The ins and outs of ligand binding to CCR2

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Chapter 5

Allosteric modulation of the chemokine receptor CCR2 by amiloride analogues and sodium ions

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Abstract

The chemokine receptor CCR2 is a G protein-coupled receptor (GPCR) that is expressed on immune cells and involved in many diseases characterized by inflammation. Previously two distinct binding pockets were reported for small molecules targeting this receptor. In the present study, we revealed yet another binding pocket via which amiloride analogues and sodium ions were discovered to modulate CCR2.

In radioligand binding studies the amiloride analogue HMA allosterically inhibited binding of the agonist ^{125}I-CCL2, the orthosteric antagonist [^{3}H]-INCB3344 and the intracellular antagonist [^{3}H]-CCR2-RA-[R]. Differently, sodium ions only allosterically inhibited ^{125}I-CCL2 binding, while they enhanced binding of [^{3}H]-CCR2-RA-[R]. Three residues located in the core of the transmembrane domain, D88$^{2.50}$, W256$^{6.48}$ and H297$^{7.45}$, turned out to be important for modulation of the antagonist radioligands, since mutation of these residues abolished or diminished the allosteric effects induced by HMA and sodium ions. Upon induced-fit docking of HMA in a homology model of CCR2, we visualized its interaction with D88$^{2.50}$, W256$^{6.48}$ and H297$^{7.45}$ and predicted additional surrounding residues of this binding pocket.

With this work we identified a third binding pocket for small molecules at CCR2, which is located in the core domain of the receptor. By means of the intracellular radioligand [^{3}H]-CCR2-RA-[R] we were able to demonstrate allosteric modulation of the intracellular CCR2 domain by HMA and sodium ions, which has not yet been reported for other GPCRs. This work contributes to our knowledge of allosteric modulation of GPCRs, and offers novel opportunities for targeting CCR2.

Introduction

G protein-coupled receptors (GPCRs) comprise the largest family of cell-surface signal-transduction proteins in mammalian cells [1]. They transfer signals into the cell upon binding of their endogenous ligand at the extracellular face of the receptor. However, during the last decades many small molecule ligands and endogenous chemical entities have been discovered to bind at sites different from where the endogenous ligand binds, so-called allosteric sites [2]. Via these sites they can activate, inhibit or modulate the response of the GPCR. In this study we reveal a novel allosteric binding site in the chemokine receptor CCR2 that allows amiloride analogues and sodium ions to modulate the receptor.

CCR2 is a member of the CC-chemokine receptors which belong to the family of class A GPCRs. It is mainly expressed on monocytes, dendritic cells and lymphocytes, and therefore this receptor is important for correct functioning of the immune system [3, 4]. However, CCR2 is also involved in a variety of diseases including atherosclerosis, rheumatoid arthritis and chronic pain, which makes it an attractive target for the pharmaceutical industry [5]. Many CCR2 small molecule antagonists have been developed, but unfortunately without any clinical success so far. Development of allosteric drugs might offer therapeutic benefits for targeting this receptor [6].

CCR2 is mainly activated by its endogenous chemokine ligand CCL2, which is a peptide of 76 amino acids that binds to the extracellular loops (ECLs) and the TM domain of CCR2 [7]. The numerous small molecule antagonists that have been synthesized so far are chemically divergent, and were recently discovered to bind to two distinct binding sites at CCR2 [8]. One class of antagonists binds in the main binding pocket, located at the upper half of the TM domain of the receptor (orthosteric site), while the other class binds at the intracellular side of the receptor (allosteric site) (Chapter 4). The presence of these multiple binding sites alerted us to investigate whether CCR2 is also prone to modulation by typical GPCR modulators such as amiloride analogues and sodium ions, and if so, via which binding site this is manifested. Amiloride analogues and sodium ions have been found to modulate several class A GPCRs, including adenosine, dopamine and opioid receptors [9-11]. The binding pocket for these ligands is located in the core of the TM-domain, surrounded by the highly conserved residues $D^{2.50}$ and $W^{6.48}$. The position of the sodium ion within this pocket has been revealed in high resolution crystal structures of the inactive A_{2A} adenosine receptor (A_{2A}AR) [12], the inactive protease-activated receptor 1 (PAR1) [13], and the inactive δ-opioid receptor (δ-OR) [11]. In addition, several biochemical studies provided proof that amiloride analogues bind to this same binding pocket in e.g., the α2-adrenergic receptor [36] and the A_{2A}AR [10, 14].

Up to date there is no evidence of the presence of such a sodium binding pocket for chemokine receptors, and neither has modulation of this receptor family by amiloride analogues been researched. In the present study, we took advantage of the availability of two different tool compounds, the orthosteric antagonist radioligand [^{3}H]-INCB3344 and the intracellular antagonist radioligand [^{3}H]-CCR2-RA-[R], to study CCR2 modulation by the sodium ions and amiloride analogues. We unmasked a third binding pocket for small molecules at CCR2, and thereby revealed that this receptor bears small molecule binding pockets throughout the entire transmembrane region. Therefore, this study offers novel opportunities for targeting CCR2.

Materials and methods

Chemicals and reagents. INCB3344 and CCR2-RA-[R] were synthesized according to published methods [15-17]. [^{3}H]-INCB3344 (specific activity 32 Ci mmol^{-1}) and [^{3}H]-CCR2-RA-[R] (specific activity 60 Ci mmol^{-1}) were custom-labeled by Vitrax (Placentia, CA). Bovine serum albumin (BSA, fraction V) was purchased from Sigma (St. Louis, MO, USA). Bicinchoninic acid (BCA) and BCA protein assay reagent were obtained from Pierce Chemical Company (Rockford, IL, USA). Tango CCR2-bla U2OS cells stably expressing human CCR2 (U2OS-CCR2) were obtained from Invitrogen (Carlsbad, CA). Chinese hamster ovary (CHO) cells were obtained from Hans den Dulk (Leiden University, the Netherlands). pcDNA3.1+ plasmid containing wild-type (WT) CCR2 with a 3x hemagglutinin (HA) epitope tag at the N-terminus was kindly provided by James Pease (Imperial College London, UK) [18]. The monoclonal rabbit anti-HA-tag antibody and the HRP-conjugated goat anti-rabbit antibody were obtained from Novus Biologicals (Cambridge UK). LiCl was obtained from Merck KGaA (Darmstadt, Germany). KCl, NaCl, amiloride (3,5-diamino-6-chloro-*N*-[diaminomethylene]pyrazine-2-carboxamide) and HMA (5-[N,N-hexamethylene]amiloride) were purchased from Sigma Aldrich (Zwijndrecht, the Netherlands). MGCMA (5-[N-methyl-N-guanodinocarbonyl-methyl]amiloride), benzamil (3,5-diamino-N-[(1E)-amino-(benzylamino)methylidene]-6-chloropyrazine-2-carboxamide), MIBA (5-[N-methyl-N-isobutyl]amiloride) and phenamil (3,5-diamino-N-[imino(phenylamino) methyl]pyrazinecarboxamide) were obtained from Dr E. J. Cragoe (Lansdale, USA) and were synthesized as described before [19]. Sodium butyrate was obtained from Fisher Scientific (Landsmeer, NL). Polyethylenimine (PEI) was obtained from Polysciences Inc (Eppelheim, Germany). All other chemicals were obtained from standard commercial sources.

Cell culture. U2OS-CCR2 cells were cultured as described before [8]. CHO cells were cultured in DMEM/Ham's F12 medium supplemented with 10% fetal calf serum, 50 IU/ml penicillin, and 50 µg/ml streptomycin, in a humidified atmosphere at 37 °C and 5% CO_2. Cells were subcultured twice a week at a ratio of 1:20 on 10 cm ø or 15 cm ø plates by trypsinization.

Site-directed mutagenesis. $D88A^{2.50}$, $W256A^{6.48}$ and $H297A^{7.45}$ mutations (superscript indicates the Ballesteros Weinstein numbering system [20], in which transmembrane residues are assigned two numbers that belong to the helix number and the residue number relative to the most conserved residue in this helix, which is assigned 50) were generated by site-directed mutagenesis using WT HA-tagged CCR2 plasmid DNA as a template for the generation of mutant plasmids by polymerase chain reaction (PCR) using the QuickChange®II site-directed mutagenesis kit (Stratagene, the Netherlands) and the appropriate oligonucleotide primers (Eurogentec, the Netherlands), under conditions recommended by the manufacturer. All mutants were verified by DNA sequencing before use (LGTC, The Netherlands).

PEI transfections. WT and mutant CCR2 receptors were transiently transfected into CHO cells using PEI. One day prior to transfection CHO cells were split in a ratio of 1:9 on 15 cm ø plates, to reach 50-60% confluence. 10 µg of plasmid DNA was diluted in a sterile 150 mM NaCl solution and subsequently mixed with PEI solution (1 mg/mL) to obtain a DNA:PEI mass ratio of 1:6. The mixture was incubated for 20 min at room temperature before transfection. The culture medium of the cells was refreshed and 1 mL of DNA/PEI mixture was added to cells and incubated for 48 hrs at 37 °C and 5% CO_2. In case of transfections with mutant CCR2 receptors, 500 µl sodium butyrate (final concentration of 5 mM) was added to each plate after 24 hrs in order to increase the receptor expression [21].

Cell surface expression by Enzyme-Linked Immunosorbent Assay (ELISA). Cell surface expression of WT and mutant CCR2 constructs, all containing a HA-tag, was measured by enzyme linked immunosorbent assay (ELISA). In a 96-well plate, $1x10^6$ cells per well were plated 24 hrs after transfection and incubated at 37 °C, 5% CO_2. 48 hrs after transfection cells were fixed with 3.7% formaldehyde. The cells were washed with DMEM and the primary antibody (monoclonal rabbit anti-HA-tag 1:5000 in DMEM) was incubated for 30 min at RT. After removal of the primary antibody, the cells were washed with DMEM/25 mM HEPES and dried for 10 min. Subsequently, a mixture of the secondary antibody (HRP-conjugated goat anti-rabbit 1:5000 in DMEM) was incubated for 30 min. The cells were washed twice with PBS (37 °C) and left to dry for 10 min at RT. 100 µl tetramethylbenzene (TMB) was added in the

dark and incubated for 5 min. The reaction was stopped after addition of 100 µl 1 M H_3PO4. After 5 min, absorbance was measured at 450 nm with a Victor2V plate reader (Perkin Elmer, Waltham, MA, USA).

Cell membrane preparation. Preparation of membranes was performed as described before [8]. Briefly, cells were scraped from 15 cm ø plates upon which the membranes and cytosolic fractions were separated during several centrifugation steps. Finally, the membrane pellet was resuspended in ice-cold 50 mM Tris-HCl buffer containing 5 mM $MgCl_2$, pH 7.4, and aliquots were stored at −80 °C. Membrane protein concentrations were measured using a BCA protein determination with BSA as a standard [22].

[^{3}H]-INCB3344 binding assays. Binding assays were performed in a 100 µL reaction volume containing 50 mM Tris-HCl buffer (pH 7.4), 5 mM $MgCl_2$, 0.1% CHAPS and 7.5 µg of membrane protein for WT CCR2 membranes and 20-40 µg for mutant receptors, at 25 °C. Saturation experiments were performed using 12 different concentrations of radioligand from 0.5 to 20 nM for 120 min of incubation. Non-specific binding was determined at three concentrations of radioligand with 10 µM BMS22. Displacement assays were carried out with 3.5 nM [^{3}H]-INCB3344 using 10 concentrations of competing ligand for 120 min of incubation. In dissociation experiments the membranes were first incubated with 3.5 nM [^{3}H]-INCB3344 for 90 min. Dissociation was initiated by addition of 10 µM BMS22 in the absence or presence of modulator at different time points during 180 min. Homologous competition experiments with mutant receptors were performed with 3.5 nM [^{3}H]-INCB3344 and increasing concentrations of unlabeled INCB3344 for an incubation time of 120 min. For assays with the mutant receptors and NaCl or HMA, a radioligand concentration of 6.0 nM was used. The assays were terminated as described before [8]. In all experiments, total radioligand binding did not exceed 10% of the amount added to prevent ligand depletion.

[^{3}H]-CCR2-RA-[R] binding assays. Assay conditions were similar as described for [^{3}H]-INCB3344 binding assays, unless otherwise stated. Saturation experiments were carried out using 12 different concentrations of radioligand from 0.1 to 75 nM for 120 min of incubation. Non-specific binding was determined at three concentrations of radioligand with 10 µM JNJ-27141491. Displacement assays were carried out with 7.0 nM [^{3}H]-CCR2-RA-[R] using 10 concentrations of competing ligand for 120 min of incubation. Kinetic experiments were performed at 15 °C. For dissociation experiments the membranes were first incubated with 7.0 nM [^{3}H]-CCR2-RA-[R] for 90 min. Dissociation was initiated upon addition of 10 µM of JNJ-

27141491 in the absence or presence of modulator at different time points during 180 min. For displacement and dissociation assays non-specific binding was determined with 10 µM JNJ-27141491. Homologous competition experiments with mutant receptors were performed with 20 nM [^{3}H]-CCR2-RA-[R] and increasing concentrations of unlabeled CCR2-RA-[R] for an incubation time of 120 min. Non-specific binding was determined with 10 µM CCR2-RA-[R]. For assays with the mutant receptors and NaCl or HMA, a radioligand concentration of 20 nM was used. For measurements with HMA, nonspecific binding was determined for every data point with a combination of 10 µM CCR2-RA-[R] and 0.1 mM HMA. The assays were terminated as described before [8]. In all experiments, total radioligand binding did not exceed 10% of the amount added to prevent ligand depletion.

^{125}I-CCL2 dissociation assay. The assay was performed in a 100 µL reaction volume containing 50 mM Tris-HCl buffer (pH 7.4), 5 mM $MgCl_2$ and 0.1% 3-((3-cholamidopropyl)-dimethylammonio)-1-propanesulfonate (CHAPS). 15 µg of U2OS-CCR2 membrane protein was incubated with 0.1 nM ^{125}I-CCL2 for 2 hrs at 37ºC. Subsequently, dissociation was initiated upon addition of 50 nM CCL2 in the absence or presence of modulator at different time points. Non-specific binding was determined with 10 µM INCB3344. The assay was terminated as described before [8]. Total radioligand binding did not exceed 10% of the amount added to prevent ligand depletion.

Homology modeling and docking. A homology model of the chemokine CCR2 receptor was constructed using the homology modeling tool within Maestro [23-25]. This model was based on the structure of the chemokine CCR5 receptor co-crystalized with maraviroc (Protein Data Bank: 4MBS). The best model was selected based on the energy-based scoring function, the alignment between CCR2 and CCR5 was performed using ClustalW as implemented within Maestro. HMA was docked using the induced fit docking protocol [26, 27]. The grid center was placed based on residues D88$^{2.50}$, W256$^{6.48}$ and H297$^{7.45}$ with an automatic box size. Visualizations were created using PyMOL version 1.6.0.0. [28].

Data analysis. All experiments were analyzed using the non-linear regression curve fitting program Prism 5 (GraphPad, San Diego, CA, U.S.A.). The pIC_{50} values of HMA and MIBA were obtained by non-linear regression analysis of the displacement curves. The pK_D, defined as $-\log_{10}K_D$, and B_{max} values of [^{3}H]-INCB3344 and [^{3}H]-CCR2-RA-[R] for WT CCR2 were obtained by computer analysis of saturation curves according to the equation bound = $(B_{max}*[L])/([L]+K_d)$ where B_{max} is the maximal number of binding sites (pmol/mg) and K_D is the concentration

of radioligand required to reach half-maximal binding. The pK_D values of [^{3}H]-INCB3344 and [^{3}H]-CCR2-RA-[R] for mutant CCR2 receptors were calculated from homologous competition experiments using the Cheng-Prusoff equation, assuming that unlabeled and labeled INCB3344 had identical affinities, and the same for unlabeled and labeled CCR2-RA-[R] [29]. The dissociation rate constant (k_{off}) was obtained by computer analysis of the exponential decay of radioligand binding to the receptor. [^{3}H]-INCB3344 experiments were fitted according to monophasic equations. Dissociation of [^{3}H]-CCR2-RA-[R] occurred in a biphasic manner. Binding data from ^{125}I-CCL2 dissociation kinetic experiments were fitted assuming monophasic or biphasic dissociation curves. By the F-test, a significant better fit for biphasic dissociation was found for curves with addition of HMA and NaCl. Data shown are the mean ± S.E.M. of at least 3 separate experiments performed in duplicate. Statistical analysis was performed with a two-tailed unpaired Student's *t*-test.

Results

Antagonist equilibrium binding assays. To determine if CCR2 was susceptible to modulation by amiloride analogues (Fig. 1) and sodium ions, we performed equilibrium binding studies with the radiolabeled orthosteric antagonist [^{3}H]-INCB3344 and allosteric antagonist [^{3}H]-CCR2-RA-[R].

Addition of 0.1 mM amiloride, benzamil, phenamil or MGCMA did not result in any radioligand displacement (data not shown). Differently, [^{3}H]-INCB3344 was displaced by HMA and MIBA with a pIC_{50} of 4.1 ± 0.1 and 3.8 ± 0.0, respectively (Fig. 2A+B). The allosteric radioligand [^{3}H]-CCR2-RA-[R] was also displaced by both HMA and MIBA with a pIC_{50} of 4.1 ± 0.1 and 3.9 ± 0.0 (Fig. 2A+B). These data indicate that CCR2 possesses a binding pocket for amiloride analogues, among which HMA binds with the highest affinity. In addition, the hill slopes of the curves deviated from unity, being -1.9 ± 0.1 (HMA) and -1.9 ± 0.5 (MIBA) for displacement of [^{3}H]-INCB3344, and -2.1 ± 0.1 (HMA) and -2.0 ± 0.3 (MIBA) for displacement of [^{3}H]-CCR2-RA-[R] (Table 1). This suggests that HMA and MIBA bind to CCR2 in an allosteric manner with respect to both antagonist radioligands.

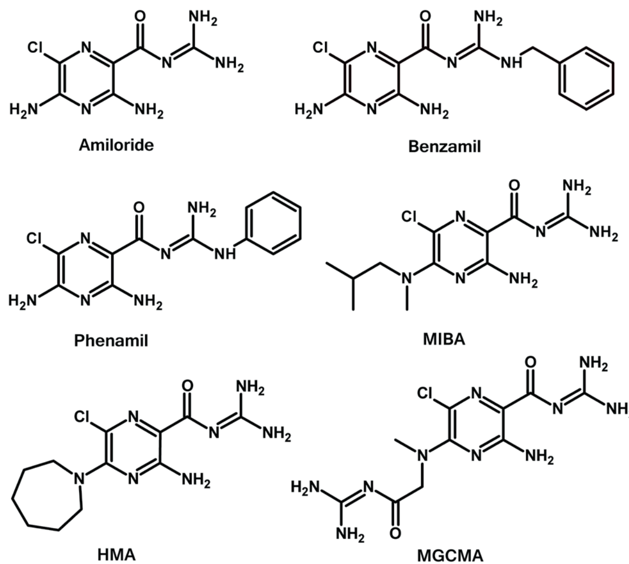

Fig. 1. Chemical structures of amiloride and the amiloride analogues HMA, MIBA, benzamil, phenamil and MGCMA.

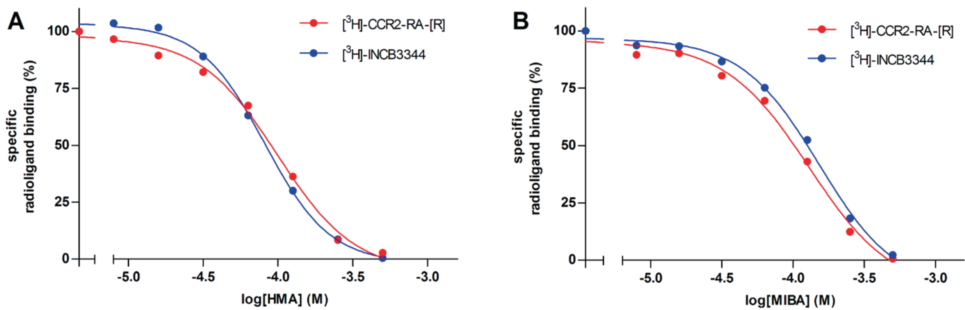

Fig. 2. Displacement of the orthosteric antagonist [^{3}H]-INCB3344 and the allosteric antagonist [^{3}H]-CCR2-RA-[R] from CHO cell membranes transiently expressing CCR2, upon addition of increasing concentrations of the amiloride analogues HMA (A) or MIBA (B). Results are presented as percentage of bound radioligand for one representative experiment performed in duplicate.

Table 1. Displacement of [^{3}H]-INCB3344 and [^{3}H]-CCR2-RA-[R] by HMA and MIBA from CHO cell membranes transiently expressing CCR2.

	orthosteric [^{3}H]-INCB3344		allosteric [^{3}H]-CCR2-RA-[R]	
amiloride analogue	**HMA**	**MIBA**	**HMA**	**MIBA**
pIC_{50} ± S.E.M.	4.1 ± 0.1	3.8 ± 0.0	4.1 ± 0.1	3.9 ± 0.0
hill slope	-1.9 ± 0.1	-1.9 ± 0.5	-2.1 ± 0.1	-2.0 ± 0.3

Data presented as pIC_{50} (mean ± S.E.M.) of three independent experiments performed in duplicate.

Besides the amiloride analogues, we also included sodium ions in the antagonist radioligand binding studies. Interestingly, high concentrations of NaCl significantly enhanced binding of the allosteric antagonist [^{3}H]-CCR2-RA-[R] up to 131 ± 7 % at 1 M, whereas binding of the orthosteric antagonist [^{3}H]-INCB3344 was not affected (Fig. 3A). To further investigate if the enhanced binding of [^{3}H]-CCR2-RA-[R] was the result of CCR2 modulation by the sodium cation or rather the chloride anion, we performed similar binding experiments in the presence of 1 M LiCl or KCl. LiCl significantly enhanced binding of [^{3}H]-CCR2-RA-[R] up to 169 ± 16 %, whereas [^{3}H]-CCR2-RA-[R] binding in the presence of 1 M KCl was not affected (Fig. 3B).

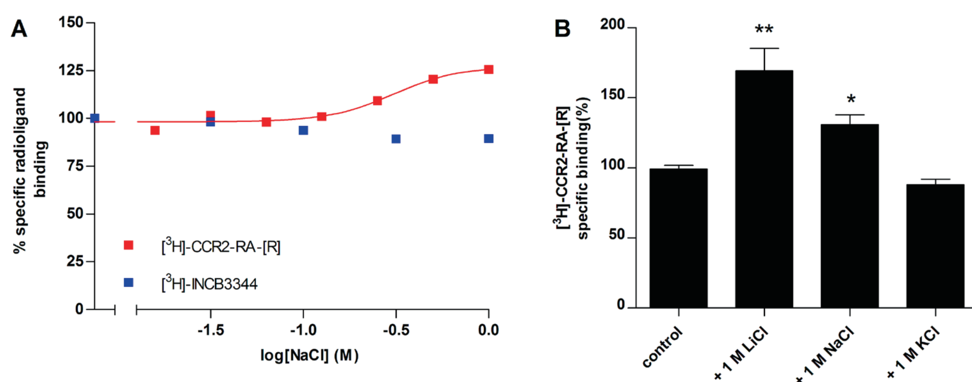

Fig. 3. (A) Modulation of binding of the orthosteric antagonist [^{3}H]-INCB3344 and the allosteric antagonist [^{3}H]-CCR2-RA-[R] to CHO cell membranes transiently expressing CCR2 by increasing concentrations of NaCl. Results are presented as percentage of bound radioligand for one representative experiment performed in duplicate. (B) Modulation of binding of the allosteric antagonist [^{3}H]-CCR2-RA-[R] by 1 M LiCl, NaCl or KCl. Results are presented as mean ± S.E.M. for at least three independent experiments performed in triplicate. * $p < 0.05$ vs. control, ** $p < 0.005$ vs. control, Student's *t*-test.

Antagonist radioligand dissociation assays. Since the equilibrium binding studies indicated allosteric interactions of the amiloride analogues and sodium ions with respect to one or both of the antagonist radioligands, we continued with kinetic binding studies to further explore their mode of action. For both radioligands [^{3}H]-INCB3344 and [^{3}H]-CCR2-RA-[R], dissociation was initiated by the addition of excess competing unlabeled ligand in the presence and absence of the most potent amiloride analogue HMA and a physiological concentration (150 mM) of sodium ions. In the presence of 0.1 mM HMA the dissociation rate of the orthosteric antagonist [^{3}H]-INCB3344 was 0.030 ± 0.001 min^{-1}, which was significantly faster than 0.024 ± 0.002 min^{-1} in the control situation (Fig. 4A, Table 2). [^{3}H]-CCR2-RA-[R] dissociated in a biphasic manner from the receptor, with the fast phase accounting for 48% of the dissociation

(Table 2). In the presence of 0.1 mM HMA we observed an increased, albeit not significant (p = 0.13), fast phase dissociation rate of 0.27 ± 0.09 min^{-1} compared to 0.10 ± 0.03 min^{-1} in the control situation (Fig. 4B, Table 2). The percentage of fast phase dissociation was not affected in the presence of HMA, nor was k_{slow}. These data suggest noncompetitive interactions of HMA with respect to both radioligands. On the other hand, addition of 150 mM NaCl did not significantly affect the dissociation rate of either one of the antagonist radioligands (Fig. 4, Table 2).

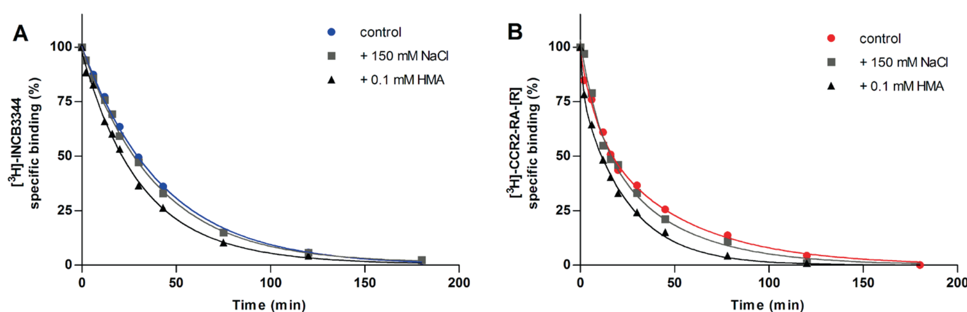

Fig. 4. Dissociation of the antagonist radioligands from CHO cell membranes transiently expressing CCR2. (A) Dissociation of [3H]-INCB3344 was initiated upon addition of 10 µM competing ligand BMS22 in the presence and absence of 0.1 mM HMA or 150 mM NaCl at various time points. (B) Dissociation of [3H]-CCR2-RA-[R] was initiated upon addition of 10 µM competing ligand JNJ-27141491 in the presence and absence of 0.1 mM HMA or 150 mM NaCl at various time points. Results are presented as percentage of bound radioligand for one representative experiment performed in duplicate.

Table 2. Dissociation rate of [3H]-INCB3344 and [3H]-CCR2-RA-[R] from CHO cell membranes transiently expressing CCR2 in the presence and absence of NaCl and HMA.

	orthosteric [3H]-INCB3344			allosteric [3H]-CCR2-RA-[R]		
	control	+ 150 mM NaCl	+ 0.1 mM HMA	control	+ 150 mM NaCl	+ 0.1 mM HMA
k_{fast} (min^{-1})	0.024 ± 0.002	0.025 ± 0.001	0.030 ± 0.001*	0.10 ± 0.03	0.16 ± 0.07	0.27 ± 0.09
k_{slow} (min^{-1})	n.a.	n.a.	n.a.	0.022 ± 0.005	0.030 ± 0.005	0.030 ± 0.009
% fast	100	100	100	48 ± 6	43 ± 9	50 ± 12

Data presented as mean ± S.E.M. of three independent experiments performed in duplicate.
n.a., not applicable
* p< 0.05 vs. control, Student's *t*-test.

Antagonist saturation binding assays. To confirm the observed noncompetitive effects of HMA in the dissociation studies, we performed saturation binding experiments of [3H]-INCB3344 and [3H]-CCR2-RA-[R] in the presence and absence of 80 µM HMA. As a control we added unlabeled INCB3344 or CCR2-RA-[R]. Addition of HMA significantly decreased

the B_{max} of the orthosteric antagonist [^{3}H]-INCB3344, from 11 ± 1 pmol/mg in the control situation to 7.9 ± 0.6 pmol/mg in the presence of HMA (Fig. 5A and Table 3). The pK_D of 8.7 ± 0.0 was decreased to 8.5 ± 0.1 in the presence of HMA. The decrease in B_{max} suggests a noncompetitive interaction of HMA and INCB3344, whereas the small but significant decrease in the affinity of [^{3}H]-INCB3344 would reflect a competitive interaction. Differently, the B_{max} of [^{3}H]-INCB3344 was not affected in the presence of INCB3344, whereas its pK_D was decreased to 8.3 ± 0.0 (Fig. 5A and Table 3), both in agreement with a competitive mode of interaction. The B_{max} for [^{3}H]-CCR2-RA-[R] of 10 ± 0.4 pmol/mg was decreased to 7.8 ± 0.9 pmol/mg in the presence of HMA (Fig. 5B, Table 3). In addition, the pK_D of 8.1 ± 0.1 of [^{3}H]-CCR2-RA-[R] was not significantly changed by HMA, which suggests a purely noncompetitive mode of interaction. In the presence of CCR2-RA-[R] the B_{max} of [^{3}H]-CCR2-RA-[R] was not affected, whereas its pK_D was decreased to 7.6 ± 0.1 (Fig. 5B and Table 3). These data are in agreement with a competitive mode of interaction.

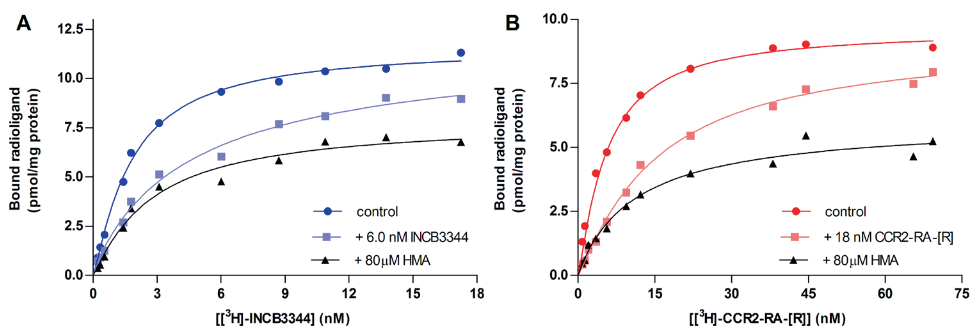

Fig. 5. Saturation binding of the antagonist radioligands to CHO cell membranes transiently expressing CCR2. (A) Binding of the orthosteric antagonist [^{3}H]-INCB3344 in the presence and absence of 6.0 nM INCB3344 or 80 µM HMA. (B) Binding of allosteric antagonist [^{3}H]-CCR2-RA-[R] from CHO membranes transiently expressing CCR2 in the presence and absence of 6.0 nM CCR2-RA-[R] or 80 µM HMA. Results are presented as amount of bound radioligand in pmol/mg protein of one representative experiment performed in duplicate.

Table 3. Saturation binding of [^{3}H]-INCB3344 and [^{3}H]-CCR2-RA-[R] to CHO cell membranes transiently expressing CCR2 in the presence and absence of HMA or competing unlabeled ligand.

	orthosteric [^{3}H]-INCB3344			allosteric [^{3}H]-CCR2-RA-[R]		
	control	+ 6.0 nM INCB3344	+ 80 µM HMA	control	+ 18 nM CCR2-RA-[R]	+ 80 µM HMA
B_{max} (pmol/mg)	11 ± 0.7	11 ± 0.8	7.9 ± 0.6*	10 ± 0.4	11 ± 0.7	7.8 ± 0.9*
pK_D	8.7 ± 0.0	8.3 ± 0.0**	8.5 ± 0.1*	8.1 ± 0.1	7.6 ± 0.1*	7.8 ± 0.2

Data presented as mean ± S.E.M. of three independent experiments performed in duplicate.
* p< 0.05 vs. control, Student's *t*-test.
** p< 0.005 vs. control, Student's *t*-test.

Agonist modulation by HMA and NaCl. Besides the modulation of antagonist binding, we also determined the effect of HMA and NaCl on the dissociation of the agonist radioligand ^{125}I-CCL2. These assays were performed with membrane preparations of U2OS cells stably expressing CCR2, since the non-specific binding of ^{125}I-CCL2 to these membranes was substantially lower compared to that for membranes of CHO cells transfected with CCR2. Dissociation of ^{125}I-CCL2 was initiated by an excess of CCL2 in the presence and absence of 0.1 mM HMA or 150 mM NaCl. The dissociation in the control situation was monophasic with a rate of 0.030 ± 0.002 min^{-1}, whereas in the presence of HMA or NaCl a biphasic dissociation pattern was observed (Fig. 6 and Table 4). Although the dissociation rates could therefore not be directly compared, enhanced ^{125}I-CCL2 dissociation was observed in both cases compared to the control situation (Fig. 6 and Table 4). These data show that the endogenous chemokine ligand is allosterically modulated by HMA and NaCl. Notably, physiological NaCl concentrations apparently increased the dissociation rate of ^{125}I-CCL2, whereas the dissociation rate of the antagonist radioligands at this concentration of NaCl was not affected.

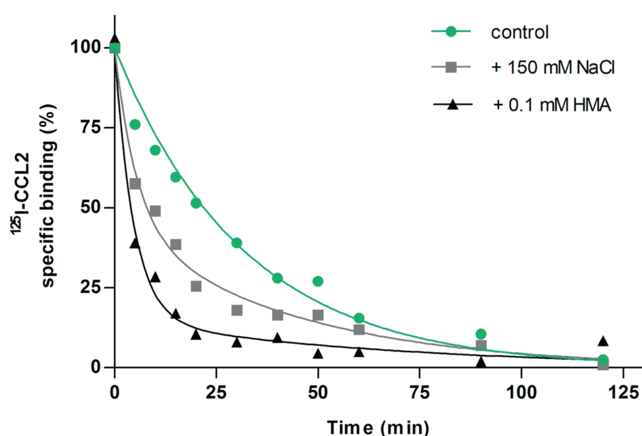

Fig. 6. Dissociation of agonist radioligand ^{125}I-CCL2 from U2OS membranes stably expressing CCR2. Dissociation was initiated upon addition of 50 nM CCL2 in the presence or absence of 0.1 mM HMA or 150 mM NaCl at different time points. Results are presented as percentage of bound radioligand for one representative experiment performed in duplicate.

Table 4. Dissociation rates of ^{125}I-CCL2 from U2OS cell membranes stably expressing CCR2 in the presence and absence of NaCl and HMA.

	control	+ 150 mM NaCl	+ 0.1 mM HMA
k_{fast} (min^{-1})	0.030 ± 0.002	0.21 ± 0.01	0.29 ± 0.03
k_{slow} (min^{-1})	n.a.	0.025 ± 0.001	0.010 ± 0.003
% fast	100	46 ± 7	86 ± 2

Data presented as mean ± S.E.M. of three independent experiments performed in duplicate.
n.a., not applicable

The effect of mutations in the core TM domain on antagonist binding. In the radioligand binding studies presented above we observed allosteric modulation of ^{125}I-CCL2, [^{3}H]-INCB3344 and [^{3}H]-CCR2-RA-[R] by HMA, and allosteric modulation of ^{125}I-CCL2 and [^{3}H]-CCR2-RA-[R] by sodium ions. This suggests that HMA and sodium ions bind to a site other than the orthosteric binding site in the TM domain and distinct from the allosteric binding site at the intracellular side of the receptor. To elucidate the location of this additional binding site, we mutated three residues in the core region of the TM domain, D88$^{2.50}$, W256$^{6.48}$ and H297$^{7.45}$.

By means of whole cell ELISA we measured the expression of the mutant receptors. Cell surface expression of D88A$^{2.50}$, W256A$^{6.48}$ and H297A$^{7.45}$ was 46 %, 19 % and 14 % compared to wild type CCR2 (Fig. 7), suggesting that mutations in the core domain affected the stability of the receptor and/or its transport to the cell membrane. Nevertheless, with sufficient amounts of membrane protein and radioligand we were able to measure the affinity of [^{3}H]-INCB3344 and [^{3}H]-CCR2-RA-[R] in homologous displacement assays. Binding of [^{3}H]-INCB3344 to W256A$^{6.48}$ could not be detected, whereas the pK_D for D88A$^{2.50}$ and H297A$^{7.45}$ was 8.5 ± 0.3 and 8.6 ± 0.1 with a B_{max} of 1.8 ± 0.7 pmol/mg and 0.49 ± 0.17 pmol/mg, respectively (Table 5). The affinity of [^{3}H]-INCB3344 for these two mutant receptors was therefore similar to the pK_D of 8.7 ± 0.0 for WT CCR2. The intracellular antagonist [^{3}H]-CCR2-RA-[R] was able to bind to all mutant receptors, with a pK_D of 7.3 ± 0.2, 6.7 ± 0.1 and 6.8 ± 0.1 for D88A$^{2.50}$, W256A$^{6.48}$ and H297A$^{7.45}$ (Table 5). Compared to the pK_D of 8.1 ± 0.1 for WT CCR2, the affinity of [^{3}H]-CCR2-RA-[R] for all three mutant receptors was substantially decreased. The B_{max} of [^{3}H]-CCR2-RA-[R] for the D88A$^{2.50}$, W256A$^{6.48}$ and H297A$^{7.45}$ mutant receptors was 2.0 ± 0.6 pmol/mg, 2.8 ± 0.5 pmol/mg and 3.1 ± 0.5 pmol/mg, respectively (Table 5).

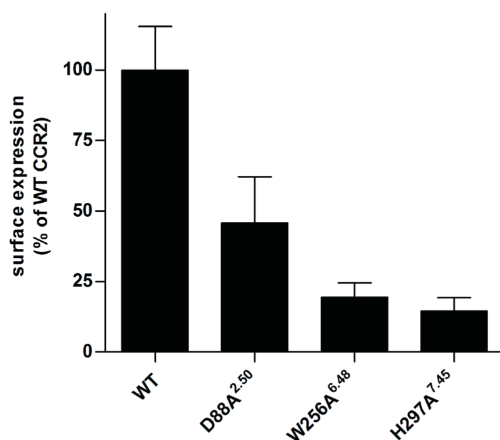

Fig. 7. Surface expression of the HA-tagged WT and mutant CCR2 receptors in CHO cells as measured by ELISA. Data was normalized for WT CCR2 expression (100%) and is presented as mean ± SD of at least two experiments performed in quadruplicate.

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Table 5. The affinity (pK_D) and maximum number of binding sites (B_{max}) of [^{3}H]-INCB3344 and [^{3}H]-CCR2-RA-[R] for three mutant CCR2 receptors that were transiently expressed on CHO cell membranes. Values were determined from homologous competition experiments with the unlabeled ligands INCB3344 and CCR2-RA-[R], respectively.

	orthosteric [^{3}H]-INCB3344		allosteric [^{3}H]-CCR2-RA-[R]	
construct	**pK_D**	**B_{max} (pmol/mg)**	**pK_D**	**B_{max} (pmol/mg)**
D88A$^{2.50}$	8.5 ± 0.3	1.8 ± 0.7	7.3 ± 0.2	2.0 ± 0.6
W256A$^{6.48}$	no binding		6.7 ± 0.1	2.8 ± 0.5
H297A$^{7.45}$	8.6 ± 0.1	0.49 ± 0.17	6.8 ± 0.1	3.1 ± 0.5

Data presented as mean ± S.E.M. of three independent experiments performed in duplicate.

The effect of mutations in the core TM domain on modulation by HMA and sodium ions. Antagonist radioligand binding was measured for WT CCR2 as well as for the mutant receptors D88A$^{2.50}$, W256A$^{6.48}$ and H297A$^{7.45}$ upon addition of 1 M NaCl or 0.1 mM HMA. The results of these experiments will be discussed per mutant in this section.

For D88A$^{2.50}$, the percentage of [^{3}H]-INCB3344 binding in the presence of 0.1 mM HMA increased to 34 ± 6 % compared to 20 ± 2 % for WT CCR2 (Fig. 8A). Similarly, the percentage of [^{3}H]-CCR2-RA-[R] binding to D88A$^{2.50}$ in presence of 0.1 mM HMA increased to 62 ± 5 % compared to 18 ± 2 % for WT CCR2 (Fig. 8B), indicating that the ability of HMA to displace both antagonists decreased upon mutation of D88$^{2.50}$. Addition of 1 M NaCl did not affect [^{3}H]-INCB3344 binding to WT CCR2, neither was a significant change observed for D88A$^{2.50}$ (Fig. 8A). However, enhanced binding of [^{3}H]-CCR2-RA-[R] was observed for WT CCR2 in the

presence of 1 M NaCl, to a percentage of 133 ± 5, which was reduced to 96 ± 4 % upon introduction of D88A$^{2.50}$ (Fig. 8B). These results indicate that D88$^{2.50}$ is involved in sodium ion binding in CCR2.

For W256A$^{6.48}$ we did not measure any binding of [^{3}H]-INCB3344, and therefore we solely used [^{3}H]-CCR2-RA-[R] to study the effects of NaCl and HMA. No displacement of [^{3}H]-CCR2-RA-[R] was observed upon addition of 0.1 mM HMA (Fig. 8B), revealing a crucial role for W256$^{6.48}$ in CCR2 modulation by HMA. In the presence of 1 M NaCl, binding of [^{3}H]-CCR2-RA-[R] was increased to 117 ± 8 %, although not significant with respect to the control situation ($p = 0.1$) (Fig. 8B). This indicates that W256A$^{6.48}$ is important for the allosteric enhancement by sodium ions that was observed for the WT receptor.

For H297A$^{7.45}$, the percentage of [^{3}H]-INCB3344 and [^{3}H]-CCR2-RA-[R] binding compared to WT in the presence of 0.1 mM HMA significantly increased to 30 ± 5 % and 82 ± 7 %, respectively (Fig. 8A+B). In addition, the ability of sodium ions to enhance [^{3}H]-CCR2-RA-[R] binding was completely abolished since only 82 ± 6 % of radioligand binding was measured compared to 133 ± 5 % for the WT receptor (Fig. 8B). These data indicate that the ability of both HMA and sodium ions to modulate CCR2 decreased upon mutation of H297$^{7.45}$.

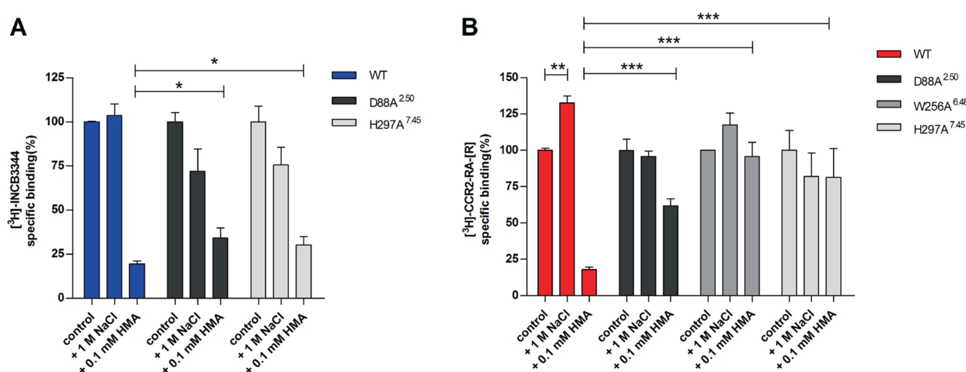

Fig. 8. Equilibrium binding of the orthosteric antagonist [^{3}H]-INCB3344 (A) and the allosteric antagonist [^{3}H]-CCR2-RA-[R] (B) in the presence and absence of 1 M NaCl or 0.1 mM HMA. For these experiments CHO cell membranes transiently expressing WT or mutant CCR2 were used. Data are presented as mean ± S.E.M of the percentage of bound radioligand of at least three independent experiments performed in triplicate. * $p < 0.05$ vs. WT, ** $p < 0.01$ vs. control, *** $p < 0.01$ vs. WT, Student's *t*-test.

Docking of HMA in a CCR2 homology model. We constructed a CCR2 homology model using the crystal structure of CCR5 (PDB: 4MBS), and performed docking of HMA therein. The positively charged guanidinium group of HMA was docked towards the bottom of the binding pocket where it formed ionic interactions with the negatively charged D88$^{2.50}$ (Fig.

9A+B). For W256$^{6.48}$, a hydrogen bonding interaction with the oxygen of HMA (Fig. 9A) as well as π-stacking interactions with the pyrimidine core (Fig. 9B) was predicted. Yet another interaction, between H297$^{7.45}$ and the oxygen of HMA, is visualized in the docking pose as well as the interaction map (Fig. 9A+B).

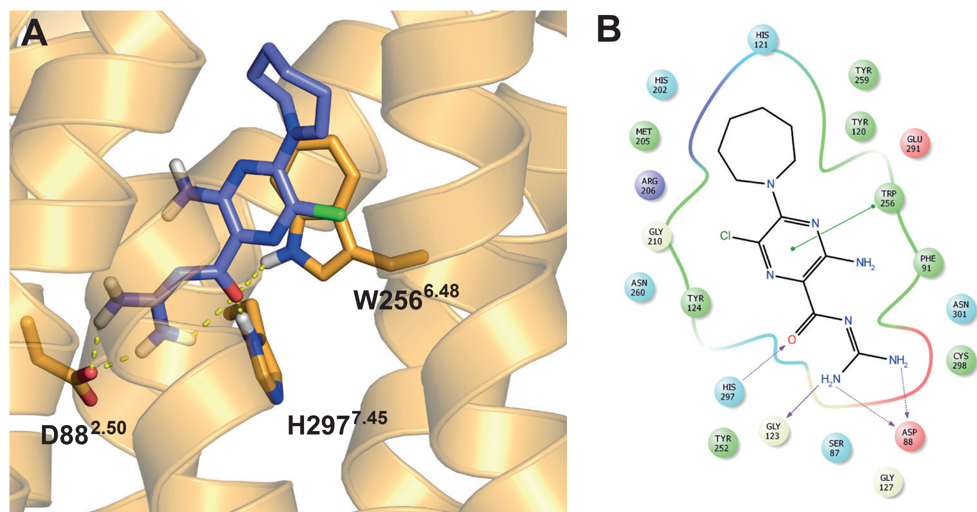

Fig. 9. (A) Induced fit docking of HMA in a homology model of CCR2 based on the crystal structure of CCR5. An ionic interaction between D88$^{2.50}$ and the guanidinium group of HMA and between H297$^{7.45}$ and the oxygen of HMA is illustrated, as well as a hydrogen bonding interaction of W256$^{6.48}$ and the oxygen of HMA. (B) The 2D interaction map that illustrates an additional π-stacking interaction between W256$^{6.48}$ and the pyrimidine core of HMA, as well as all surrounding residues.

Discussion

Modulation of GPCRs by sodium ions and amiloride analogues has been described for a number of class A GPCRs, including adrenergic receptors [30], adenosine receptors [10, 12], dopamine receptors [9] and opioid receptors [11]. This ubiquitous amount of data among various GPCRs suggests interactions with a very conserved site, providing evidence for a common allosteric mechanism [31]. With the present study we add the first chemokine receptor, CCR2, to the list of GPCRs that are modulated by amiloride analogues and sodium ions. For CCR2 two distinct small molecule binding pockets have previously been identified [8], of which one is located at the upper half of the TM domain, whereas as the other pocket resides at the intracellular side of the receptor (Chapter 4). Here we provide evidence for modulation of CCR2 by the amiloride analogue HMA and sodium ions via yet another site,

suggesting that CCR2 comprises three distinct small molecule binding pockets. Moreover, by means of the intracellular radioligand $[^3H]$-CCR2-RA-[R] as a unique tool compound, we were able to study the influence at the intracellular side of the receptor induced by HMA and sodium ions.

In radioligand binding assays we observed allosteric modulation of ^{125}I-CCL2 and $[^3H]$-CCR2-RA-[R] by sodium ions. We measured increased binding of the intracellular antagonist $[^3H]$-CCR2-RA-[R] at a high concentration of 1 M NaCl. To address the question whether sodium or chloride ions are responsible for this effect, we also examined the effects of equal concentrations of LiCl and KCl. Their differential effects strongly pointed to the involvement of monovalent cations rather than (chloride) anions. Lithium ions, which have a smaller diameter than sodium ions, even further enhanced $[^3H]$-CCR2-RA-[R] binding, while the larger potassium ions did not modulate antagonist binding at all. These data suggest that the size of the cations that are able to modulate CCR2 antagonist binding is restricted to the 116 pm diameter of sodium. A physiologically relevant NaCl concentration of 150 mM did not affect binding of $[^3H]$-CCR2-RA-[R], but increased the dissociation rate of ^{125}I-CCL2. This latter finding is in line with data for other GPCRs indicating that sodium ions stabilize the inactive state of the receptor and decreases the affinity of agonists [12, 32-34]. While this is the first study to report the effect of sodium ions on an intracellular GPCR antagonist, allosteric enhancement of orthosteric antagonists by NaCl has previously been described for among others dopamine, adenosine and α2-adrenergic receptors [9, 10, 33, 34]. Interestingly, in our study the binding of the orthosteric antagonist INCB3344 was not modulated by NaCl. It should be noted that all orthosteric GPCR antagonists for which binding was previously found to be enhanced by NaCl, behaved as inverse agonists. Therefore enhancement by NaCl, which stabilizes the inactive state of the receptor, is compliant with their mechanism of action. For INCB3344 no such inverse agonism has been detected [8, 35], which could explain the lack of effect of NaCl.

Previous studies have shown that amiloride and its analogues bind to the same binding pocket as sodium ions [12, 36]. They are known to decrease agonist binding, and in contrast to sodium ions, they also decrease antagonist binding [10, 34, 36]. For CCR2 we discovered that the amiloride analogues HMA and MIBA displaced the orthosteric antagonist $[^3H]$-INCB3344 as well as the intracellular antagonist $[^3H]$-CCR2-RA-[R] with similar potencies. In kinetic experiments in the presence of HMA we observed increased dissociation rates for both antagonist radioligands as well as for the radiolabeled agonist ^{125}I-CCL2. Saturation binding experiments in the presence of HMA revealed characteristics of a noncompetitive ligand with respect to the allosteric antagonist $[^3H]$-CCR2-RA-[R] and mixed effects with respect to the

orthosteric antagonist [^{3}H]-INCB3344. All these results together strongly indicate allosteric modulation of CCR2 by HMA, acting via yet another allosteric binding pocket for small molecules on CCR2. The mixed effect in the saturation binding assay with [^{3}H]-INCB3344 could indicate that the binding site of HMA and INCB3344 are partially overlapping. Nevertheless, whereas INCB3344 enhanced binding of [^{3}H]-CCR2-RA-[R] [8], HMA completely displaced this radioligand from CCR2. Although HMA and INCB3344 most likely bind in close proximity, these two ligands clearly induce distinct conformational changes of CCR2.

Several highly conserved amino acid residues in class A non-olfactory GPCRs have been reported to be involved in sodium ion and/or HMA binding, including $D^{2.50}$ (92% conserved), $W^{6.48}$ (78% conserved) and $N^{7.45}$ (63% conserved) [11-14]. CCR2 contains the residues $D88^{2.50}$ and $W256^{6.48}$, but at position 7.45 a histidine is present instead of an asparagine. $H^{7.45}$ is highly conserved among chemokine receptors (87%), but otherwise not prevalent in class A GPCRs (10%) (GMOS web interface. http://lmc.uab.cat/gmos/, accessed on 25-02-2014). In our studies, mutation of $W256A^{6.48}$ completely prevented [^{3}H]-INCB3344 from binding, whereas its affinity for $D88A^{2.50}$ and $H297A^{7.45}$ was only slightly decreased. Since $W^{6.48}$ is located at the top of the sodium binding pocket [11, 12] (Fig. 9A) and was previously found to interact with the CCR5 antagonist maraviroc [37], this residue in CCR2 could be an interaction partner for INCB3344. Binding studies with the intracellular radioligand [^{3}H]-CCR2-RA-[R] revealed dramatic conformational changes of the intracellular region of the receptor by mutations in the sodium binding pocket, since the affinity of CCR2-RA-[R] for all mutant receptors was drastically decreased compared to WT CCR2. $D^{2.50}$ and $W^{6.48}$ are known to be critical residues for the conformational changes at the intracellular side between inactive and active states of a GPCR. Both residues were predicted to participate in a water-mediated hydrogen-bonding network with other residues that include $N^{7.49}$ and $Y^{7.53}$ of the NPxxY motif at the bottom of TM-VII [38, 39].

Since we were able to measure binding of at least one of the antagonist radioligands for all three mutant receptors, we examined the effect of these residues on modulation of CCR2 by sodium ions and HMA. $D88^{2.50}$ revealed a critical role for modulation by sodium ions, as enhancement of the intracellular radioligand [^{3}H]-CCR2-RA-[R] was completely abolished for the $D88A^{2.50}$ mutant receptor. Recent high-resolution crystal structures of the δ-OR, A_{2A}AR and PAR1 confirm the central role of $D^{2.50}$ in the coordination of the sodium ion [11-13]. Our findings suggest that such a sodium ion binding site is present in CCR2 as well. Mutation of $W256^{6.48}$ into alanine also partially reduced the enhanced [^{3}H]-CCR2-RA-[R] binding upon addition of NaCl, indicating that $W^{6.48}$ is involved in the formation of the sodium binding pocket of CCR2. In the crystal structures of the A_{2A}AR and the δ-OR, hydrogen bonding interactions

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between $W^{6.48}$ and water molecules surrounding the sodium pocket were identified [11, 12], but the exact role of $W^{6.48}$ in antagonist modulation by sodium ions in these receptors remains to be deciphered. Additionally we found that $H297^{7.45}$ was necessary for the sodium ion to enhance binding of [^{3}H]-CCR2-RA-[R]. This residue is therefore likely to play a similar role as the conserved $N^{7.45}$ in the majority of other GPCRs in the creation of the binding pocket for the sodium ion [11, 12].

Mutation of $W256^{6.48}$ completely prevented CCR2 modulation by 0.1 mM HMA, whereas $D88A^{2.50}$ and $H297A^{7.45}$ still allowed HMA to displace both antagonist radioligands, although to a lesser extent as for WT CCR2. The important role of $W^{6.48}$ is in agreement with a study of the $A_{2A}AR$ in which extensive steric interactions between $W^{6.48}$ and HMA were predicted upon induced fit docking [14]. However, the observed decrease in the potency of HMA to displace the CCR2 antagonists is opposite to a 4-fold increase in affinity that was previously found for the A_3 adenosine receptor (A_3AR) upon mutation of $W^{6.48}$ into alanine [34]. Although this binding pocket is highly conserved among class A GPCRs, small modifications due to a few differential residues might lead to distinct positioning of HMA in this pocket. From the 15 conserved GPCR residues that are lining the sodium ion binding pocket in inactive GPCR crystal structures [14], 6 are different for CCR2, including $S87^{2.49}$, $G127^{3.39}$, $I131^{3.43}$, $Y252^{6.44}$, $H297^{7.45}$, $C298^{7.46}$. Except for $I131^{3.43}$, all these residues were predicted to be in close proximity of HMA in CCR2 (Fig. 9B), which could therefore be a reason for the observed differences in HMA and sodium ion modulation of CCR2 compared to other GPCRs.

In agreement with radioligand binding studies, molecular docking of HMA in the homology model of CCR2 revealed direct ionic and hydrogen bonding interactions with $D88^{2.50}$ and $H297^{7.45}$. In addition, hydrogen bonding as well as π-stacking interactions between HMA and $W256^{6.48}$ were predicted. The lack of affinity that was observed in the radioligand binding assays for amiloride and its analogues phenamil, benzamil and MGCMA further supports this docking pose of HMA in our model. The phenyl ring of benzamil and phenamil that is attached to the guanidinium group would cause steric hindrance, and interference with the ionic interactions between HMA and $D^{2.50}$. In addition, at the top of the binding pocket the hydrophobic hexamethylene group of HMA and the N-methyl-N-isobutyl group of MIBA seem to be involved in crucial hydrophobic interaction, since the lack of such a group in amiloride and the presence of a positively charged substituent in MGCMA prevented these ligands from binding.

In summary we have revealed that CCR2 is modulated by amiloride analogues as well as sodium ions, which is mediated via a third small molecule binding pocket in the core of the TM domain. Due to its close proximity to the orthosteric binding pocket, this finding

could potentially lead to the development of bitopic ligands binding to both sites. These ligands might have distinct pharmacological properties since they target a domain that is so important for conformational rearrangements of the entire receptor. Our findings provide further understanding of sodium ion and HMA modulation, via a highly conserved site, and offer novel opportunities for targeting CCR2 and GPCRs in general.

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