofacial core of the transmembrane domains (Kristiansen, 2004). Structural modeling of GPCRs, based on homology with the resolved crystal structures of rhodopsin (Palczewski et al., 2000; Schertler, 2005), also is used to lead rational drug design, and it is centered on targeting the extracellular orthosteric site (Kristiansen, 2004). Allosteric regulators of class A GPCRs also exist, and although some of

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ABBREVIATIONS: GPCR, G protein-coupled receptor; CCR, CC chemokine receptor; SCH-C (SCH 351125), (4-bromophenyl) {1′-[(2,4-dimethyl-1-oxido-pyridin-3-yl) carbonyl]-4′-methyl-1,4′-bipiperidin-4-yl} methanone *O*-ethyloxime; BMS-397, *N*-(2,4-dichlorobenzyl)-2-{4-[(2*R*)-piperidin-2-ylcarbonyl]piperazin-1-yl}pyrido[2,3-*d*]pyrimidin-4-amine; BSA, bovine serum albumin; FB, fluorometric microvolume assay technology-Blue; PCR, polymerase chain reaction; [^{3}H]compound **1**, 5-chloro-*N*-(5,6-dichloro-3-[1,1,1-^{3}H]methoxy-pyrazin-2-yl)thiophene-2-sulfonamide; CCR4-5T, CCR4 with the C-terminal domain of CCR5; CCR5-4T, CCR5 with the C-terminal domain of CCR4; WT, wild type; CHO, Chinese hamster ovary; G_{qi5}, G_q protein α subunit with the last five C-terminal amino acids replaced with those from G_i protein α subunit; DMEM, Dulbecco's modified Eagle's medium; HEK, human embryonic kidney; TM, transmembrane; FLIPR, fluorometric imaging plate reader; for, forward; rev, reverse.

these are thought to make interactions with outer regions of the extracellular domain, their actual sites of action are not yet known (Christopoulos, 2002; Birdsall and Lazareno, 2005).

Receptors for chemoattractant cytokines (chemokines) are class A GPCRs that are thought to play a major role in diseases that include many inflammatory conditions, viral infections, and cancers (Proudfoot, 2002; Johnson et al., 2004; Saeki, 2004). With peptide ligands almost 20 times the size of a small-molecule drug, the chemokine receptors were initially assumed to be an unlikely target for the identification of competitive antagonists. After the discovery that small molecules can target chemokine receptors selectively, presumably through either syntopic competition or allosteric modulation, along with reports of their therapeutic potential in the clinic (e.g., Haringman et al., 2003), development of ligands at these GPCRs became a focus of activity in the pharmaceutical industry (Johnson et al., 2004; Saeki, 2004).

Identification of antagonists, such as SCH-C, at the CC chemokine receptor CCR5 has been the subject of considerable effort since it was identified as a coreceptor for the M-trophic form of human immunodeficiency virus (Kazmierski et al., 2003). CCR5, having CCL3 (macrophage inflammatory protein 1α), CCL4 (macrophage inflammatory protein 1β), and CCL5 (regulated upon activation, normal T cell expressed and secreted chemokine) as ligands, is also implicated in inflammatory diseases such as rheumatoid arthritis (Prahalad, 2006) and in autoimmune diseases such as multiple sclerosis and type 1 diabetes (Proudfoot, 2002; Ajuebor et al., 2006).

As the receptor for the CC chemokine ligands CCL17 (thymus and activation-regulated chemokine) and CCL22 (macrophage-derived chemokine), CCR4 has been identified as a potentially important drug target for the treatment of T cell-mediated inflammatory diseases such as asthma and atopic dermatitis (Panina-Bordignon et al., 2001; Schuh et al., 2002; Homey et al., 2006). CCR4 is also suggested to have a role in cancer, including some T-cell lymphomas and immunomodulation of tumor immunity through regulatory T cells (Ferenczi et al., 2002; Ishida and Ueda, 2006). Progress in the discovery of small-molecule CCR4 antagonists, such as BMS-397, has been reviewed recently (Purandare and Somerville, 2006).

A novel series of CCR4 antagonists was discovered by using a high-throughput recombinant cell-based functional assay of the activity of the CCR4 agonist CCL22 (Baxter et al., 2003a,b, 2005). The general properties of this pyrazinylsulfonamide series, including their high degree of selectivity against a wide range of receptors and other molecular targets, will be described elsewhere. Further work demonstrated that these compounds, although highly selective for CCR4, were also much weaker antagonists with a very similar structure-activity relationship at CCR5. In the course of a program to develop these antagonists an emerging pattern became clear for some compounds, having properties that deviated from the typical correlation of activity when comparing assays with membranes to those with intact cells. A study of these apparent outliers suggested that this entire series of CCR4 and CCR5 antagonists acts through a novel allosteric mechanism.

Materials and Methods

Materials. HEPES, sodium chloride, magnesium chloride, sodium hydroxide, and dimethyl sulfoxide were from Sigma-Aldrich (Poole, Dorset, UK). Bovine serum albumin (BSA) was obtained from Fluka (Gillingham, UK). Polypropylene 96-well plates and vented-cap cell culture flasks were from Costar (Corning, UK). Poly-D-lysine-coated 96-well plates were obtained from BD Biosciences (Oxford, UK). Heat-inactivated fetal calf serum was from Sigma-Aldrich. All other tissue culture reagents were purchased from Invitrogen (Paisley, UK). MicroScint-O was from PerkinElmer Life and Analytical Sciences (Waltham, MA). Fluo-3 AM was from Invitrogen. FuGENE 6 was obtained from Roche Diagnostics (Lewes, UK). Recombinant human BSA-free chemokines were from R&D Systems Europe Ltd. (Abingdon, Oxfordshire, UK). Fluorometric microvolume assay technology-Blue (FB)-labeled CCL5 and CCL22 were purchased from Applera (Warrington, UK). Reagents for the polymerase chain reaction (PCR) were supplied by Abgene (Epsom, UK). All other chemical reagents were analytical grade from Fisher Scientific (Loughborough, UK).

All of the chemokine antagonists described in this article (see chemical structures in Fig. 1) were supplied by the Department of Medicinal Chemistry, AstraZeneca Research and Development. Antagonists of the pyrazinyl-sulfonamide series were synthesized by using procedures similar to those disclosed previously (Baxter et al., 2003a,b, 2005). [^{3}H]compound **1** was prepared (2830.5 GBq/mmol) by displacement of the 3-chloro-substituent of the 3,5,6-trichloropyrazine derivative using tritiated methanol. SCH-C and BMS-397 were synthesized by using methods similar to those disclosed previously (Palani et al., 2001; Purandare, 2004).

Source and Culture of Stable Recombinant Cell Lines. Cells were cultured in a humidified atmosphere of 5% (v/v) CO_2 in air at 37°C in a standard incubator. CHO cells stably expressing human recombinant CCR4 (CCR4-CHO), obtained from Euroscreen (Brussels, Belgium), were cultured in NUT.MIX.F_12(HAM) with GlutaMAX-1 (Invitrogen) containing 10% (v/v) fetal calf serum and 0.4 mg/ml G-418 (Geneticin; Invitrogen). CHO cells stably expressing human recombinant CCR5 (CCR5-CHO), prepared by ligation of CCR5 cDNA isolated from human peripheral blood lymphocytes into pcDNA3.1 (Invitrogen), were cultured in DMEM containing 10% (v/v) fetal calf serum and 1 mg/ml G-418. HEK cells expressing human recombinant CCR4 (CCR4-HEK), prepared by ligation of CCR4 cDNA isolated from human T-helper 2 lymphocytes into pIRESneo2 (Clontech, Mountain View, CA), were cultured in DMEM with 2 mM glutamine and nonessential amino acids, containing 10% (v/v) fetal calf serum and 0.5 mg/ml G-418. An HEK line stably transfected with a plasmid expressing a human chimeric Gα subunit G_{qi5} ($G_q\alpha$ with the last five C-terminal amino acids replaced with those from $G_i\alpha$), was obtained from Molecular Devices (Wokingham, UK). HEK-G_{qi5} cells were cultured in DMEM with 2 mM glutamine and nonessential amino acids, containing 10% (v/v) fetal calf serum and 0.3 mg/ml hygromycin B. HEK-G_{qi5} cells expressing human recombinant chemokine receptors were cultured in similar media but with the addition of 0.5 mg/ml G-418.

Generation of Plasmids for Transient Transfection. Chimeric CCR4 and CCR5 were assembled from DNA fragments that were generated by splice overlap extension PCR (Horton et al., 1989). The cDNA sequences of the clones that were used as templates for PCR fragment generation were the same as GenBank accession numbers AB02388 (CCR4, apart from four silent base substitutions: T24C, T279C, C1044T, and C1098T; numbering starts from the beginning of the coding sequence) and AF031237 (CCR5). Predicted protein sequences of CCR4 and CCR5 were aligned, and a region of high homology within TM7 (shown as boxed in Fig. 2) was chosen as the region of overlap used to generate the chimeras. 5′-and 3′-primers for PCR were designed to include restriction sites compatible with the expression vector (NheI and NotI; Roche Diagnostics). Joined PCR fragments corresponding to bases 1 to 911 of CCR4 fused

to bases 890 to 1059 of CCR5 and bases 1 to 864 of CCR5 fused to bases 885 to 1083 of CCR4 were cloned into the mammalian expression vector pcDNA3.1 (Invitrogen), to provide resultant protein products corresponding to CCR4 amino acids 1 to 304 fused to CCR5 amino acids 298 to 352 (designated CCR4-5T) and CCR5 amino acids 1 to 288 fused to CCR4 amino acids 296 to 360 (designated CCR5-4T). Vectors for full-length wild-type (WT) CCR4 and CCR5 were similarly prepared.

Generation of Transient Recombinant Cells. Transient expression in HEK-G_{qi5} cells was made by using FuGENE 6 with relevant plasmids, and expression was performed in 225-cm^2 flasks according to the supplier's instructions. In brief, a suspension of 13×10^6 cells in culture medium without antibiotics was added to flasks, and cells were allowed to adhere overnight before addition of 16 μg of plasmid cDNA that was premixed with Opti-MEM (Invitrogen) containing FuGENE 6. The cells were incubated with cDNA for 24 h before seeding into 96-well plates and then for a further 24 h before use in calcium signaling or antagonist binding assays.

Binding of FB-CCL22 and -CCL5 to Recombinant Cells. Binding of FB-labeled chemokines was performed essentially as described by Mellentin-Michelotti et al. (1999). Recombinant CHO cells were seeded in culture medium at 10^4 cells/well in black, clear-bottomed, 96-well plates and then incubated overnight. Cells were washed with assay buffer (20 mM HEPES, Hanks' balanced salt solution, pH 7.4, at 20°C with NaOH) before adding a compound, agonist, or appropriate vehicle. Binding was initiated by addition of FB-CCL5 or -CCL22 (0.7 nM final concentration) in assay buffer. After incubation at room temperature, the median cell-associated fluorescence in each well was measured by using an 8200 Cellular Detection System (Applied Biosystems, Foster City, CA).

Cell Membrane Preparation. Wild-type or recombinant CHO cells were removed from cell culture flasks with 2 mM EDTA in phosphate-

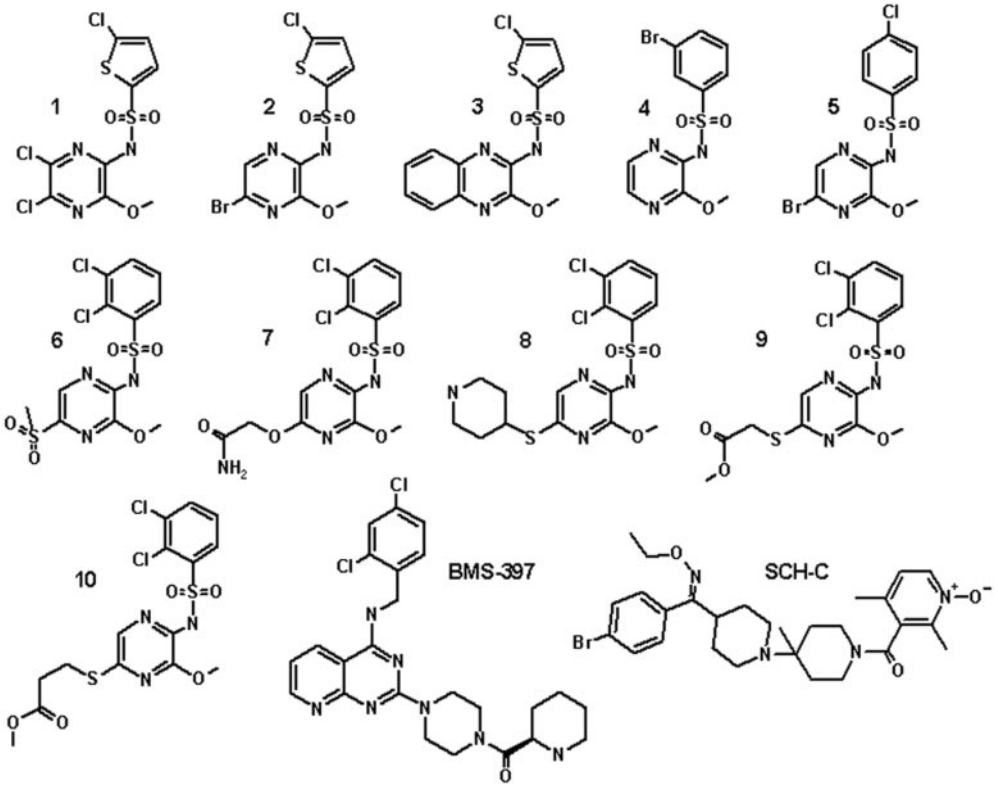

Fig. 1. Structure of CCR4 and CCR5 antagonists used in this study.

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CCR4: vElevLqdCt feryLDyAiQ aTETLafvHC ClNPIIYfFl GEKFRkYiLq
CCR5: qEffgLnnCs ssnrLDqAmQ vTETLgmtHC CiNPIIYaFv GEKFRnYlLv
Consensus: -E---L--C- ----LD-A-Q -TETL---HC C-NPIIY-F- GEKFR-Y-L-

CCR4: lF.KtcrglF vlCqyCgllQ iysadtpSSs YTqSTmdhdl hdaL
CCR5: fFqKhiakrF ..CkcCsifQ qeaperaSSv YTrSTgeqei svgL
Consensus: -F-K-----F --C--C---Q -------SS- YT-ST----- ---L
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Fig. 2. Construction of the CCR4 and CCR5 chimeras. Alignment of the C-terminal section of protein sequences for human CCR4 and CCR5 were generated using the UWGCG GAP program (Devereux et al., 1984). The predicted transmembrane domain VII is shown in bold. The region of high homology between the two sequences, including the conserved NPxxY motif, is shown boxed. Primers for splice overlap extension PCR were designed to cover this region. The primer sequences are as follows: CCR4for, CGGAATTCAAGCTTCCACCATGAACCCCACGGATATAGC; CCR4rev, GATCTTCATGATGCTCTGTAGGCGGCCGCTAGC; CCR5for, ATCTGCTAGCCACCATGGATTATCAAGTGTCAAGT; CCR5rev, ATCTGCGGCCGCTCACAAGCCCACAGATATTT; TM7for, CACTGCTGCCTTAATCCCATCATCTA; and TM7rev, TAGATGATGGGATTAAGGCAGCAGTG. The TM7for/rev primers corresponding to the region of overlap were used on both CCR4 and CCR5 templates. The primers matched perfectly with the CCR4 template and had two mismatches with CCR5 (underlined in primer sequence). TM7for and TM7rev were complementary to one another; amplification of fragments using these primers generated the 26-base pair regions of overlap necessary for the splice overlap extension PCR.

buffered saline and then harvested by centrifugation at 350*g* for 5 min at room temperature. The cell pellet was resuspended at a density of 10^7 cells/ml in ice-cold buffer (20 mM HEPES, 1 mM EDTA, 3 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 100 μg/ml bacitracin, 1 μg/ml leupeptin, and 2 μg/ml soybean trypsin inhibitor, pH 7.4, at 22°C with NaOH). The cell suspension was homogenized (2 × 30 s) by using a Polytron hand-held homogenizer (Brinkmann Instruments, Westbury, NY), and then it was centrifuged at 1000*g* for 15 min at 4°C. The resultant supernatant was centrifuged at 100,000*g* for 30 min at 4°C. The resultant membrane pellet was resuspended at 10^8 cell equivalents/ml in the homogenization buffer containing 1% (v/v) glycerol, and then it was stored at −80°C.

Antagonist Binding to Intact Recombinant Cells or Membranes. Binding buffer (20 mM HEPES, 50 mM NaCl, and 5 mM $MgCl_2$, pH 7.4, at 22°C with NaOH) containing either a test compound or appropriate vehicle was added to [^{3}H]compound **1** (1 nM final concentration) in a 96-well polypropylene plate, and binding was initiated by addition of either membranes (typically 15 μg/well) or intact cells (typically 2 × 10^4 CHO cells/well or 8 × 10^4 HEK cells/well) in binding buffer. After incubation to steady state for 1 h at room temperature, the content of each well was passed through a Packard Unifilter GF/B plate, which was washed with 4 × 350 μl of binding buffer at 4°C and then dried for at least 1 h at 50°C. Filter-bound radioactivity was measured (45% efficiency; TopCount, PerkinElmer Life and Analytical Sciences) after addition of 50 μl of MicroScint-O (PerkinElmer Life and Analytical Sciences) to each well.

Calcium Signaling in Recombinant Cells. Recombinant cells were seeded in culture medium at 5 × 10^4/well in black, clear-bottomed, poly-D-lysine-coated, 96-well plates, and then they were incubated overnight. Cells loaded with Fluo-3AM in FLIPR buffer [20 mM HEPES, 0.15 M NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM D-glucose, 0.01% (w/v) BSA, pH 7.4, at 22°C with NaOH] were briefly washed with FLIPR buffer at 37°C. Test compounds (or vehicle controls) in FLIPR buffer were then preincubated with the cells for 15 min before transfer of the plate to either a FLIPR or a TETRA (Molecular Devices), where addition of receptor agonists was made and fluorescence measured over time.

Measurement of Surface Expression of Human CCR4 on Recombinant Cells. Surface expression of CCR4 was monitored by using a phycoerythrin-tagged antibody that was specific for the N terminus of the receptor (R&D Systems Europe Ltd.); a similarly tagged IgG controlled for nonspecific antibody binding. CCR4-CHO cells were incubated overnight at 4°C with the antibody in phosphate-buffered saline supplemented with 0.1% (w/v) BSA and 10 mM sodium azide, to block antibody internalization. Cells were then incubated in 1% (w/v) paraformaldehyde (CellFix; BD Biosciences) for 30 min before washing once and then resuspending them in incubation buffer for analysis by flow cytometry on an FC500 machine (Beckman Coulter, High Wycombe, UK). Intact cells were identified by their forward- and side-scatter characteristics, and median fluorescence was measured at 575 nm using the standard FL2 channel on the device. In experiments that looked at the effect of CCR4 ligands on surface expression, cells were first incubated with increasing concentrations of CCL22 in either the absence or presence of single increasing concentrations of a CCR4 antagonist for 60 min in binding buffer at 37°C. Cells were chilled to 4°C before labeling with antibody as described above.

Data Analysis. All data were analyzed by using Prism, version 4.01 (GraphPad Software Inc., San Diego, CA), where concentration-effect curves typically were fitted to a four-parameter logistic equation, unless otherwise indicated. When appropriate, an F-test within Prism was used to determine significant differences in fitting parameters.

Results

Functional Antagonism of CCR4 Responses in Recombinant Cells. Compounds of the pyrazinyl-sulfonamide series demonstrated no agonist activity of their own across a range of functional assays for CCR4 (data not shown), but they antagonized cellular responses to CCR4 activation by either agonist. Antagonism of CCL22-mediated calcium signaling in CCR4-HEK cells (with the agonist at a concentration equivalent to 2 to 3 times the EC_{50} value for this response) is shown in Fig. 3 for two representatives from this series, along with a structurally distinct CCR4 antagonist BMS-397 (compound 16 in Purandare and Somerville, 2006; see structure in Fig. 1); similar results were obtained for antagonism of responses to CCL17 (data not shown).

Inhibition of Agonist Binding to CCR4 in Recombinant Cells. Incubation of adherent CCR4-CHO cells with the fluorescently labeled FB-CCL22 resulted in a time- and concentration-dependent increase in cell-associated fluorescence at room temperature. No binding of FB-CCL22 was detectable above background when using CHO cells either not expressing CCR4 or else expressing other chemokine receptors (e.g., CCR3 or CXC chemokine receptor 2; data not shown). Cellular association of FB-CCL22 seemed to reflect a combination of binding of the labeled agonist to CCR4 along with an irreversible uptake of the fluorescent label, presumably as internalized ligand, which proceeded at a rate that varied between experiments; a typical association curve is shown in Fig. 4A, which shows that in this experiment a steady state was approached after approximately 3 h. If a saturating concentration (100 times the estimated EC_{50} or IC_{50} value for calcium signaling) of either unlabeled CCL22 or a representative pyrazinyl-sulfonamide CCR4 antagonist was added at that time, then a time-dependent decrease in

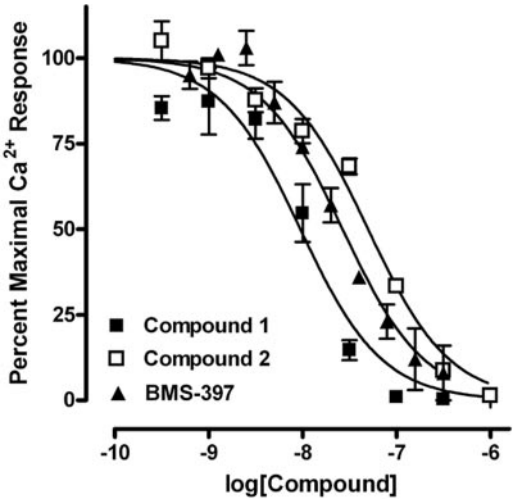

Fig. 3. Antagonism of CCR4 functional activity in recombinant cells. Calcium signaling to 0.6 nM (2–3 times the EC_{50} value) CCL22 was measured at room temperature with Fluo-3-loaded HEK-G_{qi5} cells expressing recombinant CCR4, as described under *Materials and Methods*. Responses to CCR4 activation were measured in either the absence or presence of concentrations of CCR4 antagonists as shown on the abscissa, where compounds were preincubated for 15 min before addition of the agonist. The fluorescent signal at each concentration was measured in triplicate, and then it was plotted on the ordinate as a percentage of the maximal response normalized to 100% of CCL22 signaling in the absence of CCR4 antagonist, after subtraction of the background signal in the absence of agonist. The data represent the average and standard error of four experiments. The lines represent the fit of a simple rectangular hyperbola with a slope factor of 1, constraining the top and bottom of the curves to 100 and 0, respectively. Estimates of pIC_{50} values were made for compound **1** (■; 8.02 ± 0.067), compound **2** (□; 7.30 ± 0.043), and BMS-397 (▲; 7.57 ± 0.043).

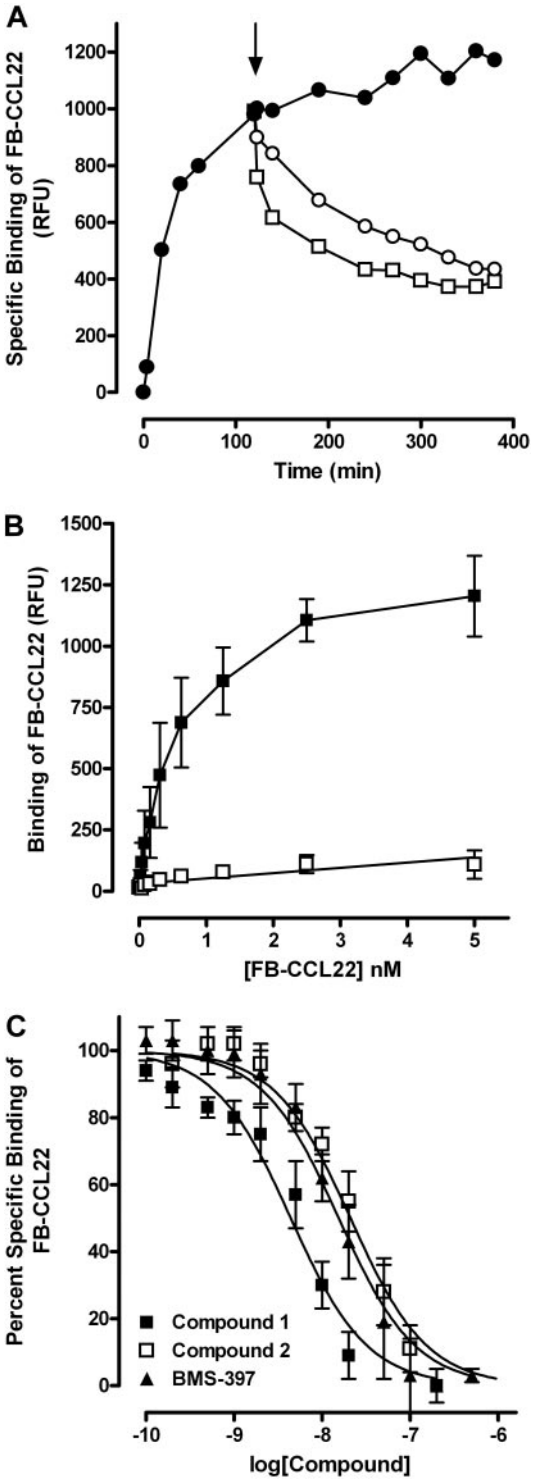

Fig. 4. Inhibition of agonist binding to CCR4 in recombinant cells. A, total binding of FB-CCL22 was measured at room temperature to CHO cells expressing recombinant CCR4, over the period of time shown on the abscissa, in the absence or presence of 10 μM compound **2** to measure nonspecific binding. Nonspecific binding was subtracted from total binding to give specific binding (●), which was plotted in relative fluorescence units on the ordinate. Addition (indicated by the arrow) of buffer in the absence or presence of either 10 μM compound **2** (□) or 100 nM unlabeled CCL22 (○) was made after 2-h preincubation with FB-CCL22 at room temperature, and cell-associated fluorescence was measured over a further 260 min. The lines simply link the data points. B, total (■) and nonspecific (in the presence of 10 μM compound **2**; □) binding of FB-CCL22 to CHO cells expressing recombinant human CCR4 was measured at concentrations as indicated on the abscissa, after incubation of the receptor for 2 h at room temperature. The data represent the average and standard deviation of three separate experiments. The lines either connect the data points (total binding) or represent the best fit of a linear regression (nonspecific binding). Specific binding was calculated as total binding subtracting the corresponding nonspecific binding (data not shown). C, total binding of FB-CCL22 to CHO cells expressing recombinant human CCR4 was measured, after incubation of the receptor with 0.7 nM FB-CCL22 for 3 h at room temperature. Binding was measured in either the absence or presence of concentrations of CCR4 antagonists as shown on the abscissa. Specific binding was calculated as described in A and plotted on the ordinate as a percentage of maximum specific FB-CCL22 binding in the absence of CCR4 antagonist. The data represent the average and standard error of four experiments. The lines represent the fit of a simple rectangular hyperbola with a slope factor of 1, constraining the top and bottom of the curves to 100 and 0, respectively. Estimates of pIC_{50} values were made for compound **1** (■; 8.36 ± 0.067), compound **2** (□; 7.68 ± 0.053), and BMS-397 (▲; 7.81 ± 0.051).

cell-associated fluorescence was observed (Fig. 4A). These results demonstrated that this assay could be used to measure reversible agonist binding, where the data are consistent with either competition or negative co-operativity by either ligand with the fluorescent probe at the receptor and its dissociation from the cell. Dissociation of FB-CCL22 from CCR4-CHO cells by either the agonist or the antagonist proceeded to a similar degree of residual fluorescence above background after 400 min, which was assumed to represent internalized FB-CCL22 after its binding to CCR4. Binding of FB-CCL22 to CCR4-CHO cells was completely blocked by prior incubation with 1 μM compound **2**, and after 3-h incubation in the absence of the antagonist it seemed to be saturable, with an estimated K_d value of 0.6 ± 0.24 nM (Fig. 4B). When measured at 3 h after simultaneous addition with the fluorescently labeled agonist (at a concentration equivalent to the K_d value), CCR4 antagonists inhibited the association of FB-CCL22 with CCR4-CHO cells in a concentration-dependent manner, with a pIC_{50} value that was consistent with their activity as antagonists of CCR4-mediated calcium signaling. Data for two representative pyrazinyl-sulfonamides and BMS-397 are shown in Fig. 4C.

Binding of a Radiolabeled Pyrazinyl-Sulfonamide to CCR4. To study the mechanism of action of the pyrazinyl-sulfonamides, a binding assay was established with a radiolabeled representative of the series. Binding of [^{3}H]compound **1** to CCR4 was reversible and linear with increasing amounts of CCR4-expressing material (data not shown). Nonspecific binding of [^{3}H]compound **1** to CCR4-expressing cells, defined as binding in the presence of 10 μM compound **2**, was linear with the concentration of the radioligand (Fig. 5A). Specific binding of [^{3}H]compound **1** was saturable at equilibrium, with an estimated K_d value that was consistent with the potency of unlabeled compound **1** in a functional assay (Fig. 5A). This estimated K_d value at CCR4 was identical when measured by using either intact cells (either HEK or CHO) or their membrane fractions (data not shown). Binding of this radioligand seemed to be specific for CCR4, in that it was not detected either in the absence of CCR4 expression or with identical host cells where other recombinant chemokine receptors were expressed (e.g., CCR3 or CXC chemokine receptor 2; data not shown). All pyrazinyl-sulfonamides competed for binding of [^{3}H]compound **1** to CCR4 with an estimated pIC_{50} value that was consistent with their measured activity to inhibit CCR4-mediated functional responses or agonist binding to CCR4. The data for representative pyrazinyl-sulfonamide antagonists is shown in Fig. 5B, along with the

structurally distinct CCR4 antagonist BMS-397, which seemed not to compete for this pyrazinyl-sulfonamide-binding site on CCR4. It is noteworthy that whereas unlabeled compound **1** competed for labeled agonist binding (Fig. 4B), the binding of [^{3}H]compound **1** to CCR4 expressed in either membranes or cells was not inhibited by unlabeled, BSA-free CCL22 and CCL17 at concentrations in excess of 10,000-fold that of their estimated K_d (data not shown).

Discordance between Cellular and Membrane Potency at CCR4. Potency of these pyrazinyl-sulfonamide antagonists typically was independent of whether it was measured with either a membrane- or a cell-based method, using either a functional response or binding with either labeled agonist or antagonist as the probe. This structure-activity relationship is shown in the top panel of Fig. 6, which correlates the pIC_{50} value for inhibition of binding of FB-CCL22 to CCR4 in cells and of [^{3}H]compound **1** to membranes. This demonstrates that over a wide range of activities most compounds in this series show an excellent correspondence for cell- and membrane-based potencies. Some compounds, however, either seemed to have much lower potency in cellular assays than expected or else the cellular potency was much higher than that measured in a membrane assay. Two examples of this discordance, highlighted as open symbols in that correlation, are represented by an ester, compound **9**, and its carboxylic acid analog. Both compounds had identical potency in the membrane binding assay, consistent with the structure-activity relationship of this chemical series, and yet the average pIC_{50} value for ester-containing compound **9** was significantly higher (~10-fold; $p < 0.01$) in the cell-based assay compared with the membrane-based assay (Fig. 6B; Table 1). A smaller, but significant difference was seen with another ester, compound **10** ($p < 0.01$; Table 1). In contrast, the pIC_{50} value for the carboxylic acid derivative of compound **9** was significantly lower (~100-fold; $p < 0.01$) in the cell-based assay (Fig. 6B; Table 1).

Requirement for Equilibration of Pyrazinyl-Sulfonamides across Cell Membranes. The results suggested that equilibration across the cell membrane might be an issue for those antagonists that displayed these properties. Consistent with that hypothesis, the discordance in cell and membrane potency of either the ester or the carboxylic acid antagonists was eliminated by permeabilizing the cells with a low concentration [0.05% (w/v)] of saponin (Fig. 6B; Table 1). No significant difference in the pIC_{50} value was calculated for the same compounds tested in the presence of saponin. It is noteworthy that this saponin treatment revealed that some relatively impermeable compounds could even differentiate a degree of heterogeneity in what could be viewed as vesicle integrity in a membrane preparation, when binding curves seem to represent a mixture of cell potency in the presence or absence of saponin (see data for the carboxylic acid derivative of compound **9** in Fig. 6B). Permeabilization with saponin did not influence the binding of the majority of compounds in this chemical series (represented by compound **5** in Table 1), including [^{3}H]compound **1** (data not shown).

Parallel investigation revealed, in contrast to what was observed with a typical pyrazinyl-sulfonamide, that not only

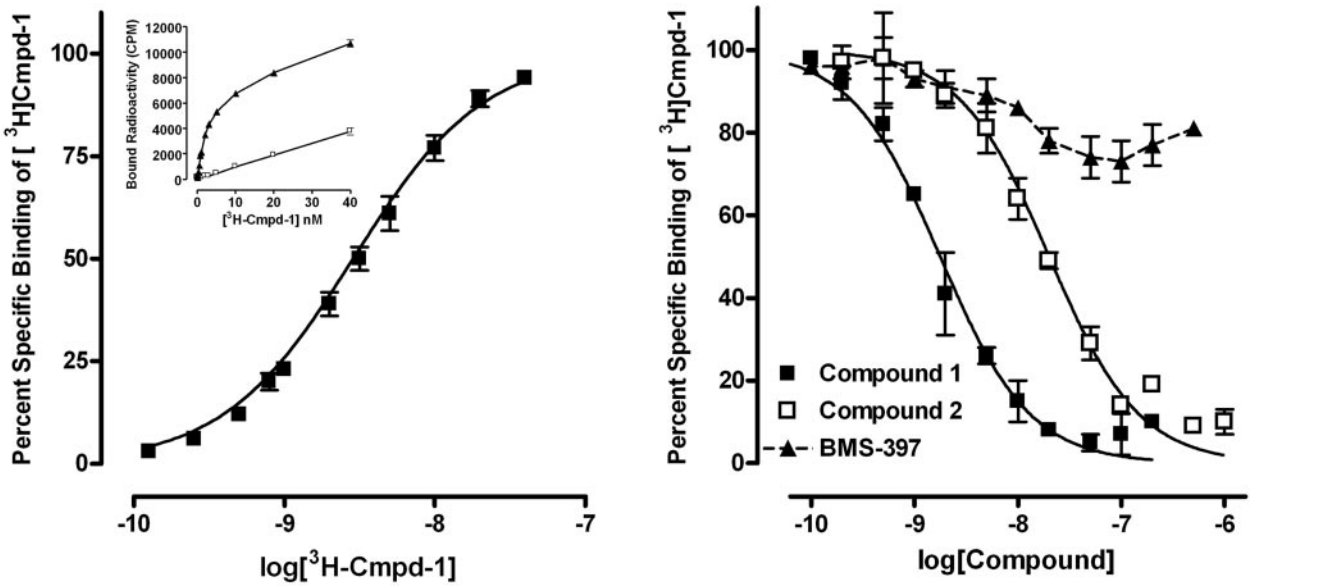

Fig. 5. Binding of CCR4 antagonists to recombinant CCR4 expressed in membranes. Left, total binding of [^{3}H]compound **1** to CCR4-CHO membranes was measured at room temperature. The radioligand was incubated at concentrations as shown on the abscissa, in the absence or presence of a saturating concentration of an unlabeled pyrazinyl-sulfonamide (differing between some experiments) to measure nonspecific binding. Nonspecific binding was subtracted from total binding to give specific binding that was then normalized to 100% of maximum and plotted on the ordinate. The data represent the average and standard error of four experiments. The line represents the fit of a simple rectangular hyperbola with a slope factor of 1. The pK_d value was estimated at 8.50 ± 0.010 (K_d = 3 nM). Inset, total and nonspecific binding from one of the four experiments is shown. Radioactivity bound to membranes in either the presence (□) or absence (▲) of a pyrazinyl-sulfonamide CCR4 antagonist was plotted on the ordinate. Right, competition for the specific binding of 1 nM [^{3}H]compound **1** was measured for the CCR4 antagonists compound **1** (■), compound **2** (□), and BMS-397 (▲), after equilibration with membranes from CCR4-CHO cells at room temperature. Compounds were tested in duplicate, at concentrations plotted on the abscissa. Specific binding was calculated as described in the left graph, and it was plotted on the ordinate as a percentage of specific [^{3}H]compound **1** binding alone. The data represent the average and standard error of four experiments. The solid lines for the first two compounds represent the fit of a simple rectangular hyperbola with a slope factor of 1, constraining the value of the top and bottom of the curves to 100 and 0, respectively. The dashed line for BMS-397 simply connects the data points. Estimates of pIC_{50} values were made for compound **1** (8.75 ± 0.038) and compound **2** (7.70 ± 0.041).

were the ester-containing compounds showing higher than expected cellular activities but also their activity to inhibit binding of [^{3}H]compound **1** to CCR4-CHO cells actually increased with time of incubation. This is represented by the ester compound **10**, when measuring a time course for inhibition of binding of [^{3}H]compound **1** to CCR4-CHO cells (Fig. 7A). To explore this further, specific binding of [^{3}H]compound **1** was measured after incubation of CCR4-CHO cells with 100 nM CCR4 antagonist for 2 h followed by extensive washing of the cells. Whereas this procedure completely reversed inhibition of binding by compound **2**, marked inhibition of binding was still observed with compound **10**. That residual

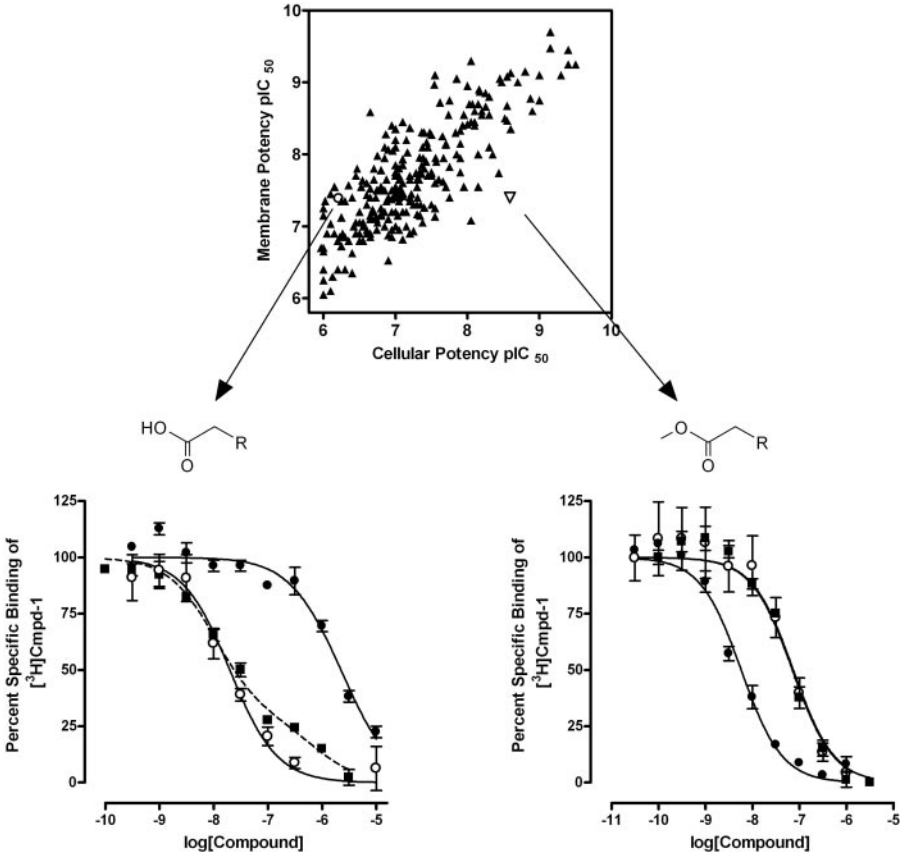

Fig. 6. Correlation of CCR4 antagonist activity in cell- and membrane-based assays and the effect of saponin permeabilization. Top, activities were compared for a wide range of a pyrazinyl-sulfonamide CCR4 antagonists (256 compounds) between inhibition of FB-CCL22 binding to CCR4-CHO cells (cellular potency) and inhibition of [^{3}H]compound **1** binding to membranes from CCR4-CHO cells (membrane potency). For estimates of cellular potency, total binding of 0.7 nM FB-CCL22 was measured after 2-h incubation in the absence and presence of at least five concentrations of a CCR4 antagonist, and the pIC_{50} value was estimated as described under *Materials and Methods*. Average pIC_{50} data calculated from at least two experiments are plotted on the abscissa. CCR4-CHO membrane-binding activity was measured by competition for the specific binding of 1 nM [^{3}H]compound **1** in the absence and presence of at least five concentrations of a CCR4 antagonist, after incubation with CCR4-CHO membranes for 1 h at room temperature, and the pIC_{50} value was estimated as described under *Materials and Methods*. Average pIC_{50} data calculated from at least two experiments are plotted on the ordinate. Highlighted on this graph are the positions in the correlation for the ester-containing compound **9** (▽) and its carboxylic acid derivative (open circle), with the arrows pointing to the relevant structural group and associated data. Competition for the specific binding of 1 nM [^{3}H]compound **1** was measured for either compound **9** (bottom right) or its carboxylic acid derivative (bottom left) at concentrations shown on the abscissa, after equilibration with CCR4-CHO cells in the presence (○) or absence (●) of 0.05% (w/v) saponin or with CCR4-CHO membranes (■). Specific [^{3}H]compound **1** binding was plotted on the ordinate as a percentage of that in the absence of competing ligand. The data represent the average and standard error of either three or four experiments. The solid lines represent the fit of a simple rectangular hyperbola with a slope factor of 1, constraining the value of the top and bottom of the curves to 100 and 0, respectively. Estimates of pIC_{50} values for binding of both compounds to either CCR4-CHO cells or membranes ± 0.05% (w/v) saponin are presented in Table 1. Data for binding of the carboxylic acid derivative to CCR4-CHO membranes were fitted to a two-site model, represented by the dashed line, with estimates of pIC_{50} values for the first site (fraction 70%) and second site of 8.0 ± 0.11 and 6.1 ± 0.26, respectively.

TABLE 1

Effect of cell permeabilization to normalize binding potencies in cells and membranes for selected pyrazinyl-sulfonamides at CCR4

Competition for the specific binding of 1 nM [^{3}H]compound **1** was measured for the CCR4 antagonists following equilibration with CCR4 in CHO membranes or cells in the presence or absence of saponin as described under *Materials and Methods*. Fitted estimates of pIC_{50} values from separate experiments were calculated, and the average and standard deviation are shown (the values in parentheses represent the number of experiments).

Compound	pIC_{50}			
	CHO Membrane	CHO Cell	Membrane + Saponin	Cell + Saponin
5	6.7 ± 0.35 (3)	6.7 ± 0.47 (3)	6.6 ± 0.49 (3)	6.7 ± 0.50 (3)
9	7.2 ± 0.10 (4)	8.3 ± 0.25 (4)	7.2 ± 0.12 (4)	7.2 ± 0.25 (4)
10	7.8 ± 0.27 (5)	8.3 ± 0.23 (5)	7.8 ± 0.36 (3)	7.9 ± 0.20 (3)
Carboxylic acid derivative of **9**	7.5 ± 0.14 (4)	5.7 ± 0.25 (3)	7.9 ± 0.17 (4)	7.8 ± 0.18 (4)

inhibition was reversed, however, by permeabilization of the cells with a low concentration [0.05% (w/v)] of saponin (Fig. 7B). This result suggested that ester-containing compounds somehow were accumulating in the cells. Chemical analysis of CCR4-CHO cells that had been incubated for 3 h with compound **10** revealed that there was a loss of the ester form and an accumulation of the corresponding carboxylic acid, indicating that the ester-containing compound had been hydrolyzed and retained as the poorly permeable carboxylic acid, active at CCR4, inside the cells (data not shown).

Apparent Cellular Activity of Antagonists at CCR4 Depended on Properties of the Host Cell. Binding activity at CCR4 in cell-based assays was independent of the nature of the host cell for the majority of pyrazinyl-sulfonamide antagonists, and yet the apparent activity for some compounds was significantly different when comparing the pIC_{50} value to inhibit binding at CCR4 expressed in CHO and HEK-G_{qi5} cells and how those estimates related to the membrane assay. Although no significant difference in pIC_{50} value was measured between either cell- or membrane-based assays for the typical pyrazinyl-sulfonamide, compound **2**, a significant difference in the pIC_{50} value between CHO cell and HEK-G_{qi5} cell assays was calculated for compound **6** at CCR4, neither estimate being comparable with the estimated membrane potency ($p < 0.001$; Table 2). The pIC_{50} value calculated for compound **8** at CCR4 in the CHO cell assay was significantly different from that measured at CCR4 in either HEK-G_{qi5} cells or CHO membranes ($p < 0.001$; Table 2). Preliminary attempts with the inhibitors probenecid or verapamil to explore a role for either an organic acid transporter or P-glycoprotein as the basis for this host cell dependence in compound activity failed to reveal any effect on compound binding to CCR4-CHO cells (data not shown).

Functional Antagonism of Agonist-Mediated CCR4 Internalization. To explore further the mechanism of action of these compounds, which seemed from previous evidence to require access to the cytoplasm, a robust functional assay was developed to monitor competitive antagonism—something not possible with the hemiequilibrium conditions of the calcium signaling assay. As described previously (Mariani et al., 2004), CCL22 promoted a rapid, dose-dependent disappearance of CCR4 from the surface of recombinant CHO cells, with a potency identical to that measured in other functional assays (Fig. 8). Antagonism of this effect with pyrazinyl-sulfonamide antagonists at concentrations up to 1000 times their binding potency showed the expected dextral shift of the agonist dose-response curve, but a Schild analysis revealed that this effect deviated from that expected for conventional competitive antagonism, especially apparent at high concentrations. Data for the representative compound **2** are shown in Fig. 8, where the fitted slope factor was significantly different from 1 ($p < 0.05$). Control experiments showed that these compounds had no effect on the specific binding of the antibody that was used to monitor surface expression of the receptor (data not shown).

Parallel Structure-Activity Relationship for Antagonists at CCR5. During the evaluation of the pyrazinyl-sulfonamides as CCR4 antagonists, it was discovered that the compounds were also weaker antagonists at the chemokine receptor CCR5. These compounds reversibly inhibited FB-CCL5 binding to CCR5-CHO cells with a structure-activity relationship similar to that measured for inhibition of FB-CCL22 binding to CCR4-CHO cells, but at ~50-fold lower potency (Fig. 10B). It was on this basis that further biochemical work was performed to explore the potential intracellular site of action at both receptors.

Generation of C-Terminal Chimeras of CCR4 and CCR5. We generated recombinant HEK-G_{qi5} cells expressing chimeric CCR4 with the C-terminal tail of CCR5 (CCR4-5T) and chimeric CCR5 with the C-terminal tail of CCR4 (CCR5-4T) (Fig. 2), to then compare their properties to the wild-type receptors. When assessed within 48 h of transient expression, both of the C-terminal chimeras were expressed on the cell surface to a magnitude that seemed to be equivalent to wild-type CCR4 and CCR5, as measured with relevant antibodies by using flow cytometry (data not shown).

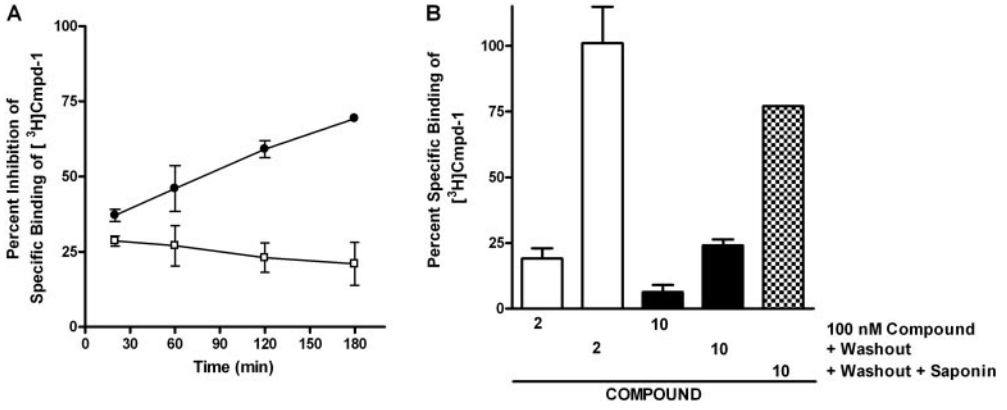

Fig. 7. Intracellular trapping of an ester-containing pyrazinyl-sulfonamide in CCR4-CHO cells and the effect of saponin permeabilization on CCR4 antagonism. A, inhibition of the specific binding of 1 nM [^{3}H]compound **1** to either CCR4-CHO cells (●) or CCR4-CHO membranes (□) was measured for 10 nM compound **10** after incubation at room temperature at time intervals as shown on the abscissa. Nonspecific binding, defined with 10 μM compound **2**, was subtracted from total binding to give specific binding; the percentage inhibition at each time was calculated and plotted on the ordinate. The data for 1- to 3-h incubations represent the mean and standard error of at least four experiments, or two experiments for the 20-min incubation data. Percentage of inhibition significantly increased in the cell-based assay between 1 and 3 h ($p < 0.05$), with values at 2 and 3 h being significantly higher than in the membrane-based assay ($p < 0.01$). B, reversibility of compound binding to CCR4 was measured in a competition assay for the specific binding of 1 nM [^{3}H]compound **1** to CCR4-CHO cells. Compounds **2** (white bars) and **10** (black bars) were preincubated at 100 nM with cells for 2 h at room temperature before washing cells extensively in binding buffer. Compounds, buffer, or 0.05% (w/v) saponin (hatched bar) was then re-added, and cells were incubated for a further 30 min after addition of 1 nM [^{3}H]compound **1**. Specific [^{3}H]compound **1** binding was plotted on the ordinate as a percentage of that in the absence of a CCR4 antagonist.

Agonist Activity at C-Terminal Chimeras of CCR4 and CCR5. After transient transfection in HEK-G_{qi5} cells, successful expression and functionality of each of the chimeras of CCR4 and CCR5 (CCR4-5T and CCR5-4T) were confirmed by measuring agonist-mediated calcium signaling, compared in parallel studies with the wild-type form of the receptors. Responses of CCR4-5T were of a similar magnitude to that observed for CCR4 WT; the normalized dose-response curves for both CCL17 and CCL22 are shown in Fig. 9A. Whereas the EC_{50} value for CCL17 at CCR4 WT was not altered by the presence of the C-terminal tail of CCR5, that for CCL22 was reduced significantly (~3-fold; $p < 0.001$) at CCR4-5T.

Responses of CCR5 expressed with the C-terminal tail of CCR4 (CCR5-4T) to either CCL4 or CCL5 were variable, but they were usually markedly attenuated from that observed for CCR5 WT, both in terms of the overall magnitude of the response and the potency of the agonist; normalized dose-response curves for both CCL4 and CCL5 are shown in Fig. 9B. Assuming that the dose-response curves did not deviate from a simple rectangular hyperbola, then if all data were analyzed assuming a common slope factor, the EC_{50} value for CCL4 was estimated to be reduced ~5-fold at CCR5-4T ($p < 0.001$), whereas that for CCL5 was estimated to be reduced ~16-fold ($p < 0.001$), compared with CCR5 WT.

Antagonist Binding of C-Terminal Chimeras of CCR4 and CCR5. Saturable, high-affinity binding of [^{3}H]compound **1** was measured to cells transiently expressing CCR4 WT (pK_d = 8.71 ± 0.023; n = 3), but binding to the chimeric CCR4-5T was not detectable (Fig. 10A). In contrast, saturable, high-affinity binding of [^{3}H]compound **1** was measured to CCR5 on cells only when expressed as a chimera with the C-terminal tail of CCR4 (CCR5-4T; Fig. 10A), with a small but significant decrease in affinity (pK_d = 8.21 ± 0.024; n = 3; $p < 0.001$ compared with CCR4 WT). In contrast to their activity at the CCR5 WT, competition by a range of pyrazinyl-sulfonamides for binding of [^{3}H]compound **1** (at a concentration below the K_d value) to cells expressing CCR5-4T was identical to that observed with CCR4 WT (Fig. 10B).

Antagonist Activity at C-Terminal Chimeras of CCR4 and CCR5. In functional assays of CCR4 and CCR5 activity, the potencies of pyrazinyl-sulfonamides at CCR4 WT were equivalent to CCR5-4T, and those at CCR5 WT were equivalent to CCR4-5T. The results for a representative compound to antagonize the response of CCR4 WT and CCR4-5T or CCR5 WT and CCR5-4T to CCL22 or CCL5, respectively, are shown in Fig. 11A; identical results were observed for responses to CCL17 or CCL4 at CCR4 WT and CCR4-5T or CCR5 WT and CCR5-4T, respectively (data not shown). In contrast, the presence of a chimeric C-terminal domain did not affect the relative selectivity of the nonpyrazinyl-sulfonamides BMS-397 and SCH-C at the wild-type forms of CCR4 and CCR5 (Fig. 11B).

TABLE 2

Dependence on host cell for binding potencies of selected pyrazinyl-sulfonamides at recombinant CCR4

Competition for the specific binding of 1 nM [^{3}H]compound **1** was measured for the CCR4 antagonists following equilibration with CCR4 in CHO membranes or HEK-G_{qi5} cells as described under *Materials and Methods*. Inhibition of the specific binding of FB-CCL22 was measured for the same CCR4 antagonists following equilibration with CCR4-CHO cells, also described under *Materials and Methods*. Fitted estimates of the pIC_{50} values were measured in separate experiments, and the average and standard error were calculated and are shown (the values in parentheses represent the number of experiments).

Compound	pIC_{50}		
	CHO Membrane	CHO Cell	HEK Cell
2	8.0 ± 0.40 (7)	7.6 ± 0.10 (6)	7.7 ± 0.20 (7)
6	7.4 ± 0.15 (4)	5.4 ± 0.10 (4)	6.4 ± 0.12 (7)
8	7.6 ± 0.19 (8)	6.5 ± 0.22 (5)	7.5 ± 0.19 (5)

Discussion

Upon discovery of the pyrazinyl-sulfonamide CCR4 antagonists through a cell-based screen, it was assumed that these molecules were active at an overlapping extracellular site for

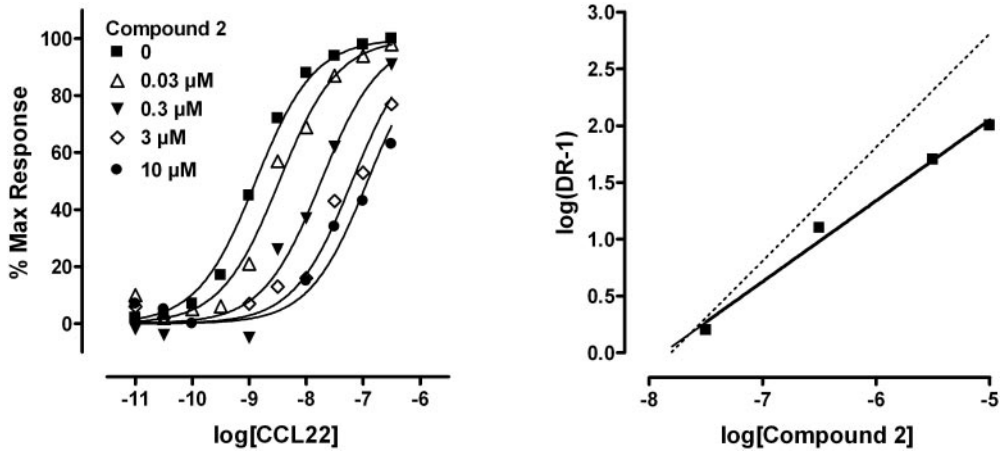

Fig. 8. Functional antagonism of CCL22-mediated internalization of CCR4 in recombinant CHO cells. Left, expression of CCR4 on recombinant CHO cells was monitored by using flow cytometry to measure the labeling of surface receptor with a CCR4-specific phycoerythrin-tagged antibody, as described under *Materials and Methods*. Agonist-mediated CCR4 internalization at 37°C was measured at CCL22 concentrations as shown on the abscissa, in either the absence or presence of compound **2** at single increasing concentrations as shown in the legend. A single measurement of the fluorescence signal at each concentration was measured and then plotted on the ordinate as a percentage of the maximal response normalized to 100% of CCL22-mediated internalization in the absence of CCR4 antagonist. The data represent one of three similar experiments. The lines represent the fit of a simple rectangular hyperbola with a shared slope factor, constraining the top and bottom of the curves to 100 and 0, respectively. Right, Schild analysis of the data shown in the left graph. The solid line represents the best fit of a linear regression, having a slope factor of 0.71. The dashed line represents the predicted behavior for a competitive interaction, plotted with a slope factor of 1 and an affinity measured independently by competition with [^{3}H]compound **1**.

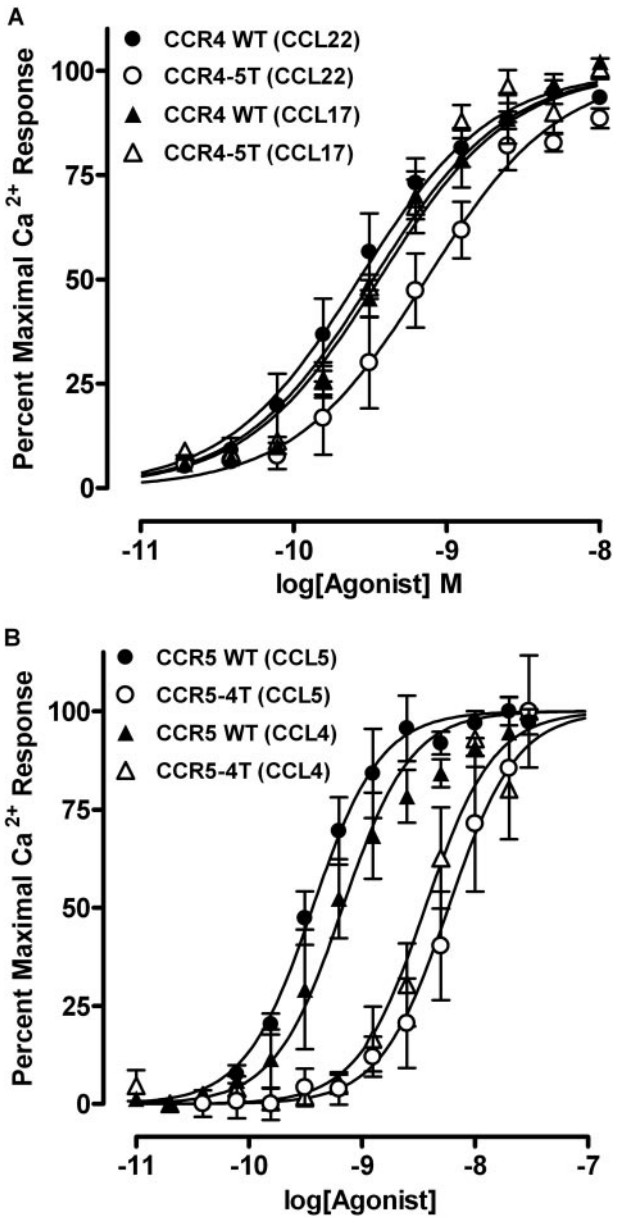

Fig. 9. Comparison of functional activity of wild-type and C-terminal chimeras of CCR4 and CCR5 in recombinant cells. A, calcium responses were measured in Fluo-3-loaded HEK-G_{qi5} cells transiently expressing either CCR4 WT (closed symbols) or chimeric CCR4 with the C-terminal tail of CCR5 (CCR4-5T; open symbols) after addition of either CCL22 (circles) or CCL17 (triangles) at concentrations as shown on the abscissa. The fluorescence signal at each concentration was measured in quadruplicate, and then it was plotted on the ordinate as a percentage of the maximal response normalized to 100% of maximal agonist signaling, after subtraction of the background signal in the absence of agonist. The data represent the average and standard error of either four (CCL22) or three (CCL17) experiments. The lines represent the fit of a simple rectangular hyperbola, constraining the slope factor at 1 and top and bottom at 100 and 0, respectively. Estimates of pEC_{50} values for CCL22 were 9.57 ± 0.023 (EC_{50} = 0.27 nM; CCR4 WT) and 9.13 ± 0.026 (EC_{50} = 0.73 nM; CCR4-5T), and for CCL17 they were 9.44 ± 0.039 (EC_{50} = 0.36 nM; CCR4 WT) and 9.47 ± 0.050 (EC_{50} = 0.33 nM; CCR4-5T). B, As in A, but measuring responses in CCR5 WT (closed symbols) or chimeric CCR5 with the C-terminal tail of CCR4 (CCR5-4T; open symbols) after addition of either CCL5 (circles) or CCL4 (triangles). The data represent the average and standard error of four experiments. The lines represent the fit of a simple rectangular hyperbola, sharing the slope factor for all curves and constraining top and bottom at 100 and 0, respectively. The common slope factor for all curves was estimated at 1.4 ± 0.11. Estimates of pEC_{50} values for CCL5 were 9.44 ± 0.052 (EC_{50} = 0.37 nM; CCR5 WT) and 8.24 ± 0.052 (EC_{50} = 5.8 nM; CCR5-4T) and for CCL4 were 9.17 ± 0.052 (EC_{50} = 0.67 nM; CCR5 WT) and 8.44 ± 0.061 (EC_{50} = 3.6 nM; CCR5-4T).

the chemokine ligands of this GPCR. The data were consistent with that interpretation. The compounds are functional antagonists at CCR4, similar in activity to another published antagonist, BMS-397, blocking calcium signaling to CCR4 agonists. The compounds promote dissociation of an agonist from CCR4-expressing cells. Binding of pyrazinyl-sulfonamides is CCR4-dependent, reversible, and seems to be to a single site on the receptor. Other compounds from this series compete for binding to this site with affinities that correlate to their functional activity, but the site is selective such that the structurally distinct BMS-397 does not seem to compete for binding.

Additional data demonstrate, however, that the pyrazinyl-sulfonamides require access to the cytoplasm for their activity, suggesting that these antagonists act via an intracellular site on CCR4. This novel finding is supported by a series of observations that relate to the property of a compound to maintain an intracellular concentration in steady state with the extracellular medium. The cell plasma membrane seemed to present a diffusion barrier for some compounds from this chemical series, which showed a significant decrease in cell potency compared with their activity in a membrane assay. This explanation also would account for the measured time-dependent increase in apparent cell potency of ester-containing compounds, because these compounds crossed the plasma membrane but they were then hydrolyzed and accumulated within cells as the poorly permeable, but active carboxylic acid. These differential cell-binding potencies were eliminated by incubation of the recombinant cells with a low concentration of saponin, a common method to permeabilize, but not solubilize, the cell plasma membrane (Brooks and Treml, 1983); this method allows otherwise poorly permeable molecules to equilibrate across the plasma membrane. Although it is possible that the saponin treatment might simply have altered membrane fluidity through complex formation with cholesterol, perhaps allowing compounds better access to an extracellular site in the context of an intact cell, this is not consistent with the other data. For example, the dependence of compound binding potency on the host cell background for CCR4 expression (Table 2) is consistent with the knowledge that intracellular steady-state compound concentrations will depend on the complement of drug transporters of the host cell, with their different substrate specificities (e.g., P-glycoprotein or the ATP-binding cassette family) that will differ in profile between cell types (Kim, 2006); further work could establish the relevant process. Finally, in support of the conclusion that the pyrazinyl-sulfonamides bind intracellularly and hence cannot act competitively, their functional antagonism suggests noncompetitiveness or negative cooperativity, as also would be concluded from their effect to promote agonist dissociation.

The parallel structure-activity relationship for the pyrazinyl-sulfonamides at both CCR4 and CCR5 infers a similar mechanism of action at conserved points of interaction, such that these receptors might share significant homology in the region of this proposed binding site[1]. This idea, taken together with the evidence that these compounds seem

[1] Protein sequence identity between human CCR4 and CCR5 is ~50% overall, approximately average across the CC chemokine receptors. If an arbitrary line were drawn through a homology model of CCR4 at the midpoint of the longitudinal axis of the predicted transmembrane domains, then extracellular and intracellular identity between the two receptors would be estimated at 45 and 53%, respectively.

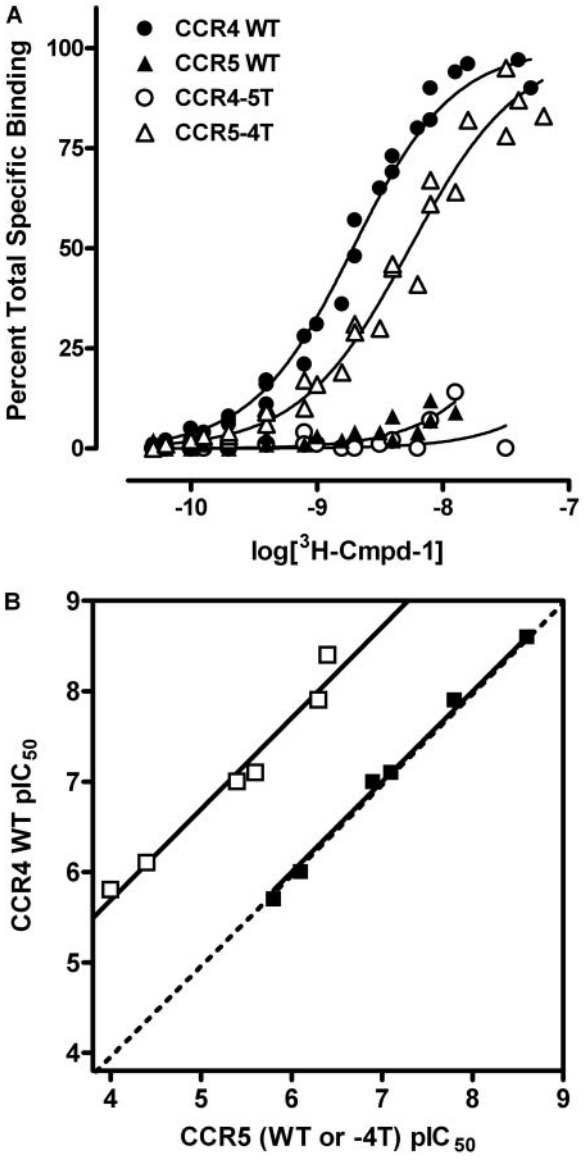

Fig. 10. A, binding of [^{3}H]compound **1** to wild-type and C-terminal chimeras of CCR4 and CCR5 in recombinant cells. Binding of [^{3}H]compound **1** was measured at concentrations as shown on the abscissa, after equilibration at room temperature with HEK-G_{qi5} cells transiently expressing either CCR4 WT (●), CCR5 WT (▲), chimeric CCR4 with the C-terminal tail of CCR5 (CCR4-5T; ○), or chimeric CCR5 with the C-terminal tail of CCR4 (CCR5-4T; △). Nonspecific binding, defined by 10 μM compound **2**, was subtracted from total binding to give specific binding that was then normalized to 100% of maximum and plotted on the ordinate. The lines represent the best fit of a simple rectangular hyperbola with a slope factor of 1 to the pooled data of three experiments. Estimates of pK_d values for the radioligand were 8.71 ± 0.020 (CCR4 WT) and 8.27 ± 0.020 (CCR5-4T). Specific binding of [^{3}H]compound **1** to CCR5 WT and CCR4-5T did not reach 50% of maximum at concentrations of 13 and 32 nM, respectively. B, correlation of antagonist binding potencies between CCR4 WT and either WT or chimeric CCR5. Inhibition of the specific binding of FB-CCL22 to CCR4-CHO cells or FB-CCL5 to CCR5-CHO cells by compounds **1**, **2**, **4**, **5**, and **7** was measured after incubation of each receptor with 0.7 nM FB-chemokine in the absence or presence of a range of antagonist concentrations for 3 h at room temperature. Average pIC_{50} data calculated from four experiments were plotted as a correlation (□), with values on the abscissa representing the pIC_{50} measured at CCR5 WT and values on the ordinate representing the pIC_{50} measured at CCR4 WT. Competition for the specific binding of [^{3}H]compound **1** to HEK-G_{qi5} cells expressing CCR4 WT and chimeric CCR5 with the C-terminal tail of CCR4 (CCR5-4T) by compounds **1**, **2**, **4**, **5**, and **7** was measured after incubation of each receptor with 1 nM [^{3}H]compound **1** for 1 h at room temperature. The average pIC_{50} values calculated from four experiments were plotted as a correlation (■), with values on the abscissa representing the pIC_{50} measured at CCR5 WT and CCR5-4T and values on the ordinate representing the pIC_{50} measured at CCR4 WT. The dashed line represents identity, whereas the solid lines represent the fit of a linear regression of each correlation, with identical slope factors and a difference in the intercept by a value of 50-fold.

to require access to an intracellular site on the receptor, suggested that the contribution of different intracellular domains of CCR4 and CCR5 to the interaction of these compounds should be considered. A first simple approach was to explore the contribution of the C-terminal region to compound activity, encompassing everything beyond the highly conserved GPCR motif, NPxxY (Fritze et al., 2003) in the modeled extracellular half of TM7, including helix 8 (Fig. 2).

Swapping the C-terminal domains between CCR4 and CCR5 resulted in chimeras (CCR5-4T and CCR5-4T) that were highly expressed in recombinant cells and functionally coupled to calcium signaling, albeit with reduced agonist potency at either receptor apart from the activity of CCL17 at CCR4 and CCR4-5T. This differential effect for CCR4 agonist potency at CCR4-5T may be related to the differential ability of CCL17 and CCL22 to promote receptor internalization (Mariani et al., 2004), which has been shown for other GPCRs to involve the C terminus (Bockaert et al., 2003). Likewise, an attenuated response and potency of CCL4 and CCL5 at CCR5-4T either could reflect a limited degree of effective receptor expression and receptor reserve for signaling or perhaps highlight a role for the integrity of the C terminus in CCR5 signaling, at least through calcium, in a recombinant cell. Notwithstanding these effects on agonist responses, it was still possible to compare the effects and interactions with the pyrazinyl-sulfonamide-binding site of the antagonists between the wild-type and chimeric receptors. The most significant result from that comparison is that potent binding and antagonist activity of the pyrazinyl-sulfonamides seem to depend on which C-terminal portion was expressed. The overall structure-activity relationship is independent of the nature of the C-terminal domain, but the presence of the C-terminal domain from CCR4 accounts for the parallel 50-fold increase in compound potency over CCR5 (Fig. 10B). The presence of different C-terminal domains does not affect the selectivity of the structurally distinct antagonists BMS-397 and SCH-C, which do not compete for binding to the site labeled by a pyrazinyl-sulfonamide and so must bind elsewhere on the receptors.

The most likely explanation for the noncompetitive functional antagonism, the promotion of agonist dissociation from the receptor, and the requirement for intracellular access is that these pyrazinyl-sulfonamide antagonists bind to an allosteric site on CCR4 and CCR5, perhaps always expected for a chemokine antagonist that was so much smaller than the natural ligand, but uniquely in that the site seems to be located in a conserved intracellular region of these receptors that involves components of the C-terminal domain. The concept of allosteric interactions in GPCRs is already well appreciated for GPCRs, and numerous allosteric ligands have been described for class A GPCRs (Christopoulos, 2002; Birdsall and Lazareno, 2005), including CCR5 (Kazmierski et al., 2003). Interaction of the pyrazinyl-sulfonamides, however, does not conform to a conventional allosteric ternary complex model with the expected reciprocation between the

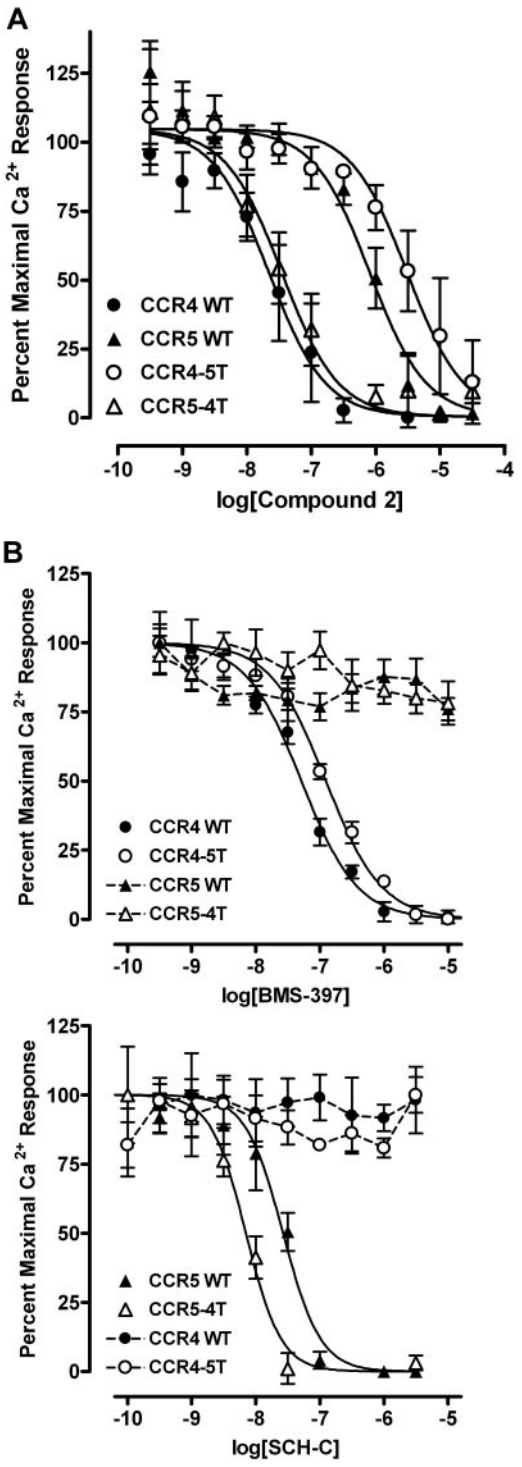

Fig. 11. Antagonism of agonist signaling with wild-type and C-terminal chimeras of CCR4 and CCR5 in recombinant cells. Calcium responses were measured in Fluo-3-loaded HEK-G_{qi5} cells transiently expressing either CCR4 WT (●), CCR5 WT (▲), chimeric CCR4 with the C-terminal tail of CCR5 (CCR4-5T; ○) or chimeric CCR5 with the C-terminal tail of CCR4 (CCR5-4T; △). Antagonism of the response to either CCL22 (0.3 nM) or CCL5 (0.6 nM for WT and 20 or 30 nM for CCR5-4T) was measured for compounds at concentrations as shown on the abscissa, after preincubation for 15 min before addition of the agonist. The fluorescence signal at each concentration was measured in quadruplicate, and then it was plotted on the ordinate as a percentage of the maximal response normalized to 100% of maximal agonist signaling, after subtraction of the background signal in the absence of agonist. The data represent the average and standard error of four experiments for each set. The solid lines represent the fit of a simple rectangular hyperbola, sharing the top for all curves and the bottom at 0. The dashed lines simply connect the data points when no inhibition was measured. Top, inhibition of signaling by compound **2**. The top of the shared curve-fit was estimated at 105 ± 2.7%, constraining the slope factor at 1. Estimates of pIC_{50} values for compound **2** were 7.6 ± 0.11 (CCR4 WT), 5.5 ± 0.11 (CCR4-5T), 6.1 ± 0.91 (CCR5 WT), and 7.5 ± 0.85 (CCR5-4T). Middle, inhibition of signaling by the CCR4 antagonist BMS-397. Fitted curves were constrained with a slope factor of 1 and the top of the curve at 100. Estimates of pIC_{50} values for BMS-397 were 7.29 ± 0.40 (CCR4 WT) and 6.91 ± 0.038 (CCR4-5T). SCH-C was inactive at both CCR4 WT and CCR4-5T. Bottom, inhibition of signaling by the CCR5 antagonist SCH-C. Fitted curves were constrained with the top of the curve at 100. Estimates of pIC_{50} values for SCH-C were 7.58 ± 0.062 (CCR5 WT) and 8.18 ± 0.063 (CCR5-4T), with the shared slope factor estimated at 1.4 ± 0.19 for both curves. BMS-397 was inactive at both CCR5 WT and CCR5-4T.

two ligands (Birdsall and Lazareno, 2005). In recombinant systems, binding of these antagonists to this intracellular allosteric site promotes the dissociation of agonists from the receptor and so must decrease the apparent affinity of the receptor for agonists, whereas agonists do not seem to affect binding of antagonists. We have not been able to determine whether this represents an artifact of the recombinant system whereby the receptor is overexpressed relative to the concentration of G protein, to yield mutually exclusive pools of coupled and uncoupled receptors. According to the simple ternary complex model (Wreggett and De Lean, 1984), such a situation is predicted to produce a significantly lower amount of high-affinity agonist binding relative to that observed for antagonists, but we have no evidence to suggest that this model is even applicable. These interactions might reflect co-operativity between monomers of an oligomeric complex, as has been described for GPCRs (Wreggett and Wells, 1995; Springael et al., 2005). The nature of this proposed allosteric interaction will be the subject of further work.

Future work will also be important to identify the location of this intracellular site. Although the sites of allosteric interaction on a class A GPCR have not yet been identified, since this work was completed a theoretical model has suggested a potential intracellular pocket in the region of TM2 and helix 8 of the M_1-muscarinic receptor (Espinoza-Fonseca and Trujillo-Ferrara, 2006). That proposal would be consistent with our experimental data, with the predicted helix 8 being contained with the C-terminal domain used to support the existence of this intracellular, allosteric site. The C-terminal domain of GPCRs in the region of helix 8 has been identified as a key area for interaction with G proteins, critical for coupling of the receptor to G protein activation (Krishna et al., 2002; Lu et al., 2002; Delos Santos et al., 2006; Lehmann et al., 2007), and with arrestins, which can be important for activation-mediated GPCR internalization or secondary signaling (Bockaert et al., 2003).

Given the selective activity of the pyrazinyl-sulfonamides as a particular class of antagonists at both CCR4 and CCR5, along with the high degree of conservation of this C-terminal region of GPCRs, it would be important to establish whether this is a generic feature of this class of molecular targets that could be used for novel drug discovery. Antagonists acting through this mechanism would provide an approach that may offer advantages and novelty over existing therapies. For example, such antagonists of chemokine receptors (e.g., CCR5) might provide an effective means to block viral entry.

This work has a significant impact on the choice of methods for screening for novel compounds and for rational drug design. Cell-based assays could miss chemical leads if those

early compounds did not have the necessary properties to equilibrate across cell membranes; within a chemical series such compounds could generate misleading structure-activity relationships. An unfortunate choice of host cell for expression of a recombinant GPCR could have the same effect if pumps or transporters influence the effective intracellular concentration. Localization of this novel binding-site will allow for the development of new molecular models and screening methods that can aid discovery of novel drugs.

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