European Journal of Pharmacology 729 (2014) 75–85

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Immunopharmacology and inflammation

Internalization of the chemokine receptor CCR4 can be evoked by orthosteric and allosteric receptor antagonists

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ARTICLE INFO

Article history:
Received 22 October 2013
Received in revised form
31 January 2014
Accepted 6 February 2014
Available online 15 February 2014

Keywords:
Chemokine
CCR4
MDC
TARC
CCL22
CCL17

ABSTRACT

The chemokine receptor CCR4 has at least two natural agonist ligands, MDC (CCL22) and TARC (CCL17) which bind to the same orthosteric site with a similar affinity. Both ligands are known to evoke chemotaxis of CCR4-bearing T cells and also elicit CCR4 receptor internalization. A series of small molecule allosteric antagonists have been described which displace the agonist ligand, and inhibit chemotaxis. The aim of this study was to determine which cellular coupling pathways are involved in internalization, and if antagonists binding to the CCR4 receptor could themselves evoke receptor internalization. CCL22 binding coupled CCR4 efficiently to β-arrestin and stimulated GTPγS binding however CCL17 did not couple to β-arrestin and only partially stimulated GTPγS binding. CCL22 potently induced internalization of almost all cell surface CCR4, while CCL17 showed only weak effects. We describe four small molecule antagonists that were demonstrated to bind to two distinct allosteric sites on the CCR4 receptor, and while both classes inhibited agonist ligand binding and chemotaxis, one of the allosteric sites also evoked receptor internalization. Furthermore, we also characterize an N-terminally truncated version of CCL22 which acts as a competitive antagonist at the orthosteric site, and surprisingly also evokes receptor internalization without demonstrating any agonist activity. Collectively this study demonstrates that orthosteric and allosteric antagonists of the CCR4 receptor are capable of evoking receptor internalization, providing a novel strategy for drug discovery against this class of target.

1. Introduction

CCR4 is expressed on a variety of functionally distinct thymocytes including skin-homing T cells (Campbell et al., 1999), $CD25^+$ T suppressor cells (Iellem et al., 2001) and T helper (Th)2 cells (Bonecchi et al., 1998), all of which have been shown to migrate to CCL22 and CCL17 released at sites of inflammation. $CCR4^+$ T cells have been shown to be elevated in several types of allergic disease (Nouri-Aria et al., 2002; Yang et al., 2004) which increases further upon allergen challenge (Panina-Bordignon et al., 2001). Taken together, these findings have driven interest in CCR4 as a therapeutic target for the treatment of inflammatory diseases with a Th2 cell component, such as asthma (Gonzalo et al., 1999; Kawasaki et al., 2001) and atopic dermatitis (Reiss et al., 2001).

Consequently, considerable effort has gone into discovering small molecule CCR4 antagonists although only one molecule has made it to human clinical trials to date (Cahn et al., 2013). A subset of these small molecules have been shown to require intracellular access for their activity (Purandare and Somerville, 2006), resulting in the identification of an intracellular allosteric site on the receptor (referred to as 'site-2' in this study) (Andrews et al., 2008). In the same study, it was apparent that one compound (BMS-397, referred to as 'Compound **2**' hereafter) did not bind to this intracellular allosteric site (Purandare, 2004) but a distinct site (referred to as 'site-1' in this study). Further work (Weston and Hall, 2008) confirmed that the interactions of these molecules with CCR4 indicate the presence of two allosteric binding sites on the receptor, both of which are spatially distinct from the orthosteric site to which CCL22 and CCL17 bind. Each of these small molecules demonstrated the ability to allosterically evoke agonist dissociation from the orthosteric site, antagonizing cellular functions such as chemotaxis and calcium mobilization. These molecular probes have been used in the present study to demonstrate biased signaling capacity of CCR4 receptors.

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http://dx.doi.org/10.1016/j.ejphar.2014.02.007

In addition to evoking chemotaxis, CCR4 ligands have also been shown to induce receptor internalization from the cell surface of human Th2 cells, resulting in a loss of functional responsiveness (Mariani et al., 2004; Sebastiani et al., 2005). GPCR internalization following agonist exposure is a component of receptor desensitization, however the role of receptor internalization in regulating the chemotactic response remains controversial.

In this study we expand the observation that the CCR4 agonists CCL22 and CCL17 can evoke receptor internalization by exploring the coupling of the CCR4 receptor to G-proteins and β-arrestin. Additionally, we confirm the presence of two allosteric binding sites and demonstrate that small molecules binding to 'site-1' are also capable of evoking receptor internalization while binders of 'site-2' do not. Lastly, we also characterize an N-terminally truncated version of CCL22 (MDC67) which acts as a competitive antagonist at the orthosteric site, and surprisingly also evokes receptor internalization without demonstrating any agonist activity. Based on these biased signaling observations we provide a novel strategy for drug discovery against this class of chemokine receptor.

2. Materials and methods

2.1. Reagents

NeuroProbe ChemoTx 3 μm-pore chemotaxis chambers were purchased from Receptor Technologies Ltd. (Leamington Spa, UK). Phycoerythrin (PE)-Mouse Anti-Human CCR4 (CD194) mAb, PE Mouse IgG Isotype control, Fluorescein Isothiocyanate (FITC) Mouse IgG Isotype Control, Purified mouse anti-human CD194 were purchased from BD Pharmingen (Beckton Dickenson UK Ltd., Oxford, UK). FITC-conjugated anti-human CD4, Alexa-fluor 647 Phalloidin and Alexa-488 conjugated rabbit anti-mouse was purchased from Invitrogen Ltd., (Paisley, UK). [^{125}I]CCL-17 (specific activity 2200 Ci/mmol) was obtained from Perkin-Elmer LAS UK Ltd. (Beaconsfield, UK). [^{3}H]compound **2** and [^{3}H]compound **3** (specific activity 37 and 53 Ci/mmol respectively) were synthesized by GE Healthcare UK Ltd. (Little Chalfont, UK). The majority of the chemokines CCL22 and CCL17 were purchased from RandD Systems Europe Ltd. (Abingdon, UK). Truncated CCL22, (MDC67) was mostly supplied by Biological Reagents and Assay Development (BRandAD), GlaxoSmithKline. Other samples of CCL17 and MDC67 were purchased from PeproTech (London, UK) while some CCL22 was supplied by BRandAD, GlaxoSmithKline. In this study we used a range of protein and small molecule ligands of the CCR4 receptor to probe aspects of its function. Compound **1** and Compound **2** are representative of a class of lipophilic amines, and Compound **3** and Compound **4** are representative of a class of arylsulfonamides (Procopiou et al., 2012), all of which were synthesized by Respiratory Therapy Area Unit Medicinal Chemistry, GlaxoSmithKline (Supplementary data Fig. S1).

2.2. Cell lines

HUT78 cells were obtained from the European Collection of Cell Cultures (ECACC), Wiltshire, UK. Chinese Hamster Ovary (CHO) cells were obtained from the ECACC, Wiltshire, UK. CHO cells were transfected with CCR4 cDNA from human basophils (Power et al., 1995) in the pCIN4 vector; stable transfectants were selected and cultured in DMEM (Hams)-F12 media supplemented with 10% heat-inactivated FBS, 1% L-glutamine and 50 μg/ml G418. Human Peripheral Blood Mononuclear Cells (PBMCs) were obtained from the peripheral blood of healthy volunteers using a Percol gradient according to standard protocol (Ulmer et al., 1983). Acquisition of the blood samples was approved by Hertfordshire Research Ethics Committee and all donors gave written informed consent prior to donation. Prior to use in all experiments, cells were re-suspended in Assay Buffer [RPMI 1640 supplemented with 1% Bovine Serum Albumin and 1 mM HEPES].

2.3. Cell culture and membrane preparation

The cells were grown in Corning CellSTACKS© (Corning Inc., NY, USA) and membrane fragments were prepared from the CHO-CCR4 cells as previously described (Slack and Hall, 2012).

2.4. Radioligand binding studies

All [^{125}I]-CCL17 and [^{3}H]antagonist radioligand binding experiments were performed using a format previously described (Slack et al., 2011; Slack and Hall, 2012) but with minor modifications. Briefly, antagonist binding assays were carried out in 96-deep well plates at ambient room temperature (20–22 °C) in binding buffer (without BSA) with either [^{3}H]compound **2** or [^{3}H]compound **3**, membranes and either vehicle or unlabeled antagonist. Non-specific binding (NSB) was determined for [^{3}H]compound **2** and [^{3}H]compound **3** by 10 μM unlabeled compounds **2** or **3** respectively. Binding was terminated by rapid vacuum filtration through a 48-well Brandel (Brandel Inc. Gaithersburg, MD, USA) harvester onto GF/B filter papers pre-soaked in 0.3% v/v poly-ethylenimine (for [^{3}H]compound **2**) or water (for [^{3}H]compound **3**). Samples were washed rapidly three times with ice cold distilled water and filters transferred into liquid scintillation (LS) vials containing 4 ml LS fluid (Ultima-Flo™ M, Perkin-Elmer LAS UK Ltd., Beaconsfield, UK). The amount of radioligand bound to receptor was measured by LS microscopy using a TriCarb 2900TR LS counter (Perkin-Elmer LAS UK Ltd., Beaconsfield, UK). For saturation binding, CCR4 membranes were incubated with increasing concentrations of [^{3}H]compound **2** or [^{3}H]compound **3** in the presence of vehicle or NSB for 2 h prior to filtration.

For competition binding displacement studies, membranes were mixed with [^{3}H]compound **2** or [^{3}H]compound **3** and increasing concentrations of unlabeled ligand for 2 h prior to filtration.

2.5. Beta-arrestin assay

The CCR4 cell line was obtained from DiscoveRx and grown in the manufacturer's suggested medium. Agonist induced coupling was detected as described (Demont et al., 2011).

2.6. GTPγS assay

CHO-CCR4 cell membranes (5 μg/ml) were mixed at a 1:1 ratio with 25 mg/ml WGA coupled PS imaging (Leadseeker) beads (Perkin-Elmer) before being incubated for 1 h at 4 °C. GDP was added to 384-well solid white plates (Nunc, FAC 4.4 μM) containing test compound. ^{35}S-GTPγS (Perkin-Elmer) was diluted 1:1200 in assay buffer and 20 μl/well added to the plates before centrifugation at 1200 rpm for 30 s. After 3 h plates were read using Viewlux (Perkin-Elmer) with a 613/55(A09) emission filter.

2.7. Chemotaxis assay

HUT78 cell chemotaxis was measured using a transwell chemotaxis chambers. Cells were loaded with 7.5 μg/ml Calcein-AM, washed and re-suspended in assay buffer [RPMI 1640, 1% BSA, 1 mM HEPES] then incubated at 37 °C for 30 min with vehicle or antagonist. Chemokines were diluted in assay buffer, loaded into the lower wells of the chemotaxis chamber and cells (1×10^7 cells/ml) were placed above the filter and the whole chamber was incubated at 37 °C for 90 min. After incubation, the filter was