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# Pharmacological characterization of the chemokine receptor, CCR5

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**1** We investigated the effects of a number of naturally occurring chemokines (MIP-1$\alpha$, MIP-1$\beta$, RANTES, MCP-2, MCP-3, MCP-4) on different processes linked to the chemokine receptor CCR5 in recombinant CHO cells expressing the receptor at different levels.

**2** Internalization of CCR5 following chemokine treatment was studied and MIP-1$\alpha$, MIP-1$\beta$ and RANTES (50 nM) were able to induce internalization ($\sim$50%) of the receptor. Internalization due to MCP-2, MCP-3 and MCP-4 was less ($\sim$20%).

**3** Phosphorylation of CCR5 following chemokine treatment was studied and MIP-1$\alpha$, MIP-1$\beta$ and RANTES (50 nM) were able to induce phosphorylation of CCR5 whereas the other chemokines did not induce CCR5 phosphorylation.

**4** MIP-1$\alpha$, MIP-1$\beta$, RANTES and MCP-2 were able to stimulate [$^{35}$S]-GTP$\gamma$S binding, an index of receptor/G protein activation, whereas MCP-3 and MCP-4 had no effect in this assay. MCP-2 was a partial agonist ($\sim$80%) compared to MIP-1$\alpha$, MIP-1$\beta$ and RANTES, which gave similar maximal stimulations in this assay.

**5** MIP-1$\alpha$, MIP-1$\beta$, RANTES, MCP-2 and MCP-4 were able to stimulate increases in intracellular calcium ions via activation of CCR5 whereas MCP-3 was without effect.

**6** It is concluded that different chemokines interacting with CCR5 mediate different patterns of cellular responses.

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**Keywords:** Chemokine receptor CCR5; chemokines; internalization; phosphorylation; [$^{35}$S]-GTP$\gamma$S binding

**Abbreviations:** CHO cells, Chinese hamster ovary cells; ECL, enhanced chemiluminescence; FACS, fluorescence activated cell sorter; FCS, foetal calf serum; FITC, fluorescein isothiocyanate; GPCR, G protein coupled receptor; GRK, G protein coupled receptor kinase; HIV, human immunodeficiency virus; IL2, interleukin 2; IP, immunoprecipitation; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; PBS, phosphate buffered saline; PE, phycoerythrin; PI3 kinase, phosphatidylinositol 3-kinase; PI4 kinase, phosphatidylinositol 4-kinase; PTX, pertussis toxin; RANTES, regulated on activation, normal T cell expressed and secreted

## Introduction

Chemokines are a large family of chemotactic proteins, which regulate leukocyte activation and recruitment to sites of inflammation. The chemokines can be divided into four families, depending on the relative position of four conserved cysteines. A large family of G protein coupled receptors (GPCR's) has been identified as receptors for chemokines. Originally the chemokine receptor CCR5 was characterized as a receptor that responds functionally to the CC-chemokines MIP-1$\alpha$, MIP-1$\beta$ and RANTES (Samson *et al*., 1996). CCR5 was subsequently described as the primary co-receptor for macrophage tropic human immunodeficiency virus type 1 (HIV-1) (Alkhatib *et al*., 1996; Choe *et al*., 1996; Doranz *et al*., 1996; Dragic *et al*., 1996). Cellular entry of HIV-1 is initiated by the interaction between the virus membrane glycoprotein, gp120, and the cell surface protein CD4. gp120-CD4 binding induces a number of conformational events leading to the exposure of a binding site for the co-receptor CCR5, resulting in fusion of the viral and cell plasma membranes (Littman, 1998). Recently, CD4 has been reported to interact directly with CCR5 (Xiao *et al*., 1999). Since CCR5 expression is largely restricted to the CD4+population of CD26$^{high}$ subset of memory T-cells (Bleul *et al*., 1997; Lee *et al*., 1999), it is relevant to address whether such an association affects CCR5 function and response to chemokines.

Chemokine interaction with CCR5 initiates a number of events: firstly, receptor association with G proteins, leading to activation of signalling, e.g. calcium influx; secondly, phosphorylation of the receptor by a family of G protein coupled receptor kinases (GRKs) (Pitcher *et al*., 1998) resulting in association of $\beta$-arrestin with the receptor and desensitization *via* uncoupling of receptor and G protein. The arrestins also act as adapters between the receptor and components of the endocytic machinery, such as AP-2 and clathrin (Carman & Benovic, 1998; Lefkowitz, 1998), leading to internalization of the receptor and a concomitant reduction in cell surface expressed CCR5 (Mack *et al*., 1998; Alkhatib *et al*., 1997).

A number of CC chemokines (MIP-1$\alpha$, MIP-1$\beta$, RANTES, MCP-1, MCP-2, MCP-3 and MCP-4) bind CCR5 with

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different affinities and abilities to activate the receptor (Blanpain *et al*., 1999). These chemokines can be divided in two subgroups based on amino acid sequence identity (Baggiolini *et al*., 1994; 1997). MIP-1$\alpha$, MIP-1$\beta$ and RANTES form one subgroup and are full agonists, whereas MCP-1, MCP-2, MCP-3 and MCP-4 form a second subgroup which share $\sim$60% amino acid identity within the group and $\sim$30% identity with MIP-1$\alpha$, MIP-1$\beta$ and RANTES. MCP-3 has been reported to bind CCR5 but is unable to activate the receptor in a number of tests (Blanpain *et al*., 1999). MCP-2 and MCP-4 are full agonists in some tests, with MCP-4 demonstrating a reduced potency. The diverse effects of these chemokines suggest that they interact differently with CCR5 as compared with MIP-1$\alpha$, MIP-1$\beta$ and RANTES. Thus, the outcome of chemokine-receptor interactions may reflect the affinity of the chemokine for a receptor and its ability to induce conformational change(s), thereby affecting G protein interactions. To further elucidate these interactions we studied a range of chemokines that can bind to CCR5 for their ability to affect receptor/G protein interaction, downstream signalling (increased intracellular calcium ions), receptor phosphorylation and internalization.

## Methods

### *Cells and materials*

CHO cells were transfected with pcDNA3 encoding CCR5 and selected for stable expression in 10% FCS/DMEM/Glutamine (2 mM) in the presence of G418. CHO.CCR5 cells were transfected with pREP.CD4 and selected for expression of both CD4 and CCR5 in 10% FCS/DMEM/Glutamine (2 mM) in the presence of hygromycin and G418. HeLa cells expressing CCR5 at different levels (RC-4, RC-49) were obtained from D. Kabat and described previously (Platt *et al*., 1998). Protein Phosphatase 1 (PP1) was purchased from New England Biolabs (Beverly, MA, U.S.A.), Protein G-Sepharose, pertussis toxin (PTX), staurosporine, LY294002 and wortmannin were from Sigma (Poole, U.K.), protease inhibitor cocktail was obtained from Roche (Roche Diagnostics, Lewes, U.K.). Chemokines were purchased from PeproTech (PeproTech, Inc, Rocky Hill, NJ, U.S.A.) or the MRC Aids Reagent Repository Programme (Potters Bar, U.K.). Secondary antibodies were obtained from Sigma or Harlan Sera-Lab (Loughborough, U.K.). Interleukin-2, anti CD4 antibody ARP318 were from the MRC Aids Reagent Repository Programme. Anti CCR5 antibodies HEK/1/85a/7a and 1/74/3j were raised against intact CHO.CCR5 and the CCR5 N-terminal peptide, respectively and were deposited in the MRC Aids Reagent Repository Programme. All other chemicals were from Sigma.

### *Internalization assay and flow cytometry analysis*

CHO.CCR5 and CHO.CCR5.CD4 cells respectively were incubated with serum-free medium for 2 h at 37°C harvested with 2 mM EDTA/PBS and then resuspended in medium without serum at $5\times10^6$ cells ml$^{-1}$. Cells were then incubated with chemokines (50 nM) for various times at 37°C, and washed in ice cold PBS or PBS containing 1% FCS and 1% $NaN_3$ for FACS analysis. Cell surface expressed CCR5 was detected by flow cytometry using anti-CCR5 mAB HEK/1/85a/7a and phycoerythrin (PE) conjugated anti-rat IgG. Cells were incubated for 1 h at room temperature with HEK/1/85a/7a (saturating amounts of hybridoma supernatant), washed three times with PBS buffer containing 1% FCS and 1% $NaN_3$ and incubated for 1 h with PE-labelled anti-rat IgG. Samples were quantified on a FACScan and data analysed with CellQuest software version 3.1 (Becton Dickinson, San Jose, CA, U.S.A.). The relative CCR5 surface expression was calculated as $100\times$(mean channel of fluorescence (stimulated) $-$ mean channel of fluorescence (negative control)/mean channel of fluorescence (medium) $-$ mean channel of fluorescence (negative control)) (%). CHO cells not expressing CCR5 as well as irrelevant monoclonal antibodies were used for negative controls with similar results. Cell surface expressed CD4 was detected in the same way, using the anti-CD4 mAB ARP318 (5 $\mu$g per stain) and a corresponding secondary PE labelled antibody.

### *Immunoprecipitation, SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot*

Cells were washed in PBS and then resuspended in cold (4°C) PBS at a final density of $10^7$ cells ml$^{-1}$. Antibodies (HEK/1/85a/7a, saturating amounts of hybridoma supernatant) were added to the cell suspension and incubated for 1 h at 37°C. Cells were collected by centrifugation and resuspended in lysis buffer (1% Brij97, 5 mM iodoacetamide (added immediately before use), 150 mM NaCl, 20 mM Tris (pH 8.2), 20 mM EDTA and protease inhibitors) at 4°C for 40 min with gentle mixing. The nuclei were separated by centrifugation at $13{,}000\times g$ for 25 min in a refrigerated centrifuge. Protein G-Sepharose (Sigma), pre-washed with PBS, was added to the samples and incubated at 4°C overnight. The Sepharose was washed four times with 1 ml of ice-cold lysis buffer and samples eluted by adding $2\times$sample buffer (75 mM TrisHCl, pH 6.8, 6% SDS, 10% mercaptoethanol, 10% glycerol) and heated to 90°C for 1 h. The samples were separated on a 12.5% SDS–PAGE and electrophoretically transferred to a nitro-cellulose membrane. The membranes were blocked with 5% non-fat powdered milk in PBS. For Western blotting, these membranes were incubated with respective antibodies, then washed and incubated with horseradish peroxidase conjugated secondary antibodies. The blots were developed by enhanced chemiluminescence (Amersham, Buckinghamshire, U.K.).

### *Treatment with pertussis toxin, wortmannin, LY 294002, staurosporine and sodium butyrate*

Pertussis toxin was used to uncouple the G proteins from the receptor at 100 ng ml$^{-1}$ PTX for 2 or 15 h as indicated before the addition of various chemokines for internalization or [$^{35}$S]-GTP$\gamma$S-binding assays. Wortmannin (100 nM and 1 $\mu$M), LY 294002 (10 $\mu$M) or staurosporine (1.4 ng ml$^{-1}$) were incubated for 1 h with the cells before assaying. These agents were then present during the agonist incubation. Cells were incubated in culture for 18 h with 5 mM sodium butyrate to increase receptor expression and then washed and prepared for assays.

*Phosphorylation and dephosphorylation*

Cells were treated as described with different chemokines and the CCR5 immunoprecipitated and analysed by SDS–PAGE. Phosphorylation of CCR5 was detected by decreased mobility in SDS–PAGE and confirmed by treatment with protein phosphatase 1. For phosphatase treatment, cells were treated with chemokines before addition of the corresponding antibodies and cells were lysed in lysis buffer without EDTA. Phosphatase was added according to the manufacturers instructions and incubated 2 h at 30°C before adding the Protein G-Sepharose and completing the immunoprecipitation as above.

*Immunofluorescence*

Cells were grown on coverslips and incubated in medium without serum for 2 h before treatment with chemokines for 1 h. The cells were then washed with medium and incubated with the CCR5 antibody (HEK/1/85a/7a) for 1 h at 37°C. After washing, the cells were incubated with the corresponding secondary FITC-labelled antibody for 1 h, washed and fixed in ice-cold methanol and mounted on glass slides. Images were taken using a Leica NT Confocal Imaging system.

*Membrane preparation*

Membranes were prepared from confluent cells. Cells were harvested in ice-cold HEPES buffer (mM: HEPES 20, $MgCl_2$ 6, EDTA 1 and EGTA 1, pH 7.4) and homogenized with an ultra-turrax ($4 \times 4$ s). The cell debris was collected by centrifugation for 10 min at $1700 \times g$, the resulting supernatant was centrifuged for 1 h at $48{,}000 \times g$ at 4°C and resuspended in HEPES buffer, aliquoted and stored at −70°C. Protein levels were determined using the method of Lowry *et al*. (1951).

*[$^{35}$S]-GTPγS-binding assays*

The [$^{35}$S]-GTPγS-binding assays were carried out essentially as described (Gardner *et al*., 1996). Thirty micrograms of cell membranes were incubated in triplicate in [$^{35}$S]-GTPγS-binding buffer containing mM: HEPES 20, NaCl 100, $MgCl_2$ 10 and 0.1% BSA, pH 7.4 and GDP 10 μM in a final volume of 0.9 ml in the absence or presence of an agonist to measure the basal and stimulated nucleotide exchange, respectively. The reaction was initiated by the addition of cell membranes and the tubes were incubated at 30°C for 30 min. This preincubation ensured that ligand binding was at equilibrium before addition of 100 μl of [$^{35}$S]-GTPγS to give a final [$^{35}$S]-GTPγS concentration of 100 pM. The assay was incubated for a further 30 min before termination by rapid filtration through GF/C filters with four washes of ice-cold PBS using a Brandel cell harvester (Gaithersburg, MD, U.S.A.). The filters were soaked for at least 6 h in 2 ml of LKB optiphase 'Hisafe'3 scintillation fluid after which bound radioactivity was determined by liquid scintillation counting.

*Analysis of intracellular calcium ion concentration*

Cells were harvested with 2 mM EDTA/PBS and washed twice in buffer (mM: NaCl 148, KCl 5, $CaCl_2$ 2.5, HEPES 10, Glucose 1, probenecid 2.5, 0.1% BSA, pH 7.4) and incubated with 4 μM Fura-2 (Molecular Probes, Eugene, OR, U.S.A.) at 37°C. After washing cells with buffer, cells were resuspended at $2 \times 10^6$ cells ml$^{-1}$ of buffer. Chemokine-induced intracellular calcium mobilization was determined by spectrofluorimetry using a Perkin Elmer LS50 fluorimeter. The peak values of intracellular calcium ion concentration following the chemokine challenge were determined as described in Grynkiewicz *et al*. (1985).

*Data analysis*

Data were analysed using GraphPad Prism (GraphPad Software, San Diego, CA, U.S.A.). Concentration/response curves for MIP-1α, MIP-1β and RANTES in [$^{35}$S]-GTPγS binding assays were fitted well by models assuming a Hill coefficient of 1 and so data with MCP-2 were analysed similarly. Statistical analysis were performed using Student's *t*-test with a $P$ value $<0.05$. Data represent the mean ± s.e.mean of at least three independent experiments.

## Results

*Cell lines and antibodies used*

Two recombinant CHO cell lines were used for this study, one expressing CCR5 alone and one expressing CCR5 and CD4. Expression of CCR5 and CD4 on the CHO cells was confirmed by FACS analysis and Western blot (Figure 1). Cell surface CCR5 expression was greater on the CHO.CCR5 cell line compared to the CHO.CCR5.CD4 cell line with mean fluorescence intensities of 453.3 and 56.1, respectively. In the majority of studies described below CCR5 was detected using the monoclonal antibody HEK/1/85a/7a. Stimulation of [$^{35}$S]-GTPγS binding by MIP-1α was unaffected by the presence of antibody HEK/1/85a/7a (data not shown) showing that this antibody binds to sites on CCR5 unrelated to chemokine binding sites. In some studies another antibody (1/74/3j) was used. This is specific for a linear epitope within the N-terminal region of CCR5 not involved in chemokine binding. Use of this antibody gave essentially identical data to those obtained with antibody HEK/1/85a/7a. CD4 was shown to associate with CCR5 in the CHO.CCR5.CD4 cells by co-immunoprecipitation (Figure 2). The extent of this interaction was not influenced by receptor activation after treatment of cells with MIP-1β (50 nM) before lysis and immunoprecipitation of CD4 (data not shown).

*Internalization of CCR5*

Cells were incubated in serum-free medium and treated with MIP-1α, MIP-1β and RANTES (50 nM) for various times and cell surface CCR5 expression determined with mAB HEK/1/85a/7a (Figure 3a, Table 1). After 1 h MIP-1α and RANTES were able to reduce cell surface CCR5 expression by up to ~50%, whereas MIP-1β was less effective (Figure 3a). In all subsequent experiments, CHO cells were incubated with chemokine for 1 h to assess different internalization rates.

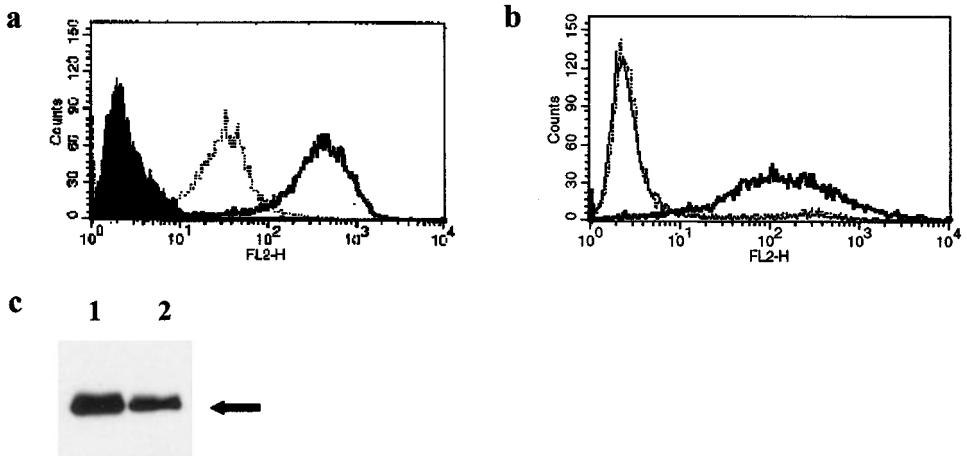


**Figure 1** Expression of CCR5 and CD4 on CHO.CCR5 and CHO.CCR5.CD4 cells. Expression was determined using FACS and Western blot analysis as described in the Methods section. (a) FACS analysis for expression of CCR5: black: negative control, bold line: CHO.CCR5 cells, dotted line: CHO.CCR5.CD4 cells. (b) FACS analysis for expression of CD4: black and dotted line: CHO and CHO.CCR5 cells, bold line: CHO.CCR5.CD4 cells. (c) Membranes prepared from the cells were subjected to SDS–PAGE and Western blot analysis using a CCR5 specific antibody (HEK/1/85a/7a): 1: CHO.CCR5 cells, 2: CHO.CCR5.CD4 cells, the same amount of membrane preparation (10 $\mu$g) was analysed for both cell types. Arrow indicates CCR5.

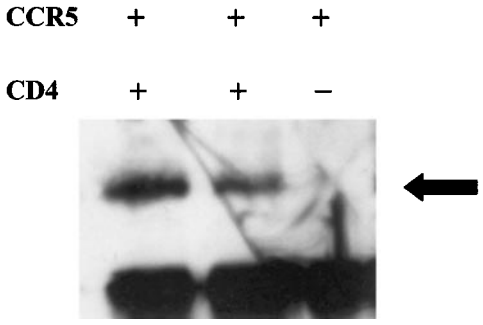


**Figure 2** Interaction of CD4 and CCR5. Cells were subjected to an immunoprecipitation with an anti-CD4 antibody, separated on SDS–PAGE and transferred to nitrocellulose membrane. CCR5 was detected by ECL using a CCR5 specific antibody (HEK/1/85a/7a) and a corresponding secondary antibody. Experiments on CD4 and CCR5 interaction (two left lanes) were done in CHO.CCR5.CD4 cells and control experiments (right lane) in CHO.CCR5 cells. Arrow indicates CCR5. This is one representative result from three independent experiments performed.

To investigate whether G protein coupling is necessary for receptor internalization, assays were performed with cells pre-treated with pertussis toxin (PTX), for 2 h and 15 h. PTX treatment inhibited CCR5 internalization induced by MIP-1$\alpha$ (PTX 2/15 h) and RANTES (PTX 15 h), but not by MIP-1$\beta$ (Table 1). The initiation of Gi/Go dependent signalling events in response to CCR5 activation in CHO.CCR5 cells was also inhibited by PTX using the same conditions (see below). In a similar set of experiments wortmannin (1 $\mu$M) significantly increased CCR5 internalization due to MIP-1$\beta$, and the same trend was seen with RANTES (Table 1). A lower concentration of wortmannin (100 nM) did not increase the extent of internalization due to MIP-1$\beta$ (data not shown). Also treatment of the cells with LY 294002 (10 $\mu$M), which specifically inhibits PI3 kinases, did not increase the MIP-1$\beta$ induced internalization in CHO.CCR5 cells (data not shown). Treatment with PTX or wortmannin, in the absence of chemokine treatment, did not affect expression levels of CCR5 on the cells. In order to examine whether protein kinase C contributed to regulation of internalization of CCR5 we tested the effects of staurosporine (1.4 ng ml$^{-1}$), but this compound had no effect on chemokine-induced internalization of CCR5 (data not shown).

Internalization of CCR5 following chemokine treatment was compared in CHO cells co-expressing CD4. In CHO.CCR5.CD4 cells, treatment with either MIP-1$\alpha$, RANTES or MIP-1$\beta$ elicited similar levels of internalisation (Table 1), whereas in CHO.CCR5 cells MIP-1$\beta$ was less effective than MIP-1$\alpha$ or RANTES (Table 1). Dose-response experiments for MIP-1$\alpha$ and RANTES (Figure 3) showed that for both chemokines the effect on internalization was half maximal at about 20 nM chemokine. The difference in the effects of MIP-1$\beta$ in the two cell lines is probably attributable to the reduced level of CCR5 expression in the CHO.CCR5.CD4 cells rather than a direct effect of CD4. When experiments were performed on CHO.CCR5 cells using 200 nM MIP-1$\beta$ the internalization rate was similar to that seen when the lower concentration of chemokine was used (data not shown), so that the concentration of MIP-1$\beta$ is not limiting in these experiments. In order to investigate whether soluble CD4 is able to change the conformational state of CCR5 and therefore influence the effects of MIP-1$\beta$, MIP-1$\beta$ was added together with soluble CD4 for initiating internalization in CHO.CCR5 cells. There was only a small increase in the internalisation rate (Figure 4a). This could, however, be because CD4 has to be anchored in the cell membrane to actually affect CCR5 or MIP-1$\beta$. Therefore internalization experiments were performed in CHO.CCR5.CD4 cells where CCR5 expression had been increased by sodium butyrate treatment (5 mM, 18 h).

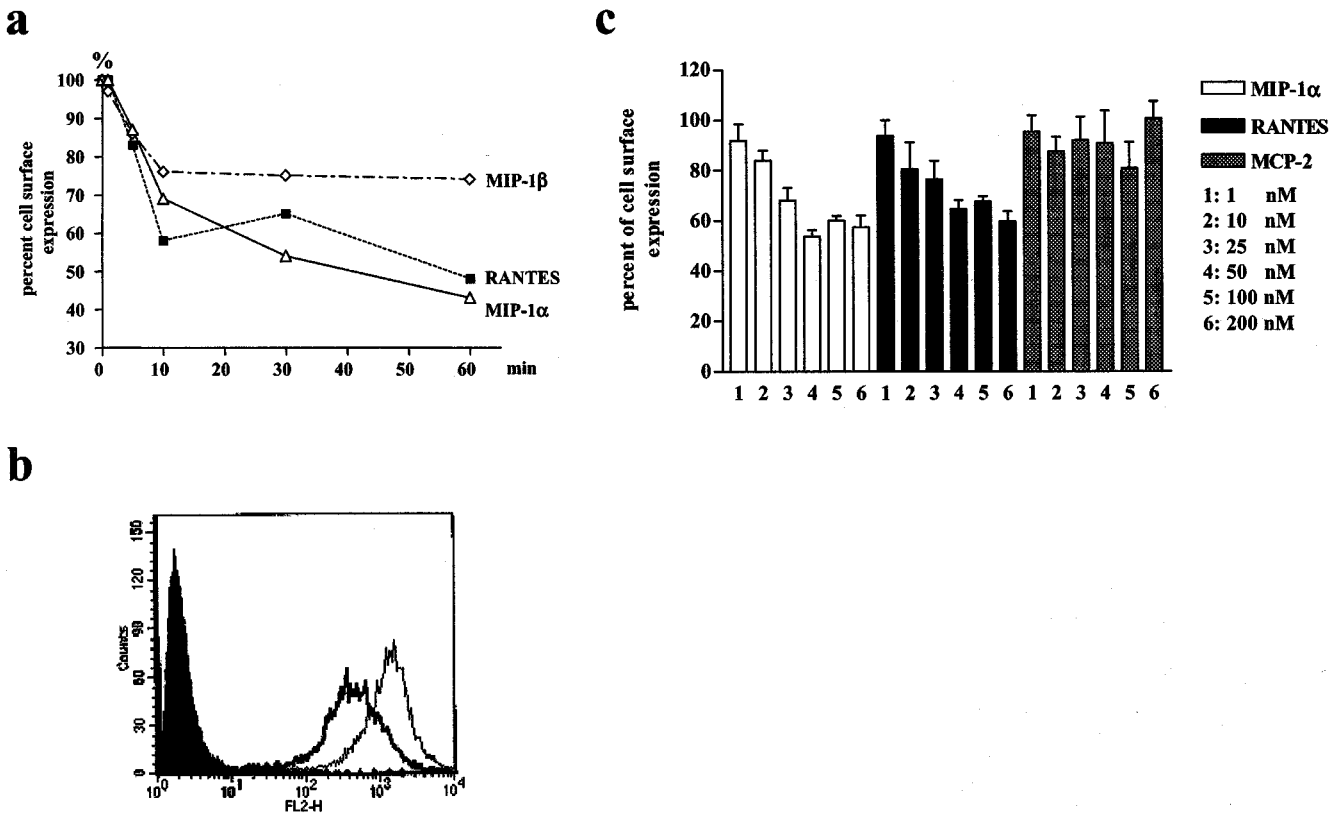


**Figure 3** Internalization of CCR5. (a) Time course for CCR5 internalization. CHO.CCR5 cells were harvested in PBS/EDTA and incubated in serum-free medium for 2 h. Cells were then incubated with 50 nM chemokines for various times. After the incubation, the cells were washed in ice-cold PBS +1% FCS +1% $NaN_3$, and stained for FACS analysis with anti CCR5 HEK/1/85a/7a antibody and anti-rat PE antibody. Internalization rates were determined as described in the Methods section. The graph shows one representative experiment which has been replicated three times. (b) Representative FACS data for an internalization experiment with RANTES, black: negative control, bold line: cells treated with RANTES, dashed line: untreated cells. (c) Dose response experiments for CCR5 internalization. CHO.CCR5.CD4 cells were treated with different amounts of chemokines for 1 h and internalization of the receptor was determined.

**Table 1** Internalization of CCR5 following treatment of CHO.CCR5 and CHO.CCR5.CD4 cells with chemokines

| | | | *Cell surface CCR5* (% of control untreated cells) | | |
|---|---|---|---|---|---|
| *Cells* | *Chemokine* | *Treated control* | *PTX* (2 h) | *PTX* (15 h) | *Wortmannin* |
| CHO.CCR5 | MIP-1α | 52.3±4.0** (4) | 76.0±6.2* (13) | 98.2±4.0** (6) | 61.1±4.1 (8) |
| CHO.CCR5 | RANTES | 54.4±3.1*** (5) | 58.8±8.3 (9) | 83.7±4.7** (12) | 39.1±11.5 (5) |
| CHO.CCR5 | MIP-1β | 85.8±5.3 (4) | 88.3±4.0 (8) | – | 61.1±4.1** (8) |
| CHO.CCR5.CD4 | MIP-1α | 48.6±3.6*** (7) | 65.0±9.4 (9) | 117.7±7.7** (4) | 54.6±8.3 (10) |
| CHO.CCR5.CD4 | RANTES | 59.6±4.8*** (8) | 63.2±8.1 (8) | 82.9±4.6** (8) | 47.1±8.0 (10) |
| CHO.CCR5.CD4 | MIP-1β | 42.2±10.9** (5) | 70.0±6.2** (9) | 116.5±5.9** (3) | 41.2±11.2 (7) |

Cell surface CCR5 was detected by FACS analysis as described in the Methods section. Cell surface CCR5 is expressed as mean±s.e.mean ($n$) as a percentage of values in untreated cells. Statistical comparisons were made between values for treated control cells (MIP-1α, MIP-1β, RANTES) and values for cells treated with interleukin-2 as a negative control (**$P<0.05$; ***$P<0.001$). To study effects of pertussis toxin and wortmannin in internalization, cells were incubated with 100 ng $ml^{-1}$ PTX for 2 or 15 h as indicated, and 1 μM wortmannin for 1 h at 37°C respectively prior to the assay. Statistical comparisons were made between values from PTX or wortmannin treated cells and treated control cells (*$P<0.06$, **$P<0.05$).

Treatment with sodium butyrate increased CCR5 and CD4 expression on CHO.CCR5.CD4 cells (as determined in flow cytometry analysis, Figure 4c(1,2); based on the mean fluorescence intensity (Figure 4) there was approximately 2 fold more CCR5 after butyrate treatment). In butyrate-treated cells the rate of internalisation was specifically decreased for MIP-1β but unaffected for MIP-1α (Figure 4a) such that receptor density may be affecting the internalization of CCR5 due to MIP-1β. To investigate further the effect of receptor density on CCR5 internalization, we used two HeLa cell lines that both expressed the same amount of CD4 (data not shown) but different levels of CCR5 as determined by flow cytometry (Figure 4c, panels 3 and 4). Based on the fluorescence intensity in flow cytometry

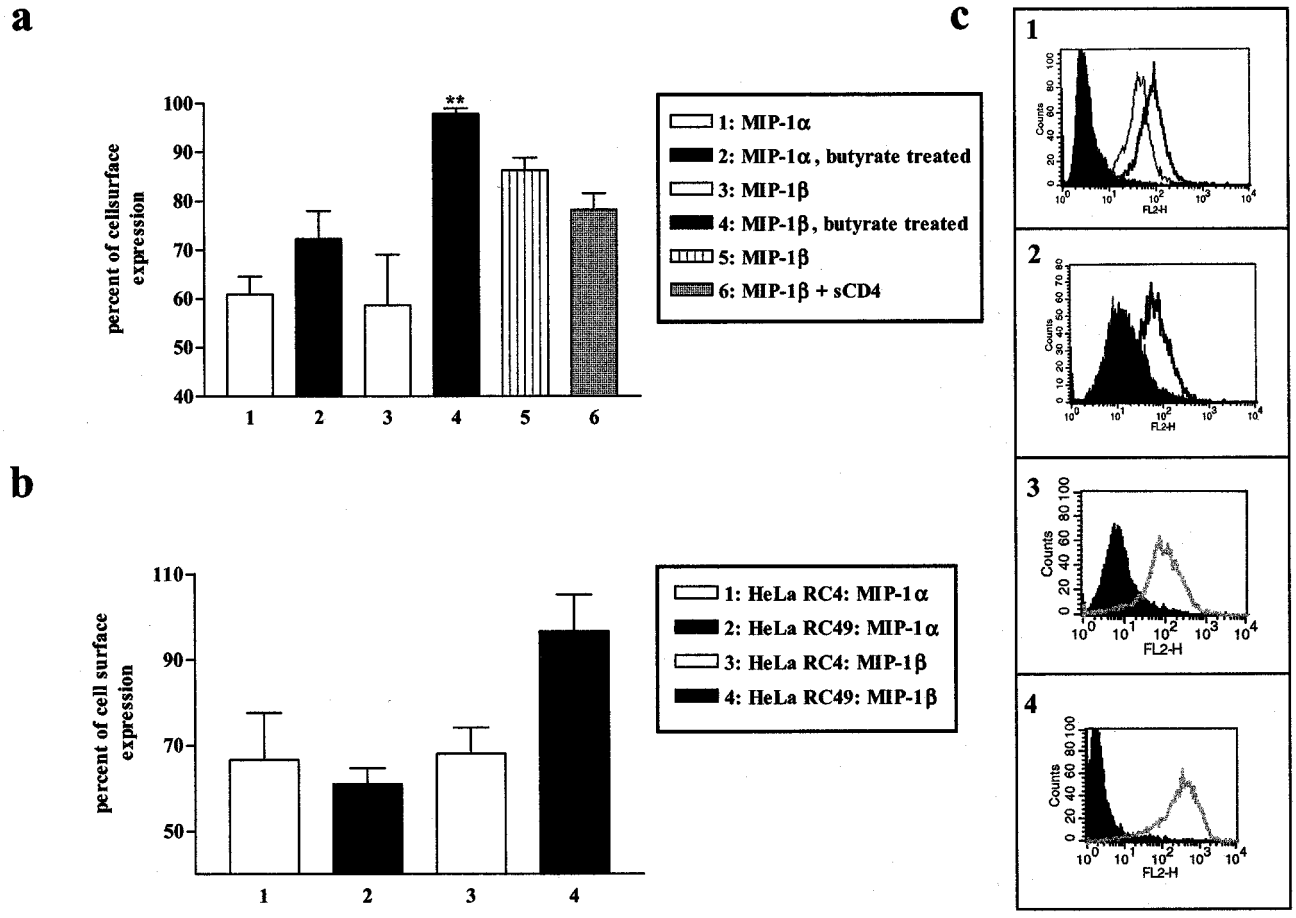


**Figure 4** Internalization of CCR5 in cells expressing different levels of CCR5. (a) Bars 1–4 show data from control CHO.CCR5.CD4 cells and cells that were incubated with 5 mM sodium butyrate for 18 h to increase the receptor density on the cell surface before an internalisation assay was performed as described in the Methods section. Bars 5 and 6 show CHO.CCR5 cells that were treated with MIP-1$\beta$ alone or MIP-1$\beta$ incubated with 1 $\mu$g of soluble CD4 (sCD4). Data represent the means $\pm$ range from two independent experiments performed (**$P<0.05$ relative to control). (b) Bars 1 and 3 show HeLa RC-4 cells treated with MIP-1$\alpha$ and MIP-1$\beta$ respectively, bars 2 and 4 show treatment of HeLa RC-49 cells. (c) (1) FACS analysis to determine the cell surface expression of CCR5 in CHO.CCR5.CD4 cells. black: negative control, thin line: CHO.CCR5.CD4 cells (mean fluorescence: 47.14), bold line: CHO.CCR5.CD4 cells treated with sodium butyrate (mean fluorescence: 89). (2) FACS analysis to determine the cell surface expression of CD4, black CHO.CCR5.CD4 cells (mean fluorescence: 21.77), bold line: CHO.CCR5.CD4 cells treated with sodium butyrate as described (mean fluorescence: 69.53). (3) FACS analysis to determine the cell surface expression of CCR5 in HeLa RC-4 cells: black: negative control, thin line HeLa RC-4 (mean fluorescence 45.7). (4) FACS analysis to determine the cell surface expression of CCR5 in HeLa RC-49 cells: black: negative control, thin line HeLa RC-49 (mean fluorescence 171.6).

analysis there was approximately four times more CCR5 in RC-49 cells as compared with RC-4 cells. Internalization assays in these cells with MIP-1$\alpha$ and MIP-1$\beta$ gave similar results to sodium butyrate treated CHO.CCR5.CD4 cells. In cells that expressed higher levels of CCR5 (HeLa RC-49), MIP-1$\beta$ induced internalization was decreased (Figure 4b).

In CHO.CCR5.CD4 cells PTX treatment inhibited MIP-1$\alpha$ (PTX 15 h), MIP-1$\beta$ (PTX 2 h, 15 h) and RANTES (PTX 15 h) induced internalisation. In these cells a slight increase (non significant) in the internalization rate induced by RANTES was seen following treatment with wortmannin (Table 1).

MCP-2, MCP-3 and MCP-4 appear to have smaller effects on CCR5 internalization (Table 2). Since these chemokines exhibit smaller effects, we attempted to control for non-specific effects by inclusion of an unrelated protein of a similar size (interleukin 2 (IL2)). No significant internalization initiated by MCP-2 or MCP-3 could be observed in CHO.CCR5 cells relative to IL2, whereas MCP-4 induced a low but significant internalisation ($P<0.05$) compared to IL2 as negative control. Pertussis toxin (15 h) treatment inhibited the internalization seen with MCP-4. After treating the cells with wortmannin an increase in the internalization rate was seen for MCP-2, MCP-3 and MCP-4 although the overall effect of the chemokines was less than for MIP-1$\alpha$ or RANTES (Table 1). In CHO.CCR5.CD4 cells the same results were seen as in CHO.CCR5 cells (data not shown).

The FACS data were confirmed using immunofluorescence and confocal laser scanning microscopy (Figure 5). Cells that had not been treated with chemokines showed a bright fluorescence on the cell surface, whereas treated cells showed a different pattern. Receptor clustering and internalization were observed in cells incubated for 1 h with MIP-1$\alpha$, MIP-1$\beta$ or RANTES, but not with cells treated with MCP-2,

**Table 2** Internalization of CCR5 in CHO.CCR5 cells due to MCP-2, MCP-3 and MCP-4; effects of pertussis toxin and wortmannin

| | *Cell surface CCR5* (% of control untreated cells) | | |
|---|---|---|---|
| *Chemokine* | *Treated control* | *PTX* | *Wortmannin* |
| MCP-2 | 85.5±6.8 (6) | 81.2±4.4 (8) | 67.8±3.8** |
| MCP-3 | 85.9±5.5 (7) | 90.0±5.1 (7) | 67.2±3.8** (6) |
| MCP-4 | 79.2±2.5** (6) | 95.4±2.9 (9) | 70.0±3.0** (5) |
| IL2 | 100.9±7.5 (4) | – | – |

Internalization of CCR5 was determined as described in the Methods section. Cell surface CCR5 is expressed as mean±s.e.mean ($n$) as a percentage of values in untreated cells. Cells were incubated with 100 ng ml$^{-1}$ PTX for 15 h, and 1 $\mu$M wortmannin for 1 h at 37°C respectively where indicated. Statistical comparisons were made between treated control values for MCP-2, MCP-3 or MCP-4 and values for cells treated with interleukin-2 (IL2) as a negative control for internalization (**$P<0.05$). Only values for MCP-4 were significantly different compared to interleukin-2. For the effects of PTX and wortmannin, comparisons were made with the corresponding chemokine control values. Wortmannin treatment significantly increased the internalization compared to control incubation with chemokines (**$P<0.05$)

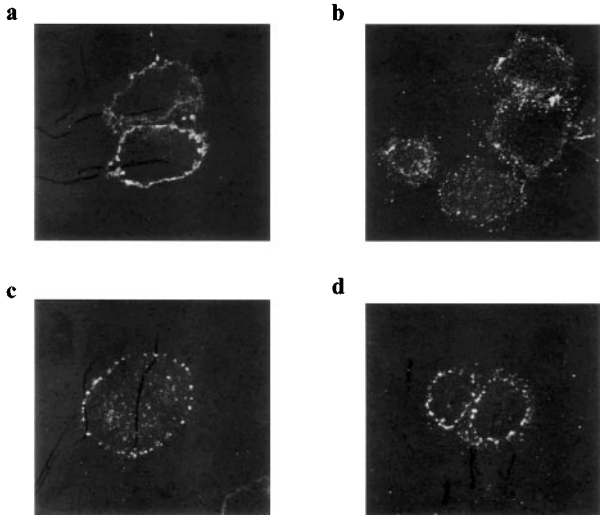


**Figure 5** Effects of chemokines on CCR5 determined using confocal microscopy. Cells were grown on coverslips overnight, incubated in medium without serum for 2 h and then incubated with 50 nM chemokines or vehicle control for 1 h (a) control, (b) MIP-1$\alpha$, (c) MIP-1$\beta$, (d) RANTES. Cells were washed and a stain was performed using anti-CCR5 HEK/1/85a/7a antibody and anti-rat-FITC. Pictures were taken using Leica NT Confocal Imaging system. Data show representative cells.

MCP-3 or MCP-4 (data not shown). After longer time periods (8 h) some internalization was seen for MCP-2, MCP-3 and MCP-4 (data not shown), however this was in a restricted number of cells, whereas the majority of cells responded to MIP-1$\alpha$, MIP-1$\beta$ and RANTES.

### *CCR5 receptor phosphorylation*

Cells were treated with chemokines and CCR5 receptor was immunoprecipitated. CCR5 phosphorylation was detected as a shift in the apparent molecular weight of the receptor on SDS–PAGE (Figure 6). MIP-1$\alpha$, MIP-1$\beta$ and RANTES were able to induce receptor phosphorylation after incubation for 1 min. A time course showed that this phosphorylation was stable up to 1 h after initiation (Figure 6a). Treatment with protein phosphatase 1, a serine/threonine-specific phosphatase, abolished the shift in apparent molecular weight (Figure 6b,c) confirming that this was due to phosphorylation. MCP-2 (50 nM, 150 nM), MCP-3 (50 nM) and MCP-4 (50 nM) were unable to induce receptor phosphorylation.

### *Stimulation of [$^{35}$S]-GTP$\gamma$S binding after CCR5 activation*

In order to investigate the activation of G proteins via CCR5, the stimulation of [$^{35}$S]-GTP$\gamma$S binding was examined. MIP-1$\alpha$, MIP-1$\beta$, RANTES and MCP-2 were able to induce a concentration-dependent increase in [$^{35}$S]-GTP$\gamma$S binding in membranes from both CHO.CCR5 and CHO.CCR5.CD4 cells (Figure 7, Table 3). The maximal stimulation due to MIP-1$\alpha$ was smaller in membranes from CHO.CCR5.CD4 cells (~100% over basal) as compared to membranes from CHO.CCR5 cells (~300% over basal) reflecting the different CCR5 levels. Data for the $EC_{50}$ and maximal agonist effect for different chemokines are given in Table 3. RANTES is the most potent chemokine tested here and MCP-2 exhibits a lower potency response (Table 3). MIP-1$\alpha$, MIP-1$\beta$ and RANTES all produce the same maximal response in this assay i.e. they are full agonists whereas MCP-2 produces a response that is ~80% of this maximal response. MCP-3 and MCP-4 do not activate CCR5 at all in this assay. The calculated $EC_{50}$ values and relative maximal effects for agonists are similar in CHO.CCR5 and CHO.CCR5.CD4 cells. PTX treatment (15 h) abolished the stimulation of [$^{35}$S]-GTP$\gamma$S binding by chemokines, whereas a 2 h treatment with the toxin elicited only about 30–50% inhibition of the [$^{35}$S]-GTP$\gamma$S binding response (data not shown). The effects observed were similar for MIP-1$\alpha$, MIP-1$\beta$ and RANTES.

### *Changes in intracellular calcium ions*

In order to investigate signalling events downstream of CCR5, the effects of chemokines on changes in intracellular calcium ions were investigated in CHO.CCR5.CD4 cells. The concentration of intracellular calcium ions was increased by MIP-1$\alpha$, RANTES and MCP-2 (10 nM) and by MIP-1$\beta$ and MCP-4 (100 nM). The extent of change in intracellular calcium ions was similar for the different ligands. MIP-1$\beta$ and MCP-4, when used at 10 nM, did not significantly alter intracellular calcium ion concentration. MCP-3 did not increase intracellular calcium ions in the cells even when used at 100 nM (Figure 8).

## Discussion

In this study we have examined the pharmacological profile of the chemokine receptor, CCR5. Six naturally occurring chemokines were tested (MIP-1$\alpha$, MIP-1$\beta$, RANTES, MCP-2, MCP-3, MCP-4) and although it has been shown

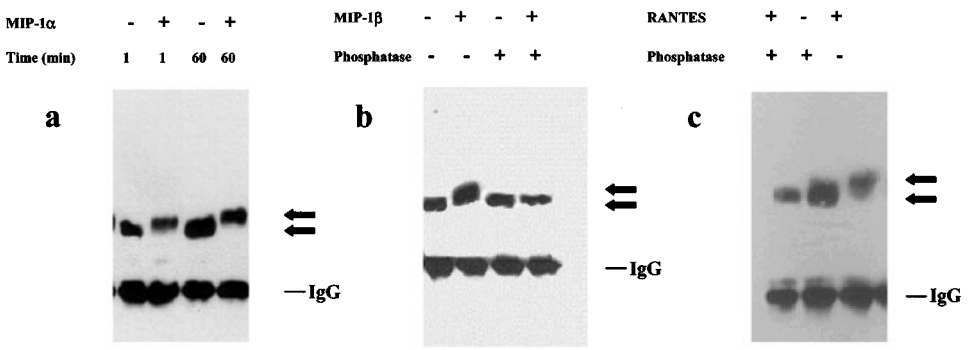


**Figure 6** Detection of chemokine induced phosphorylation in CHO.CCR5 cells. Cells were incubated in medium without serum and incubated with chemokines (50 nM) as indicated and then subjected to an immunoprecipitation with an anti CCR5 antibody. CCR5 was detected on a Western blot using a specific antibody. Arrows indicate CCR5. (a) MIP-1$\alpha$ treatment at different time points. Results for MIP-1$\beta$ and RANTES treatment were similar (data not shown). (b,c) Treatment with protein phosphatase 1. Results for MIP-1$\alpha$ were similar (data not shown). Data show one representative blot from three independent experiments with similar results.

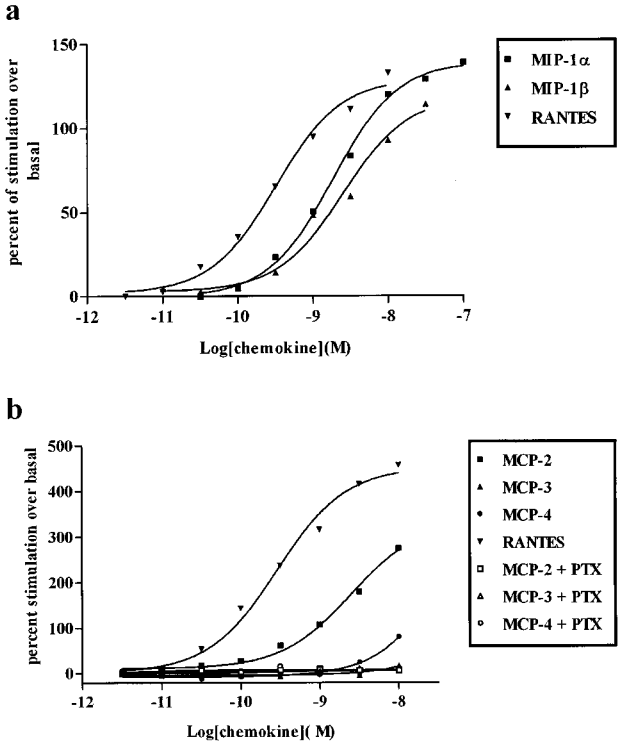


**Figure 7** Stimulation of [$^{35}$S]-GTP$\gamma$S binding in CHO.CCR5 and CHO.CCR5.CD4 membranes by natural chemokines. [$^{35}$S]-GTP$\gamma$S binding was determined after 30 min incubation in the presence of increasing concentrations of each agent as described in the Methods section. Results are expressed as a percentage of the unstimulated basal level of binding. Data shown are the means of triplicate determination from a representative of at least three separate experiments. Summary data are provided in Table 1. (a) Stimulation in CHO.CCR5.CD4 cells induced by MIP-1$\alpha$, MIP-1$\beta$ and RANTES. (b) Stimulation in CHO.CCR5 cells induced by RANTES, MCP-2, MCP-3, MCP-4 (closed symbols) and CHO.CCR5 cells after PTX (15 h) treatment (open symbols).

previously that these are able to bind to CCR5 and in some cases induce calcium influx (Blanpain *et al*., 1999) it is not known whether the mechanisms for receptor activation are the same. We therefore compared the different chemokines for their ability to stimulate G protein activation *via* CCR5 as determined using the stimulation of [$^{35}$S]-GTP$\gamma$S binding, to increase intracellular calcium ion concentration, to induce CCR5 phosphorylation and to induce CCR5 internalization. These activities have different time courses but they reflect different aspects of CCR5 activation. The data described below show that different patterns of activation were seen in the different assays suggesting that the different chemokines lead to different cellular responses.

Internalization of CCR5 was seen after treatment of CHO.CCR5 cells with either MIP-1$\alpha$, RANTES or MIP-1$\beta$ although this occurred to a greater extent for MIP-1$\alpha$ and RANTES and did not reach significance for MIP-1$\beta$. The time course of internalization was biphasic with a large part of the effect occurring in the first 10 min followed by a much slower response. In CHO.CCR5.CD4 cells internalization occurred to a similar extent with MIP-1$\alpha$, RANTES and MIP-1$\beta$. This difference between the two cell types is unlikely to reflect an effect of CD4 as soluble CD4 did not affect internalisation triggered by MIP-1$\beta$ and it is more likely to reflect the different expression levels in the cells containing CD4. Indeed when expression of CCR5 was raised in these cells, using sodium butyrate treatment, the effect of MIP-1$\beta$ was much less. Similar results were obtained in HeLa cells that express different amounts of CCR5 supporting the idea that differences in receptor density affect the rate of MIP-1$\beta$ induced internalization. It seems that there may be some kinetic difference for the internalization of CCR5 triggered by MIP-1$\beta$. The level of CCR5 expressed at the cell surface will depend on the rate of internalization and the rate of recycling of CCR5. The balance between these two processes is altered by the chemokine and only with a moderate expression level of CCR5 is MIP-1$\beta$ able to achieve the same extent of internalization as MIP-1$\alpha$ and RANTES.

The extent of internalization induced by MCP-2, MCP-3 and MCP-4 ($<20\%$) was much less than for the other chemokines (e.g. $\sim 50\%$ for MIP-1$\alpha$), in either cell type used suggesting a difference in behaviour between the two groups of ligand. PTX treatment of cells reduced CCR5 internalization induced by MCP-4. We tested the effects of wortmannin on CCR5 internalization as wortmannin has been shown to

**Table 3** Stimulation of [$^{35}$S]-GTP$\gamma$S binding

| | *CHO.CCR5 cells* | | | | *CHO.CCR5.CD4 cells* | | | |
|---|---|---|---|---|---|---|---|---|
| *Chemokine* | *$pEC_{50}\pm$s.e.mean* | *$EC_{50}$* (nM) | *% MIP-1α response* | n | *$pEC_{50}\pm$s.e.mean* | *$EC_{50}$* (nM) | *% MIP-1α response* | (n) |
| MIP-1α | 8.90±0.13 | 1.3 | 100 | 11 | 8.64±0.04 | 2.3 | 100 | 7 |
| MIP-1β | 8.46±0.10 | 3.4 | 97±8 | 11 | 8.35±0.21 | 4.5 | 100±5 | 4 |
| RANTES | 9.48±0.10 | 0.33 | 94±7 | 11 | 9.29±0.16 | 0.50 | 94±12 | 7 |
| MCP-2 | 8.26±0.10 | 5.5 | 78±12 | 3 | nd | | | |

Stimulation of [$^{35}$S]-GTP$\gamma$S binding was determined on membranes from cells that expressed CCR5 (CHO.CCR5) alone or both CCCR5 and CD4 (CHO.CCR5.CD4) as described in the Methods section. Data were analysed to provide the $EC_{50}$ and the maximal response, expressed as a % of that given by MIP-1α.

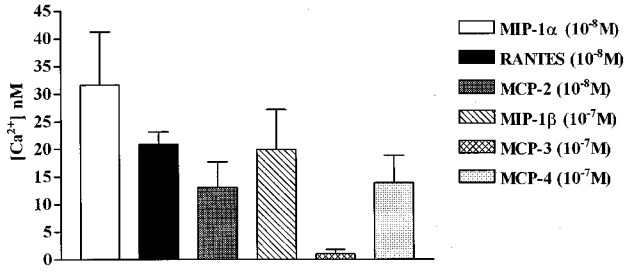


**Figure 8** Intracellular calcium ion concentrations in CHO.CCR5.CD4 cells. Intracellular calcium ion concentrations were determined after stimulation of CCR5 with the indicated chemokines as described in the Methods section. Data are expressed as mean±s.e.mean (three or more experiments).

increase internalization of the related chemokine receptor, CXCR2 (Feniger-Barish *et al*., 2000). Wortmannin (1 μM) treatment of CHO cells was found to increase the levels of CCR5 internalization by those ligands exhibiting minimal effects under control conditions, e.g. MIP-1β, MCP-2, MCP-3 in cells expressing high levels of CCR5 (CHO.CCR5 cells). Feniger-Barish *et al*. (2000) have suggested that these effects are mediated *via* wortmannin-inhibition of PI4-kinases. PI4-kinases are inhibited by wortmannin at concentrations that are significantly higher than those required to inhibit PI3-kinases (Xue *et al*., 1999). In the present study, no effect was seen with 100 nM wortmannin or with the specific PI3-kinase inhibitor LY294002, so that the effects of the higher concentration of wortmannin cannot be due to inhibition of PI3-kinase. Based on the speculations of Feniger-Barish *et al*. (2000), therefore, wortmannin may be exerting its effect at the higher concentration in the present study by inhibiting PI4-kinase, increasing the formation of clathrin coated pits at the cell surface and hence allowing the cell machinery to internalize more CCR5.

The reduced ability of MCP-4 to cause internalization of CCR5 could be attributable to its lower affinity for CCR5 although the concentrations of chemokine used should have occupied a significant fraction of the receptors (Blanpain *et al*., 1999). MCP-2, however, has a comparable affinity to MIP-1α for CCR5 (Blanpain *et al*., 1999) so this cannot be the explanation for the lower extent of internalization seen for this chemokine. MCP-3 also has a high affinity for CCR5 ($K_i\sim3$ nM (Blanpain *et al*., 1999)) but has been reported to be an antagonist for CCR5 (Blanpain *et al*., 1999), so that receptor internalization by this ligand may represent a novel effect for an antagonist. However, there is a precedent for antagonists affecting receptor internalization, the RANTES derivative (RANTES9-68) has been shown to induce internalization of CCR5 but to act as an antagonist in functional tests (Amara *et al*., 1997). Also, for the CCK receptor, an antagonist ligand was able to induce partial receptor internalization (Roettger *et al*., 1997).

The effect of PTX treatment is generally thought to be due to the uncoupling of receptor and G proteins of the Gi/o class and the effects of PTX on agonist stimulated [$^{35}$S]-GTP$\gamma$S binding in the present study support this conclusion (see below). MIP-1α and RANTES-induced internalization of CCR5 was inhibited by PTX treatment, suggesting that receptor-G protein coupling is critical for receptor internalization. Indeed, a truncated form of CCR5 lacking the C-terminal cytoplasmic tail does not internalize upon ligand binding, is deficient in signalling and yet can still allow HIV entry into target cells (Alkhatib *et al*., 1997). The data of the present study do not, however, distinguish between direct effects of receptor/G protein uncoupling by PTX on CCR5 internalization and indirect effects subsequent to changes in second messengers. We also examined a potential role of protein kinase C in the internalization process using the inhibitor staurosporine. There was no effect of staurosporine on chemokine-induced internalization of CCR5 so that protein kinase C does not contribute to this process.

For many GPCR's a consequence of receptor activation is the subsequent phosphorylation of the receptor leading to desensitization. Phosphorylation of CCR5 at serine residues has been demonstrated after MIP-1α or RANTES activation using radiolabelled phosphate (Oppermann *et al*., 1999; Vila-Coro *et al*., 1999). In the present study we have shown phosphorylation of CCR5 after stimulation of CHO.CCR5 cells with MIP-1α, RANTES and MIP-1β. This was demonstrated by the alteration in mobility on electrophoresis and it was confirmed that this was due to phosphorylation of CCR5 by reversal after treatment with protein phosphatase 1 (selective for phosphorylation at serine/threonine residues). This phosphorylation was detectable after 1 min treatment with chemokine and was stable for up to 1 h. By contrast no phosphorylation was detectable after treatment with MCP-2, MCP-3 or MCP-4 (even at 150 nM (MCP-2)), this is consistent with the low level of internalization seen with these chemokines since receptor phosphorylation is thought to precede internalization.

Signalling events downstream of CCR5 were assessed by determining the effects of chemokines on changes in intracellular calcium ion concentration in CHO.CCR5.CD4 cells. Increases in intracellular calcium ion concentration were observed following activation of CCR5 with 10 nM MIP-1α,

RANTES or MCP-2, whereas for MIP-1$\beta$ and MCP-4 increases were seen only at 100 nM chemokine. The extent of increase in intracellular calcium ion concentration was similar for the different ligands. MCP-3 did not elicit any changes in intracellular calcium ions in these assays.

A further index of CCR5 activation was obtained by examining the stimulation of [$^{35}$S]-GTP$\gamma$S binding. This assay has been used for a number of GPCR's to provide a measure of agonist potency and efficacy (Gardner *et al*., 1996; Hall *et al*., 1999). In CHO.CCR5 membranes MIP-1$\alpha$, RANTES and MIP-1$\beta$ were able to induce stimulation ($\sim$300%) of [$^{35}$S]-GTP$\gamma$S binding over the basal level and although the different chemokines exhibited different potencies the maximum stimulation of [$^{35}$S]-GTP$\gamma$S binding was similar in each case. Of the other chemokines tested only MCP-2 was able to stimulate [$^{35}$S]-GTP$\gamma$S binding. MCP-2 exhibited both lower potency and efficacy ($\sim$80%) as compared to MIP-1$\alpha$, RANTES and MIP-1$\beta$. MCP-3 and MCP-4, therefore, do not elicit a measurable response in this system. Similar data were obtained in CHO.CCR5.CD4 cells using the same range of chemokines although the maximal stimulation achieved by MIP-1$\alpha$ was lower reflecting the lower CCR5 expression in these cells. Treatment with PTX for 15 h completely abolished the chemokine induced signalling, whereas 2 h treatment only inhibited signalling by 30–50%. Although a 2 h treatment has an effect on the signalling, it does not seem to be sufficient to uncouple receptor from Gi/o G protein completely. This agrees with the internalization results, where a 2 h treatment mostly failed to inhibit internalization induced by RANTES and MIP-1$\alpha$. The correspondence between these effects supports the model that the effects of PTX on internalization are due to uncoupling of receptor from signalling systems.

From the four measures of CCR5 activation, therefore, different profiles emerge concerning the effects of the different chemokines. For example, MCP-2 is clearly an agonist in the [$^{35}$S]-GTP$\gamma$S binding and intracellular calcium assay but is unable to stimulate receptor phosphorylation or strong internalization under conditions where MIP-1$\alpha$, MIP-1$\beta$ and RANTES are able to. The affinity of MCP-2 for binding to CCR5 is comparable to that of MIP-1$\alpha$ (Blanpain *et al*., 1999). These data suggest that MCP-2 interacts with CCR5 in a different manner to the other chemokines and that the conformational change(s) induced in the receptor may be able to induce some processes linked to the receptor but not others. MCP-3 also binds to CCR5 with a high affinity (Blanpain *et al*., 1999) but is unable to activate the receptor and appears to be an antagonist in each assay. MCP-4 exhibits weak activity in the internalization assay, but is a low potency agonist in the intracellular calcium assay. It seems that the division of the chemokines examined in this study in to two groups (MIP-1$\alpha$, MIP-1$\beta$, RANTES and MCP-2, MCP-3, MCP-4) on the basis of amino acid sequence identity (Baggiolini *et al*., 1994; 1997) has functional consequences for their effects on CCR5 as well. The former group are able to activate the receptor fully leading to G protein activation, intracellular calcium ion release and CCR5 phosphorylation and internalization. The latter group have much more diverse effects (MCP-2 is an agonist whereas MCP-3 is an antagonist in some tests) and this must indicate a fundamental difference in the manner in which they interact with CCR5. Further support for the division of the chemokines in to different functional groups with different interactions with CCR5 comes from studies of naturally occurring mutants of CCR5 (Blanpain *et al*., 2000). The A29S mutant of CCR5 was severely impaired with respect of responses to MIP-1$\alpha$, MIP-1$\beta$ and RANTES, whereas effects of MCP-2 were comparable to those of the native receptor.

In this study we examined the responses of CCR5 in CHO cells expressing CCR5 alone and in CHO cells expressing CCR5 together with CD4. We were able to confirm CD4-CCR5 association in the latter cell system by co-immunoprecipitation from the CHO.CCR5.CD4 cells (Figure 2), although only a small proportion of cell surface expressed CCR5 was found to co-localize with CD4 using confocal microscopy (unpublished observations). After activation of CCR5 with different chemokines we were unable to detect any difference in the amount of CCR5 that could be precipitated with CD4 so that receptor activation does not alter the CCR5-CD4 complex formation. It seems, therefore, that most of the CCR5 in CHO.CCR5.CD4 cells does not interact with CD4 so we were unable to determine the effects of CD4 on CCR5 signalling using this system.

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